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Characterization of the serum-induced growth response in selected Gram-positive and Gram-negative bacterial pathogens.

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CHARACTERIZATION OF THE SERUM-INDUCED GROWTH RESPONSE IN SELECTED GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIAL PATHOGENS

by

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ABSTRACT OF DISSERTATION
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Title Characterization of the Serum-Induced Growth Response in Selected Gram-Positive and Gram-Negative Bacterial Pathogens.

Blood-borne infections caused by bacteria are serious and potentially fatal diseases. The physiological and genetic responses of the blood-borne pathogens *Corynebacterium jeikeium* and *Vibrio vulnificus* were studied in growth medium supplemented with calf serum. The growth response of these pathogens in the presence of serum was investigated; the possible role of iron and other cations, such as manganese, zinc, copper, calcium, and magnesium, that are essential for bacterial growth was determined. The growth of *C. jeikeium* in a serum-supplemented medium exhibited a doubling in cell density in 3 h when compared with the culture grown without serum. It was determined that Mg^{2+} in the growth medium along with serum is essential for rapid growth. Culturing of *V. vulnificus* in the serum medium significantly increased the growth rate by approximately 3-log in 2 h. Unlike the case of *C. jeikeium*, it was observed that Fe^{2+} along with serum supplementation was necessary for enhanced growth in *V. vulnificus*. Analysis of radiolabeled total cellular proteins following growth in serum medium revealed differential expression of a 36-kDa protein, CSIP36, in both *C. jeikeium* and *V. vulnificus*. The N-terminal protein microsequencing of CSIP36 generated amino acid residues of AlaProAlaGly(Cys/Ser)LeuGlyGlyLeu, which were found to be unique when compared with the protein database. A degenerate oligonucleotide probe was deduced from these amino acid residues and used to identify the gene encoding this protein by using a

acid residues and used to identify the gene encoding this protein by using a TOPO™ Walker method. Cloning followed by nucleotide sequence analysis of the gene exhibited conserved domains of an aminotransferase, which is known to function during enhanced growth of other microorganisms. Southern blot DNA-DNA hybridization using this degenerate oligonucleotide probe exhibited positive hybridization, suggesting that the gene coding for the CSIP is located on the chromosome. Also, positive hybridization signals from the Northern blot hybridization experiments suggested this aminotransferase gene is inducible when grown in medium supplemented with serum. The results from this study contribute to our understanding of rapid growth of *C. jeikeium* and *V. vulnificus* and, possibly, their pathogenic behavior following infection in a human host.

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LIST OF ABBREVIATIONS

APW	Alkaline peptone water
CPS	capsular polysaccharide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
PCR	Polymerase chain reaction
PLP	Pyridoxal-5'-phosphate
SDS	Sodium dodecylsulfate
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
SSC	Standard saline citrate
TE buffer	Tris·Cl, EDTA buffer
TSB	Tryptic soy broth

INTRODUCTION

Blood-borne infections caused by bacteria are serious and potentially fatal diseases. Analysis of the genetic and physiological responses of blood-borne bacteria would improve our understanding of diseases caused by these pathogens. A study of *Staphylococcus aureus* demonstrated growth in serum was suitable as a model for infection in a host's bloodstream [70]. Several genes induced by growth in serum were identified in this pathogen. Elevated expression of amino acid biosynthesis genes was identified in *S. aureus* when grown in medium supplemented with blood serum. Identified were a glutamate synthase gene and four genes putatively involved in the biosynthesis of the aspartate family amino acids, lysine, methionine, threonine, and isoleucine. Glutamate synthase is involved in an important step in the production of amino acids. Two of the enzymes are involved in the synthesis of all four aspartate family amino acids. The other two are involved only in the biosynthesis of lysine or methionine. Lysine biosynthetic components have been shown to have roles in pathogenesis because they have been identified during signature-tagged mutagenesis screening of *S. aureus* using bacteremia models of infection [44]. In addition, a surface protein that appears to be an adhesin, a lantibiotic precursor-encoding gene, and a gene with unknown function were reported. Lantibiotics are a group of antibiotic peptides that are produced by and primarily act on Gram-positive bacteria [70]. The response was identical when using either human serum or pig serum, suggesting that other mammalian serum sources should serve as a suitable substitute for human serum in this type of study.

In this work, two pathogenic microorganisms, *Corynebacterium jeikeium* and *Vibrio vulnificus* were studied. Both of these bacteria are known to cause bacteremia and septicemia in humans. Bacteremia is the presence of bacteria in the blood and is typically asymptomatic. Septicemia is a systemic disease in which organisms rapidly multiply in the blood and is a very serious condition that is often fatal. There is little known about the ability of bacteria to grow rapidly in a host's blood stream. The effect of blood serum on *C. jeikeium* and *V. vulnificus* was studied to better understand possible mechanisms involved in causing septicemia. The physiological and genetic responses of these bloodborne pathogens were studied by growing them in calf serum. In addition, we investigated the roles of Fe^{2-} and other divalent cations essential for bacterial growth [46] in serum-supplemented media. The other cations used in this study were Mn^{2-}, Zn^{2-}, Cu^{2-}, Ca^{2-}, and Mg^{2-}. Also, protein expression during serum-induced growth was examined.

The micronutrients Mn^{2-}, Zn^{2-}, and Cu^{2-} are used for redox processes, for stabilization of molecules through electrostatic interactions, as components of various enzymes, and for regulation of osmotic pressure [11]. Mn^{2-} plays an important role in glycolysis, gluconeogenesis, and the metabolism of sugars and amino acids [30]. Zn^{2-} has been associated with transcriptional regulation and with pathogenesis in bacteria. A Zn^{2-} finger transcription factor that regulates prokaryotic promoters has recently been found in bacteria [8]. It was originally believed that Zn^{2-} finger transcription factors occurred only in eukaryotes. Some enterotoxins or neurotoxins secreted by pathogenic microorganisms are Zn^{2-} metalloproteases [45]. These metalloproteases cause proteolytic tissue damage in hosts. Cu^{2-} is an essential trace element utilized in a number of oxygenases and electron transport proteins in bacteria [10]. Ca^{2-} is involved in a variety of prokaryotic cellular

processes, including cell cycle control and cell division [75]. Ca^{2+} has also been implicated in bacterial pathogenesis [64]. A Ca^{2+}-responsive virulence regulon has been studied in human-pathogenic Yersiniae bacteria. This regulon is thought to function when Yersiniae infect their mammalian host, and all evidence points to the role of this regulon in protecting the bacteria against the host immune response. In addition, the activity and stability of several enzymes are directly controlled by binding to Ca^{2+}. One such enzyme is an adenosine triphosphate (ATP)-synthase described in *Escherichia coli* [76]. Mg^{2+} is a cofactor in more than 300 enzymatic reactions involving energy metabolism and protein and nucleic acid synthesis [18]. The generation and use of ATP is dependent on the presence of Mg^{2+} [58]. The role of Mg^{2+} has been extensively studied in *Salmonella typhimurium*. It has been found that Mg^{2+} plays a role in resistance to host immune responses [19] and in the expression of virulence genes [47] in *S. typhimurium*. Mg^{2+} is the regulatory signal for the *phoP/phoQ* two-component signal transduction system. The *phoP/phoQ* system has been shown to regulate over 40 genes in *S. typhimurium*, including genes required for virulence and survival in a host [23]. The importance of Fe^{2+} for growth of microorganisms has long been recognized, and pathogenic bacteria have evolved mechanisms to scavenge Fe^{2+} from the Fe^{2+} transport proteins, transferrin and lactoferrin [43]. This scavenging is accomplished through the production of siderophores, which can acquire Fe^{2+} from transferrin or lactoferrin and deliver it to the bacterial cell. *V. vulnificus* does produce siderophores, and it has been shown that the inability to produce significant siderophores has been associated with reduced virulence in *V. vulnificus* [41]. The role of Fe^{2+} in *V. vulnificus* pathogenicity is discussed in detail in a later section of this manuscript. There have been no reports of siderophore production in *C. jeikeium*.

However, it was determined that virulence is enhanced in *Corynebacterium* species by injecting Fe^{2+} compounds into animal hosts [14].

Coryneform bacteria are a group of aerobic and facultatively anaerobic Gram-positive, slightly curved bacilli that are generally non-motile, catalase positive, and non-acid fast [7]. These organisms are among the most ubiquitous in the environment and are found in fresh and salt water, soil, and air and on both plants and animals [40]. *Corynebacterium diphtheriae*, the organism that causes diphtheria, is the most recognizable and well characterized member of this genus. Most of the other *Corynebacteria* are considered to be mostly non-virulent and of little clinical significance. A pathogenic *Corynebacterium* species, now known as *C. jeikeium*, was first described in the 1970s. *C. jeikeium* is a commensal organism of the normal human skin flora. However, *C. jeikeium* is an opportunistic pathogen known to cause infection in immunocompromised hosts and in those who have had open-heart surgery [59]. Endocarditis, bacteremia, and septicemia are serious blood-borne infections caused by this pathogen. Human infection by this pathogen was reported in 1976 in the US [24]. At this time, several patients developed bacteremia due to a *Corynebacterium* species that was highly resistant to most antibiotics. In 1979 this pathogen was further characterized by the Centers for Disease Control in Atlanta and designated as *Corynebacterium* Group JK, after Johnson and Kaye, who had earlier reported serious infections with Corynebacteriae [31]. The *Corynebacterium* Group JK nomenclature was changed to *C. jeikeium* in 1987 and was officially validated by the International Committee on Systematic Bacteriology [28]. *C. jeikeium* has become increasingly recognized as a nosocomial pathogen in immunocompromised hosts, in patients with prosthetic devices, and in those with underlying ma-

lignant disease [1]. Immunocompromised patients or those with prolonged disease and/or multiple or extended courses of antimicrobial therapy are particularly susceptible to *C. jeikeium* infection. It was determined that the presence of *C. jeikeium* on the skin is highest in patients with malignancies or severe immune disorders. The rates of *C. jeikeium* colonization in these patients range from 40 to 82%, in comparison with 13 to 73% in other patients. The colonization rates of hospital personnel tested ranged from 12 to 36% [37, 69]. Skin colonization is most commonly found in the inguinal, axillary, and rectal areas [67]. Person-to-person contact and contaminated air have been implicated in spreading the organism in the hospital [59]. In an investigation following a *C. jeikeium* oubreak in a hematology ward, researchers found this pathogen on surfaces and in the air of most of the ward's rooms and on the hands of 18% of the staff [55]. The sensitivity of this pathogen to various antiseptics was investigated, and it was determined that the mean inhibitory concentrations for the *C. jeikeium* isolates tested were significantly higher than those for the non-*C. jeikeium* strains [38]. The antiseptic resistance of this microorganism may play a significant role in its high association with nosocomial infections. Most reported strains of *C. jeikeium* are highly resistant to all antibiotics except vancomycin. Resistance to antibiotics appears to be a stable trait. However, the mechanisms of resistance are unknown [37]. Vancomycin therapy is effective for the treatment of *C. jeikeium* infections when such infections are promptly diagnosed [16]. Virulence factors have not been identified in *C. jeikeium*. This pathogenic microorganism is resistant to multiple antibiotics and is often implicated in causing infection in patients on antibiotic therapy. One factor for pathogenicity is the ability of some bacteria to resist opsonophagocytosis. A protocol to study opsonophagocytosis of bacteria using chemiluminescence was em-

ployed to determine a possible mechanism of pathogenicity in *C. jeikeium*. This study revealed *C. jeikeium* is readily phagocytized and that, the ability to resist opsonophagocytosis is not a factor for the pathogenicity of this microorganism [25]. *C. jeikeium* was one microbial pathogen studied in my dissertation research.

The other microorganism studied was *V. vulnificus*. *V. vulnificus* is a natural inhabitant of marine and estuarine environments [60] and is a halophilic, Gram-negative, comma-shaped bacterium [7]. In the US, this bacterium is predominantly found along the coastlines of the Gulf of Mexico. There is a seasonal fluctuation in the average numbers of this microorganism in the environment. *V. vulnificus* has been isolated from waters where temperatures range from 9 to 31°C. The greatest numbers are typically found when the water temperature exceeds 18°C in the warmer months [32]. When temperatures drop below 10°C, this microorganism is rarely recovered from the environment [33, 52]. At these temperatures, *V. vulnificus* enters into a viable but non-culturable state. In this state, the cells undergo a morphological change from rods to cocci [39] and will not grow or divide on culture media. Cells can be resuscitated to a culturable state by gradually increasing the temperature [53]. *V. vulnificus* is most often isolated from waters with a salinity of 15-25 ppt, although this microorganism has been isolated in waters with salinities as low as 1 ppt and as high as 34 ppt [49]. It was determined that salinities above 25 ppt result in decreased survival of this pathogen [50].

In 1976, the bacterium now known as *V. vulnificus* was classified in the genus *Beneckea* [57]. Previously, this bacterium was part of a poorly defined group known as non-cholera *Vibrios* [4]. The name change from *Beneckea vulnifica* to *V. vulnificus* was formally accepted in 1980 when genetic investigation revealed there were no unique dif-

ferences between the *Vibrio* and *Beneckea* genera [3]. The first report of human illness caused by *V. vulnificus* was published in 1979 [5]. During this incident, 39 total cases of septicemia and wound infections were described in patients who were infected by this pathogen. All 24 patients with septicemia had ingested raw oysters, and all but one had an underlying chronic illness; 11 of these patients died as a result of the infection, despite treatment with antibiotics. The other 15 patients developed a wound infection resulting from crab bites or exposure of a pre-existing wound to seawater. It was noted that a majority of these cases occurred in the warmer months (June-August). A study of *Vibrio* infections in Florida from 1981 to 1993 discovered that *Vibrio parahaemolyticus*, *Vibrio cholerae* and *V. vulnificus* were the most common causes of human infection. *V. vulnificus* was the most common cause for septicemia, accounting for 64% of reported cases. Over half of the patients who acquired septicemia died [27].

V. vulnificus is responsible for a significant percentage of vibrio-related illnesses in the US [6] and is the probable leading cause of seafood-associated fatalities in the US [48]. In the US, most cases of *V. vulnificus* infections occur in the Gulf Coast region. *V. vulnificus* is taken up by filter-feeding mollusks, such as oysters, and becomes concentrated in the gut and other tissues. *V. vulnificus* can also be found in the intestines of estuarine fish species that inhabit oyster reefs of the US Gulf Coast. It is believed that these fish may serve as a reservoir for transport of the organism between oyster beds. Handling mollusks or fish contaminated with *V. vulnificus* can cause wound infections. Since vibrios proliferate in warm water, most infections occur during the warmer months of the year, when the numbers of *V. vulnificus* are highest. Concentrations as high as 1×10^6 bacteria per gram of oyster meat can be reached in the warm waters during the summer

months in the Gulf of Mexico [49]. Increased numbers are also found in fish during the summer months when the water temperature is warmer [17]. Gastroenteritis often results from the consumption of *V. vulnificus* in raw seafood, particularly raw oysters. Symptoms of this disease include fever, diarrhea, cramping, and nausea. Thorough cooking is the only way to completely destroy this pathogen in contaminated seafood.

V. vulnificus gastroenteritis may be largely unreported since the condition is normally not life threatening and symptoms are rarely severe enough to warrant medical assistance. Therefore, it is likely there are a much higher number of shellfish-related cases of gastroenteritis due to *V. vulnificus* than reported. People who are most susceptible to *V. vulnificus* infection usually suffer from chronic disease that affects either liver function or the immune system [66]. Damage to the liver causes release of Fe^{2-} stores into the blood. The presence of excess Fe^{2-} in blood is associated with increased virulence in *V. vulnificus*.

There are many strains of *V. vulnificus* isolated from the environment, but it appears only particular strains cause human illness [29]. It has been shown that oysters can be colonized with several different *V. vulnificus* strains [12]. Evidence has accumulated to suggest human infections are caused by only a few of the strains present in the heterogeneous populations found in shellfish [29]. Virulence assays using the most sensitive animal model, the iron-overloaded mouse bioassay model, demonstrate that there are virulent and avirulent strains [68]. In this model, serum iron levels in mice are elevated by intraperitoneal injection with ferric ammonium citrate or by treatment with carbon tetrachloride [72]. Carbon tetrachloride treatment causes release of stored iron from the liver into the serum. The iron-overloaded mice are then challenged with various dilutions

of *V. vulnificus* cells. The iron-overloaded mouse bioassay was implemented to mimic the septicemic state of a patient with high serum iron levels due to the importance of available iron for the pathogenicity of *V. vulnificus* in a host. Injection of mice with avirulent *V. vulnificus* strains requires greater than 1×10^6 bacteria for a lethal dose 50%. However, the lethal dose 50% drops dramatically for virulent strains to values ranging from a few to 1×10^3 bacteria.

Several virulence factors have been identified in this human pathogen. Avirulent strains of *V. vulnificus* are sensitive to human serum and are phagocytosed by macrophages. However, it was determined that virulent strains are able to resist phagocytosis and the bactericidal action of human serum [35, 36] due to the presence of a capsular polysaccharide (CPS) [2, 36]. The CPS that surrounds the bacterium has been definitively shown to have a positive correlation with virulence [74]. While essential for virulence, the capsule is not the only factor important for pathogenesis; it is clear that virulence is dependent upon multiple factors. Since the presence of CPS is an important virulence determinant in many bacterial pathogens, the CPS of *V. vulnificus* has been extensively studied. Encapsulated isolates have an opaque colonial morphology but undergo a reversible phase variation to a translucent colony phenotype that is correlated with reduced or patchy expression of CPS [71]. The presence and amount of CPS on any given virulent isolate have been positively correlated with quantitative measures of virulence of the organism in the mouse model [72]. Translucent-phase variants are less virulent than opaque isolates, and strains with mutations that result in loss of encapsulation lose their virulence properties. Recently, several genes have been isolated that are required for CPS expression and localization, including genes involved in CPS biosynthesis [77] and the

wza gene encoding a CPS outer membrane transporter [71]. Mutations in any gene that adversely affects CPS biosynthesis or transport results in a measurable decrease in virulence. *V. vulnificus* has multiple capsule types, and analysis of CPS by gas chromatography or by high-performance anion-exchange chromatography has shown there are many different CPS types [13, 61]. There has been no correlation made between CPS type and virulence [26]. Expression of CPS can vary depending on growth phase and temperature [72]. With a wild-type strain, it was shown that cell surface expression of CPS increased during log-phase growth but declined during stationary phase. It was also determined that CPS expression was greater during growth at 30°C than at 37°C.

Pili are involved in the attachment and colonization of host tissues. Binding of pili to host cells is mediated by the interactions between the bacterial pilus and specific host surface receptors [65]. It was reported clinical isolates from blood or wounds of infected individuals averaged higher numbers of individual pilus fibers per cell than environmental isolates [21]. The environmental isolates, on average, showed fewer bacteria adhering to individual epithelial cells. In recent studies, genes encoding components required for the biogenesis of type IV pili in *V. vulnificus* were isolated [54]. Type IV pili are of a class common to many Gram-negative bacteria [65]. Inactivation of a gene involved in type IV pili biosynthesis resulted in a loss of observable pili on the bacterial surface and a decrease in adherence to host cells [54].

V. vulnificus secretes several degradative enzymes and toxins, of which are important for the organism's ability to survive in the estuarine environment. For example, *V. vulnificus* secretes chitinase that helps the bacterium colonize and adhere to the chitin

exoskeletons of zooplankton. It is believed that a metalloprotease may be important for the organism to colonize and multiply in molluscan shellfish.

Other secreted enzymes and toxins play a role in causing human infection. Two proteins with hemolytic activity have been described from *V. vulnificus*. The most studied is the hemolysin termed cytolysin, a heat-labile, 56-kDa lytic enzyme that lyses mammalian erythrocytes and also is extremely cytotoxic to a variety of tissue culture cell lines, such as Chinese hamster ovary cells [22]. This protein has been shown to cause vascular permeability in guinea pigs and is lethal in mice, in which it causes extensive extracellular edema and damage to capillary endothelial cells [22]. Study of the activity of cytolysin suggests that it activates guanylate cyclase to increase the concentration of intracellular cyclic guanosine monophosphate levels, resulting in vasodilation [34]. The gene for the cytolysin has been cloned, sequenced, and designated *vvhA*. The role of this cytolysin in causing *V. vulnificus* infections is unclear because of the demonstration that there is no difference in the virulence between wild-type and strains unable to express the cytolysin [73]. This finding suggests that *V. vulnificus* expresses other degradative enzyme(s) that have the potential to cause the tissue destruction observed during *V. vulnificus* infections. It is apparent virulence of *V. vulnificus*, as it is in the majority of bacterial pathogens, is attributed to a combination of different virulence factors.

A second novel hemolysin, termed *vllY*, which encodes an approximately 40-kDa polypeptide, was cloned and sequenced from a clinical *V. vulnificus* isolate [15]. The deduced amino acid sequence of VllY shows that this hemolysin is related to legiolysin from *Legionella pneumophila*, a protein required for hemolysis, pigment production, and fluorescence. Expression of this gene in non-hemolytic *E. coli* results in zones of hemo-

lysis and production of green color on human or sheep blood agar. It has not been determined what role VllY might have in *V. vulnificus* pathogenesis. It is possible the VllY hemolysin causes the cytoxicity observed in strains that do not express the VvhA cytolysin.

It is well documented that the uptake of Fe^{2+} is associated with the pathogenicity of blood-borne microorganisms [56]. The importance of Fe^{2+} for growth of microorganisms has long been recognized; because free Fe^{2+} is virtually absent in the human body, pathogenic bacteria have evolved mechanisms to scavenge Fe^{2+} from the Fe^{2+} transport proteins, transferrin and lactoferrin [43]. This scavenging is accomplished through the production of siderophores, which can acquire Fe^{2+} from transferrin or lactoferrin and deliver it to the bacterial cell. *V. vulnificus* produces both hydroxymate and phenolate siderophores [62].

Iron is important for the virulence of *V. vulnificus*. The inability to produce significant siderophores has been associated with reduced virulence in *V. vulnificus*. It was shown that a mutant unable to express the catechol siderophore VenB was considerably less virulent in mice [41]. VenB, also known as vulnibactin, may also require the activity of a metalloprotease, VVP, to efficiently utilize iron bound to transferrin or lactoferrin. Analysis of iron acquisition by a VVP mutant suggests that the protease causes cleavage of transferrin and lactoferrin, making bound iron more accessible to the VenB siderophore [51]. It is possible that bacteria can sense they are in a host based on the low Fe^{2+} concentration. Many bacterial virulence genes are regulated by Fe^{2+}, with increased gene expression under low iron conditions [42]. One important factor in the transcriptional regulation by iron in many pathogens is the product of the *fur* gene. *V. vulnificus*

was found to contain a *fur* homologue, which was shown to functionally complement a *V. cholerae fur* mutant [42].

In the present study, growth in serum was investigated for the pathogenic microorganisms *V. vulnificus* and *C. jeikeium*. It was found that the growth rates of each of these pathogens are significantly increased in the presence of serum. The effects of nutritionally important cations were determined for growth in serum-supplemented media. It was found that Mg^{2-} was necessary for the increased growth rate of *C. jeikeium* in serum-supplemented media. Fe^{2-} seems to play a significant role in the enhanced growth rate of *V. vulnificus* in serum-supplemented media. To determine whether serum-inducible genes may play a role in the observed rapid growth response, protein expression was compared for cells grown with or without serum. Differential expression of several proteins was detected when cells were exposed to a medium supplemented with serum in both microorganisms. One protein of an approximate molecular weight of 36 kDa was observed in both *V. vulnificus* and *C. jeikeium*. This protein was isolated and partially sequenced from the N-terminal end. The sequence data were utilized in a DNA walking procedure to identify the gene encoding this protein. Sequence analysis suggests that the gene is an aminotransferase. Aminotransferases are involved in the biosynthesis of most of the amino acids. This novel aminotransferase likely has a similar biosynthetic function and together with other enzymes prepares the serum-induced cells for rapid growth and division.

The results of the current study will aid in understanding the mechanisms of rapid growth characteristic of bacterial septicemia. In addition, the increased growth rate of these pathogenic microorganisms in serum could be used to enhance their detection.

STUDY OF ENHANCED GROWTH IN *CORYNEBACTERIUM JEIKEIUM* FOLLOWING SERUM INDUCTION

by

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Abstract

Corynebacterium jeikeium is a Gram-positive, rod-shaped, opportunistic microbial pathogen known to cause blood-borne infections, such as endocarditis and septicemia, primarily to immunocompromised hosts and to those who have had open-heart surgery. The growth and physiology of this pathogen were studied in media supplemented with calf serum. The growth response of *C. jeikeium* in serum-supplemented medium exhibited a doubling of cell density in 3 h as compared to the culture grown without serum. The role of Mg^{2+} and other divalent cations essential for growth showed that Mg^{2+} is essential for this increased cell density in serum-supplemented media. Analysis of radiolabeled total cellular proteins following growth in serum-supplemented medium exhibited differential expression of at least two proteins of molecular weights of 34 kDa (CSIP34) and 36 kDa (CSIP36) in this pathogen. The N-terminal microsequencing of CSIP36 protein generated amino acid residues of AlaProAlaGly(Cys/Ser)LeuGlyGlyLeu, which was found to be unique when compared with the protein database. Southern blot DNA-DNA hybridization using this degenerate oligonucleotide probe exhibited positive hybridization, suggesting that the gene coding for the CSIP36 is located on the chromosome. These results suggest that elevated expression of proteins in *C. jeikeium* encoded by a series of genes may be necessary for increased cell density following infection.

Introduction

Corynebacterium jeikeium, a Gram-positive, rod-shaped bacterium, is a commensal microorganism of the normal human skin flora. However, *C. jeikeium* is an opportunistic pathogen known to cause infection in immunocompromised hosts and in those who

have had open-heart surgery [16], causing serious blood-borne infections, such as endocarditis, bacteremia, and septicemia. *C. jeikeium* has become increasingly recognized as a nosocomial pathogen in immunodeficient hosts, in patients with prosthetic devices, and in those with underlying malignant disease or on extended courses of antimicrobial therapy [1]. *C. jeikeium* grows at an increased rate and density on sheep's blood agar (5% vol/vol) when compared with media without blood or serum. It is possible that the growth response of *C. jeikeium* in serum mimics the response of this pathogen in the host bloodstream. Therefore, it would be beneficial to investigate the physiological state that promotes this rapid growth.

Upon infection, the uptake of free iron is associated with the pathogenicity of blood-borne microorganisms [13]. Enhanced virulence by *Corynebacterium* species has been reported in animal hosts when injected with iron compounds [5]. A serum-inducible gene has been identified in mammalian fibroblast cells and shown to cause rapid growth of these cells [9, 18]. Recently, expression of six biosynthesis genes, primarily of the aminotransferase gene family, as well as two surface proteins, one antimicrobial peptide, and one gene of unknown function have been identified in *Staphylococcus aureus* grown in medium supplemented with serum [19].

In this study, we have investigated the physiological role of enhanced growth of *C. jeikeium* in media supplemented with serum. Also, the effects of physiologically important metal cations, such as iron, manganese, zinc, copper, calcium, and magnesium [4], on the growth of *C. jeikeium* in a medium supplemented with serum have been investigated. Calcium is involved in a variety of prokaryotic cellular processes, including cell cycle control and cell division [20]. Mg^{2+} is a cofactor in more than 300 enzymatic reac-

tions involving energy metabolism and protein and nucleic acid synthesis [6]. We have investigated differential expression of proteins following growth in serum-supplemented medium. We have also identified a gene on the chromosome that codes for elevated expression of a protein. The enhanced growth rate in the presence of serum, the role of divalent cations, and differential expression of proteins could improve insight into the mechanism of pathogenicity exerted by this organism in humans.

Materials and Methods

Bacterial strains and microbiological media. *C. jeikeium* strain 43216 (ATCC) was used in this study. This strain was cultured in tryptic soy broth (TSB) or tryptic soy agar (TSA) medium (Difco, Detroit, MI, USA) [2]. Also, in some experiments, M9 minimal salt medium [2] supplemented with 0.02% glucose was used [2].

DNA extraction. Genomic DNA was extracted and purified as described by Ausubel et al. [3]. Cells were suspended in 567 µl of TE buffer (10 mM of Tris·HCl pH 8.0, 1 mM of EDTA) with 30 µl of 10% (wt/vol) sodium dodecyl sulfate (SDS) and 3 µl of proteinase K (20 mg/ml) (Sigma, St. Louis, MO, USA) and lysed for 1 h at 37°C. Next, 100 µl of 5 M NaCl and 80 µl of CTAB/NaCl were added and incubated for 10 min at 65°C. DNA was purified by extraction with chloroform-isoamyl alcohol (24:1), followed by purification with phenol-chloroform-isoamyl alcohol (25:24:1). DNA was then precipitated with isopropanol, centrifuged for 5 min at 10,000 g, washed with cold 70% (vol/vol) ethanol, and dried in a DNA SpeedVac (Savant, Holbrook, NY, USA). The dried DNA was resuspended in 25 µl of TE buffer and the DNA concentration measured

with a Lambda II spectrophotometer (Perkin-Elmer, Wellesley, MA, USA) at a wavelength of 260 nm.

Growth of *C. jeikeium* in serum-supplemented media. *C. jeikeium* was grown in 500 ml of M9 minimal salt [2] or TSB (Difco) medium in a 1000-ml screw-cap Erlenmeyer flask until the OD_{450nm} reached 0.2. The culture was dispensed into aliquots in 500-ml screw-capped Erlenmeyer flasks. One flask contained 100 ml of the culture grown in M9 or TSB medium. The rest of the culture was separated into four other flasks and supplemented with final concentrations of 0.1%, 1%, 10%, and 50% (vol/vol) calf serum (Colorado Serum Company, Denver, CO, USA). All cultures were incubated at 37°C on a rotary shaker set at 170 rpm, and growth was monitored at OD_{450nm} using a spectrophotometer (Perkin-Elmer Lambda II).

Effect of iron (Fe^{2+}) on *C. jeikeium* growth. Iron in serum was chelated with the iron chelator 2,2-dipyridyl (Sigma). A 300-ml culture of *C. jeikeium* was grown to an OD_{450nm} of 0.2 in M9 minimal salt medium supplemented with 10% (vol/vol) serum. Three aliquots of 100 ml each were separated into 500-ml screw-capped Erlenmeyer flasks. Then, 2,2-dipyridyl was added to two of the flasks to a final concentration of 100 or 200 μM. Cultures were incubated at 37°C on a rotary shaker set at 170 rpm, and growth was monitored at various time intervals.

Effect of important divalent cations on *C. jeikeium* growth. A 700-ml culture of *C. jeikeium* was grown to an OD_{450nm} of 0.2 in M9 minimal salt medium supplemented

with 10% (vol/vol) serum. Then, Na_2EDTA was added to the culture to a final concentration of 10 mM to chelate divalent and trivalent cations in the medium. The culture was transferred into 100-ml aliquots and incubated at 37°C on a rotary shaker set at 170 rpm for 2 h. The following divalent cations, Ca^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}, were added individually to separate flasks to a final concentration of 11 mM (1 mM free cation) to determine each one's effect on growth. The cultures were incubated at 37°C on a rotary shaker set at 170 rpm. The growth was monitored at various intervals at an OD_{450} nm.

Effect of Mg^{2+} on *C. jeikeium* growth in M9 minimal medium and TSB medium. A 200-ml culture of *C. jeikeium* in M9 minimal medium or TSB medium was grown to an OD_{450} of approximately 0.2. Two aliquots of 100 ml each were separated into 500-ml screw-capped Erlenmeyer flasks. Then, $MgCl_2$ was added to one of the flasks to a final concentration of 1 mM. Cultures were incubated at 37°C on a rotary shaker. Growth was monitored at an OD_{450nm} at various intervals.

***In situ* labeling of *C. jeikeium* total cellular proteins.** *C. jeikeium* was grown in M9 minimal medium to an OD_{450} of approximately 0.2. The culture was then separated into three equal portions. 1% or 10% (vol/vol) serum was added to two of the aliquots, and total cellular proteins were radioactively labeled with 1 μCi ^{35}S-methionine/cysteine per ml [Celltrac-35 (10.0 mCi/ml; 259Mbq; Andotek Life Sciences, Tustin, CA, USA)]. The differential expressions of protein in *C. jeikeium* cultures grown with serum or without serum were compared by a denaturing SDS polyacrylamide gel. Following sodium

dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the gel was dried in a gel dryer (Bio-Rad, Hercules, CA, USA) and autoradiography was performed using a Kodak BIOMAX™ MR film.

Isolation and sequencing of a serum-induced protein. After SDS-PAGE, proteins were transferred onto an Immobilon P^{SQ} polyvinylidene difluoride protein sequencing membrane (Millipore) using the CAPS (Fisher, Hampton, NH, USA) buffer an electroblot apparatus (Bio-Rad, Hercules, CA, USA) set at 500 mV for 30 min [15]. The proteins on the membrane were then stained for 1 min with Ponceau S dye (Fisher, Hampton, NH, USA). The desired 36-kDa protein band (CSIP-36) on the membrane was cut out using a sterile razor and transferred to a clean microcentrifuge tube. The isolated protein was sequenced at the N-terminal end using the Edman degradation method [10, 17] at the University of Alabama at Birmingham Central Protein Sequencing Facility.

Probe design and synthesis. A 27-nt degenerate oligonucleotide probe (P-CSIP-36) was designed using the *Escherichia coli* codon usage table [12] for Southern blot DNA-DNA hybridization experiment. The probe was custom synthesized by IDT, Inc. (Coralville, ID).

Southern blot DNA-DNA hybridization analysis. Genomic DNA from *C. jeikeium* was isolated and purified by the method described by Ausubel et al. [3] and subsequently treated with *NotI*, *PstI*, or *Sau3AI* restriction endonucleases (New England Biolabs, Beverly, MA, USA) using the protocol described by Ausubel et al. [3]. The restric-

tion endonuclease-treated DNA fragments were then separated in an agarose (0.9% wt/vol) gel at a constant voltage of 5 V/cm in Tris·HCl pH 8.3-acetate-ethylenediamine triacetic acid (EDTA) buffer [3]. The separated DNA was then capillary transferred onto a Zeta-Probe™ nylon membrane by the alkaline blotting method (Bio-Rad, Hercules, CA, USA). The DNA blot was first pre-hybridized with blocking solution (Bio-Rad, Hercules, CA, USA) and then hybridized overnight in a hybridization buffer (BioRad, Hercules, CA, USA) at 65°C with P-CSIP-36 probe. The probe was labeled with 1 μCi ^{32}P[α]dCTP (10.0 mCi/ml; 800 Ci/mmol; 259Mbq) (NEN DuPont, Boston, MA, USA) at the 3'-OH end using terminal deoxy-ribonucleotidyl transferase enzyme and 1x $CoCl_2$ buffer [3]. After hybridization, the membrane was washed two times at 55°C for 15 min in hybridization wash solution I and II (Bio-Rad, Hercules, CA, USA). For autoradiography, the membrane was wrapped in a Saran wrap and placed in an autoradiography cassette (Fisher, Hampton, NH, USA) with Kodak BioMax MS™ film at -80°C overnight.

Results

DNA extraction. DNA extraction procedures using the PUREGENE Yeast and Gram-Positive Bacteria Kit and acetone pretreatment did not provide a higher yield of DNA than the standard method described by Ausubel et al. [3].

Growth of *C. jeikeium* in serum-supplemented media. The cell density of *C. jeikeium* in M9 minimal salt medium supplemented with 1% (vol/vol) calf serum increased from 0.192 to 0.279. However, when the serum concentration was increased to 10% (vol/vol), the cell density doubled from an OD_{450nm} of 0.2 to 0.4 after 3 h; and at 8 h,

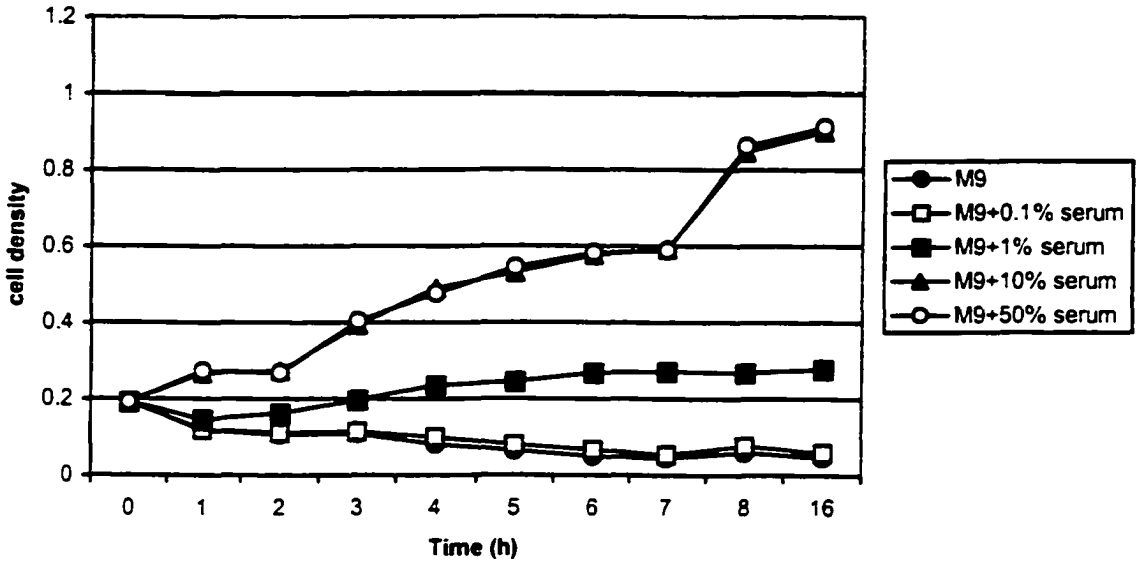

Fig. 1. Growth curve of *C. jeikeium* in M9 minimal medium and M9 medium supplemented with 10% serum. Each data point is an average of three readings. Standard error for each data point ranges from ± 0.004 to 0.010.

the OD_{450nm} was increased to 0.847 in the serum-enriched culture, which is more than four times greater than the culture without serum (Fig. 1). The 0.1% serum did not exhibit a significant increase in the cell density. Also, there was no increase in the cells density when serum was added >10% (vol/vol).

Cell density of *C. jeikeium* in TSB medium supplemented with 10% serum was significantly greater than growth in TSB without the serum. After 4 h, the cell density increased from 0.2 to 0.352 in the serum-supplemented culture. The OD_{450nm} reading of the control culture without the serum remained 0.21 during the first 4 h of incubation. After 5 h of growth, the cell density in the serum-supplemented culture became doubled (OD450nm = 0.491) in comparison with the culture without the serum (OD_{450nm} = 0.226). The serum-supplemented culture reached 2.091 after 8 h, whereas the culture without serum remained 0.279 (Fig. 2). The culture supplemented with 1% (vol/vol) serum exhibited a 1.5-fold increase in cell density in 5 h and a 2-fold increase in cell density in 8 h. The culture supplemented with 0.1% serum did not exhibit significant increase in the cell density. Additional increase in the cell density was not evident when the cultures were supplemented with >10% serum (Fig. 2).

Effect of iron on growth. Blocking the iron available in serum with the iron chelator 2.2-dipyridyl appears to have no effect on the growth of *C. jeikeium* in a 10% serum culture (Fig. 3).

Effect of nutritionally important metal cations on the growth of *C. jeikeium* in serum-supplemented media. Addition of Na_2EDTA to a culture of *C. jeikeium* in

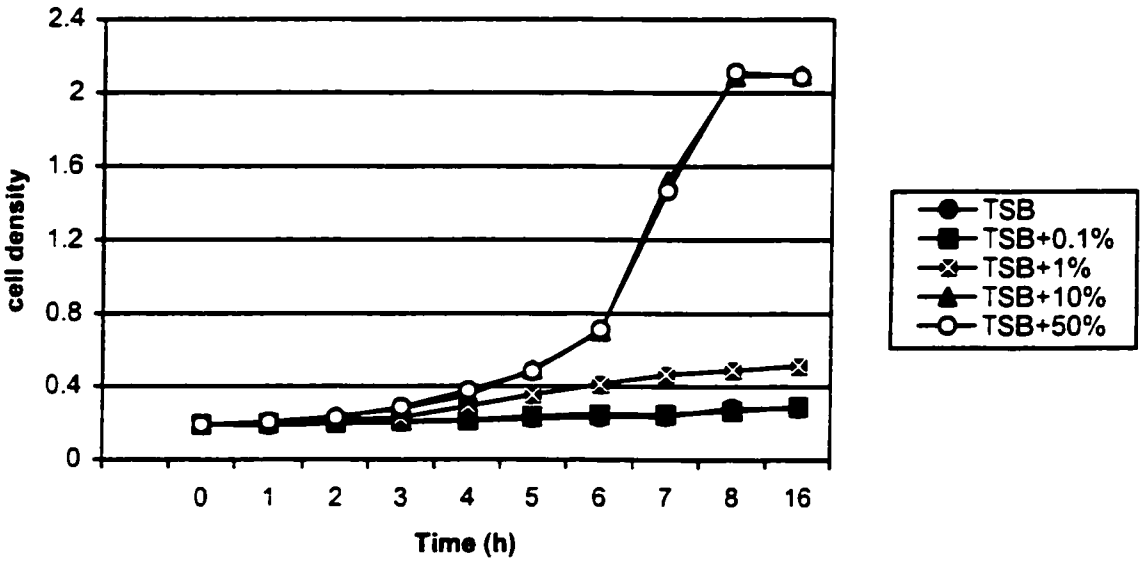

Fig. 2. Growth curve of *C. jeikeium* in TSB-enriched medium and TSB medium + 10% serum. Each data point is an average of three readings. Standard error for each data point ranges from ± 0.004 to 0.010.

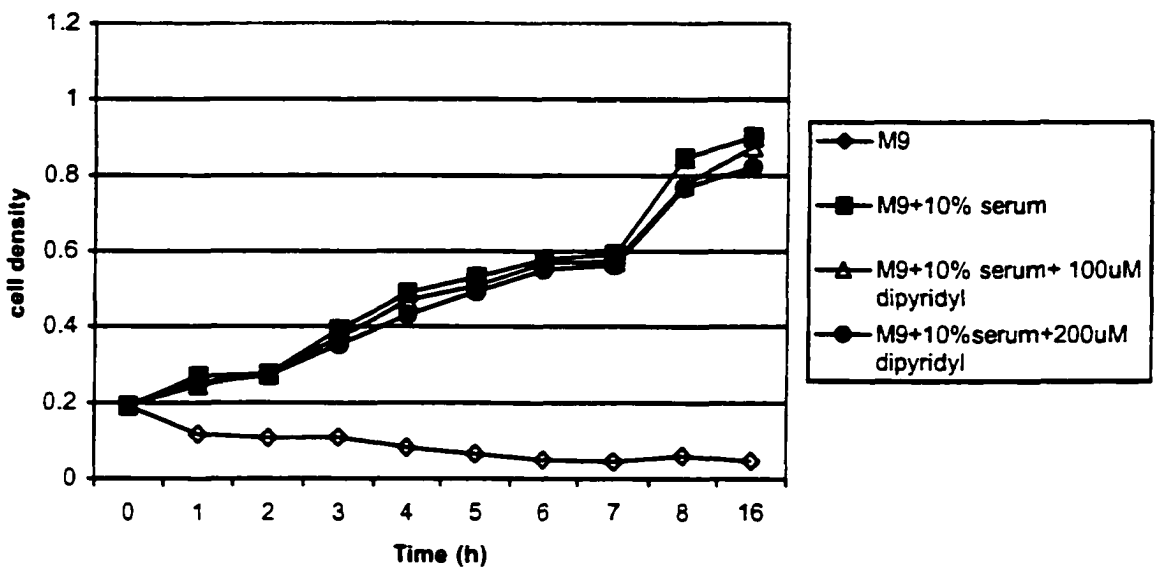

Fig. 3. Effect of the iron chelator dipyridyl on the growth of *C. jeikeium* in M9 minimal medium + 10% serum. Each data point is an average of three readings. Standard error for each data point ranges from ± 0.004 to 0.010.

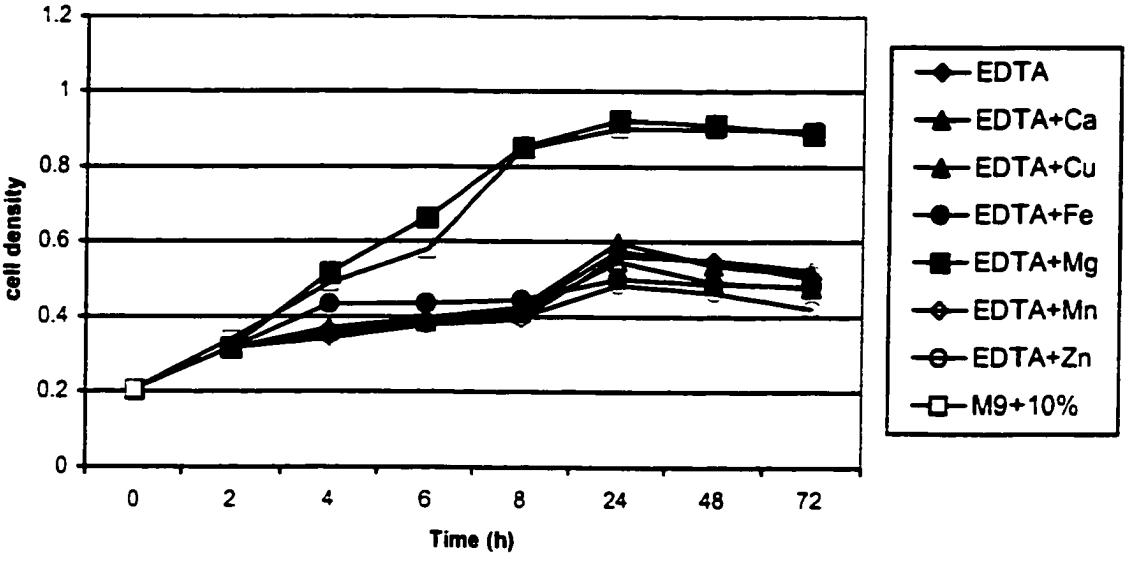

Fig. 4. Effect of the important metal cations on the growth of *C. jeikeium* in M9 minimal medium + 10% serum. Each data point is an average of three readings. Standard error for each data point ranges from ± 0.004 to 0.010.

10% (vol/vol) serum effectively reduced the enhanced growth normally observed (Fig. 4). After 4 h and 8 h of growth, the cell density at OD_{450nm} in the Na_2EDTA culture was 0.125 and 0.429, respectively, less than the culture without the Na_2EDTA. Addition of excess Ca^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, or Zn^{2+} did not exhibit restorative effect of the cell density. However, addition of Mg^{2+} to the culture with the Na_2EDTA restored the cell density to a level comparable with the cell density observed in the culture without the Na_2EDTA. The OD_{450nm} readings were the same or slightly higher at all times tested for the *C. jeikeium* culture with added Mg^{2+} when compared with the culture with 10% (vol/vol) serum without Na_2EDTA (Fig. 4). Addition of excess Mg^{2+} appears to have no effect on the growth rate of *C. jeikeium* grown in M9 medium or TSB medium without serum (Fig. 5).

Serum-induced protein expression. Elevated expressions of at least two proteins of approximate molecular weights of 45 kDa (CSIP-45) and 36 kDa (CSIP-36) from a culture of *C. jeikeium* grown in M9 minimal salt medium supplemented with 1% (vol/vol) or 10% (vol/vol) serum were identified (Fig. 6).

Isolation and sequencing of a serum-induced protein. A protein with an approximate molecular weight of 36-kDa was isolated and sequenced. The deduced amino acid sequence from the N-terminal end is AlaProAlaGly(Cys/Ser)LeuGlyGlyLeu. Using the NCBI database, a BLAST search of the deduced partial amino acid sequence of the serum-induced protein revealed no significant homology to any known proteins in the database. A degenerate oligonucleotide probe, PCSIP-36, was designed from the amino acid

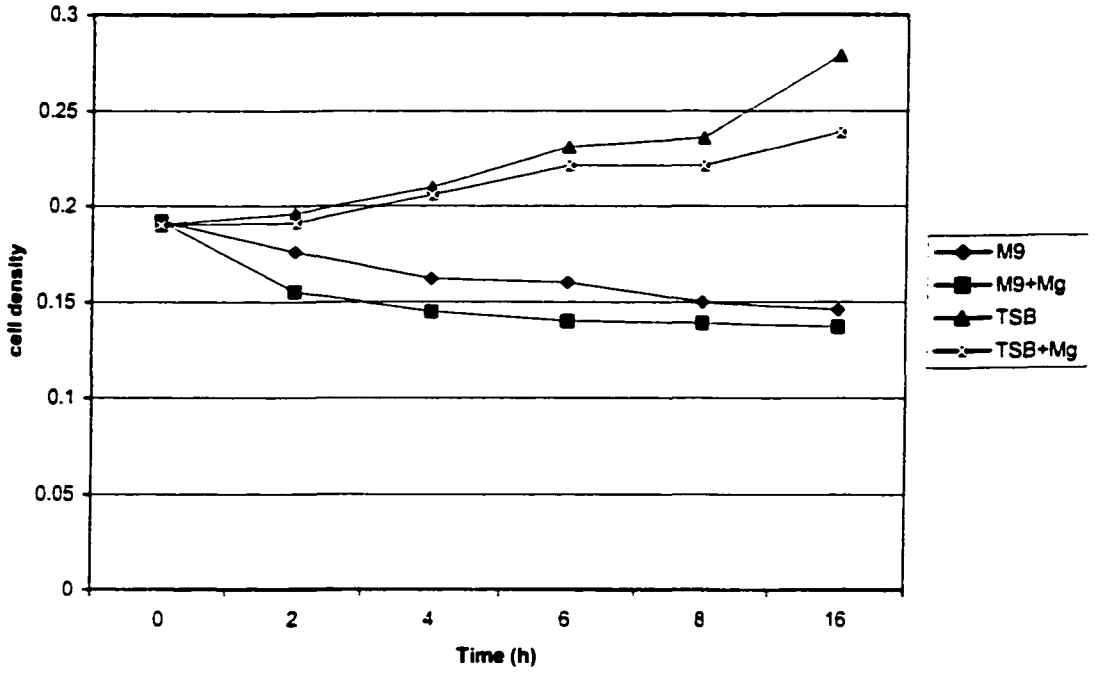

Fig. 5. Effect of magnesium on the growth of *C. jeikeium* in M9 minimal medium or TSB medium. Each data point is an average of three readings. Standard error for each data point ranges from ± 0.004 to 0.010.

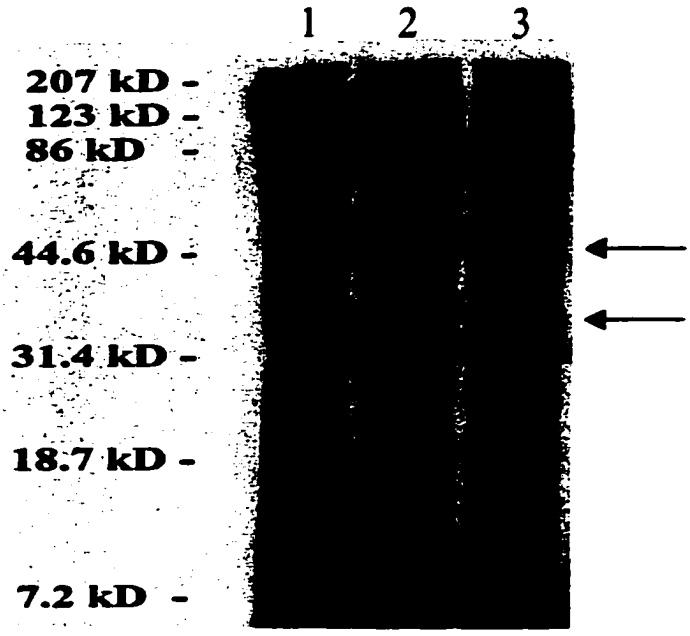

Fig. 6. Autoradiogram of ^{35}S methionine-labeled proteins in *C. jeikeium*. Lane 1, M9 medium; lane 2, M9 medium supplemented with 1% serum; lane 3, M9 medium supplemented with 10% serum. Two differentially expressed proteins of approximately 36 kDa and 45 kDa are shown by arrows.

data. The sequence of PCSIP-36 is 5'gcgccggcgggywgcttgggyggyttg3' (y = c or t, w = t or a).

Southern Blot DNA-DNA analysis. Southern blot DNA-DNA hybridization using the radiolabeled PCSIP-36 probe exhibited positive hybridization signals with *C. jeikeium* genomic DNA that was treated with restriction endonucleases (Fig. 7). Positive DNA-DNA hybridization with *C. jeikeium* genomic DNA suggests the gene that codes for the CSIP-36 protein is, in fact, from *C. jeikeium* and not from residual calf serum in the sample.

Discussion

C. jeikeium is a commensal skin bacterium with significant clinical importance. This opportunistic pathogen is a serious concern due to its ability to resist most antibiotics. Nosocomial infections are the most common form of infections. *C. jeikeium* exhibits a rapid growth response in serum-supplemented media. The rapid growth response of *C. jeikeim* in serum could be an indication of how this pathogenic organism responds in the host blood stream. It is possible that this pathogen may utilize the available serum components for rapid growth or, perhaps, can sense the presence of serum and induce rapid growth.

It was determined that Mg^{2+} was necessary for the observed rapid growth response of *C. jeikeium* in serum. It has been reported that Mg^{2+} is needed as a cofactor in more than 300 enzymatic reactions involving energy metabolism and protein and nucleic acid synthesis [6]. The cellular adenosine triphosphate generation and its use are depend-

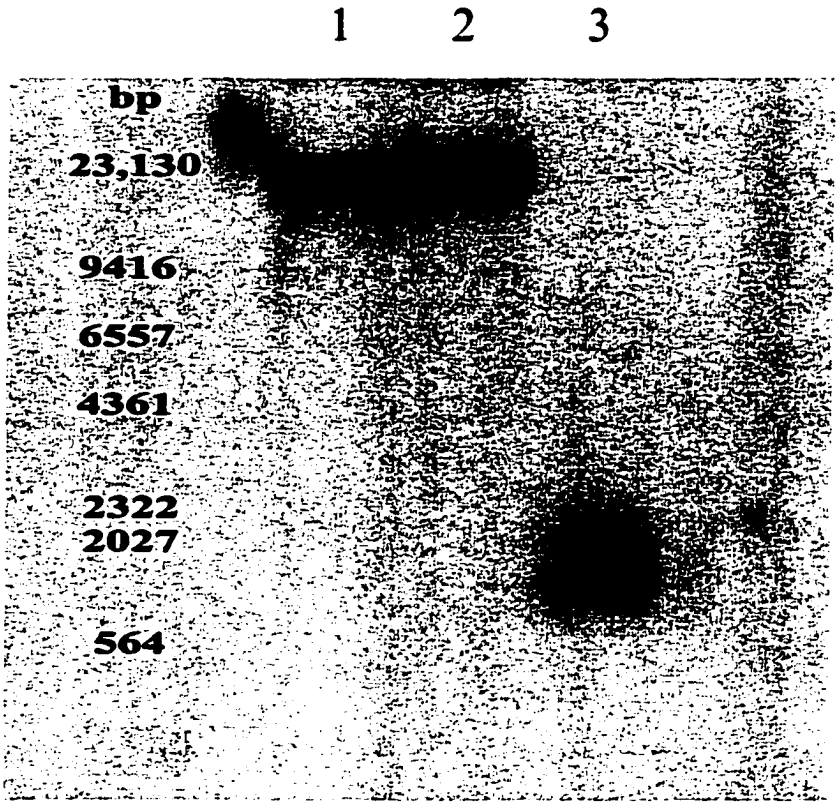

Fig. 7. Autoradiogram of Southern blot hybridization of probe PCSIP-36 to purified genomic DNA from *C. jeikeium*. Lane 1, *C. jeikeium* genomic DNA digested with *Not*I; lane 2, *C. jeikeium* genomic DNA digested with *Pst*I; lane 3, *C. jeikeium* genomic DNA digested with *Sau3*AI.

ent on the presence of Mg^{2+} [14]. The current experimental data showed that Mg^{2+} is essential in the serum-supplemented medium for an increased cell density. It is possible that Mg^{2+} plays important role in the rapid utilization of the serum components by enhancing the cellular enzymatic reactions, and increasing ATP generation and DNA replication which are coordinated with the rapid cell division.

In *Salmonella typhimurium*, Mg^{2+} plays a role in resistance to host immune responses [7] and in expression of the virulence genes [11]. It has also been reported that the Mg^{2+} is the regulatory signal for the *phoP/phoQ* two-component signal transduction system. The *phoP/phoQ* system has been shown to regulate over 40 genes in *S. typhimurium*, including genes required for virulence and survival in a host [8].

The virulence factor in *C. jeikeium* is unknown. Also, it is not clear how *C. jeikeium* resists host defenses following infection. Possibly, there is an Mg^{2+}-dependent system involved in infection and in persistence in the host's bloodstream similar to that found in *S. typhimurium*.

Several genes were identified in *Staphylococcus aureus* that are only expressed in a serum-containing medium. The majority of the genes identified in *S. aureus* were involved in amino acid biosynthesis [19]. At least two proteins, CSIP-45 and CSIP-36, are distinctly up regulated in *C. jeikeium* when exposed to serum-supplemented media. Therefore, at least the gene that codes for the CSIP-36 is likely to be involved in the observed increased cell density of *C. jeikeium* in serum-supplemented. In our knowledge, this is the first study describing a protein and its gene that could be involved in the rapid increase in the cell density when cultured in serum-supplemented growth media.

Acknowledgments

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RAPID GROWTH RESPONSE OF *VIBRIO VULNIFICUS* FOLLOWING SERUM - INDUCTION

by

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In preparation for *Current Microbiology*

Format adapted for dissertation

Abstract

Almost every year there are outbreaks of *Vibrio vulnificus* infections resulting from consumption of raw or undercooked shellfish, particularly raw oysters. Development of a method to rapidly detect this pathogen in oysters could help prevent such outbreaks. Culturing of this bacterium in medium supplemented with serum (10% vol/vol) significantly increases its growth rate. After only 2 h, a 3-log increase in viability was observed in serum-supplemented medium when compared with equivalent medium without serum. Addition of serum to medium used to enrich *V. vulnificus* could decrease the time necessary to increase cell densities, enabling more rapid and sensitive detection of this organism in oyster samples. Possible mechanisms for rapid growth when induce by serum were investigated. Fe^{2-} was found to be necessary for the increased growth rate, and differential expression of several proteins was observed in response to serum exposure. In addition, a degenerate oligonucleotide probe synthesized from the deduced amino acid sequence of a serum-induced protein isolated from the bacterium *Corynebacterium jeikeium* hybridized to purified *V. vulnificus* genomic DNA. This sequence information was used to design specific oligonucleotide primers for use with the TOPO® Walker kit to determine the identity of the gene encoding this protein. The DNA fragment generated in the DNA-walking experiment was cloned and sequenced. Sequence analysis suggests the gene is an aminotransferase.

Introduction

Vibrio vulnificus is a marine bacterium that can cause gastroenteritis, wound infection, and septicemia [16]. Gastroenteritis is caused by the ingestion of raw or under-

cooked seafood and is a self-limiting illness. Wound infection and septicemia can be caused by contact with marine water or by ingesting raw or undercooked seafood [14]. Septicemia is a serious and often fatal infection in individuals with a compromised immune system or underlying liver disease [17]. *V. vulnificus* is the probable leading cause of seafood-associated fatalities in the US [12]. Outbreaks of *V. vulnificus* infections due to shellfish consumption have a negative effect on the shellfish market. To help prevent such illnesses, it is important to develop a method for rapid detection of this pathogen in the marine environment. Conventional microbiological detection methods are laborious and time consuming, often requiring several days. The recommended most probable number method requires overnight growth followed by DNA-DNA hybridization. Increasing the growth rate of this microorganism would decrease the time necessary for its detection using conventional microbiological methods [6] as well as genetic based methods [3].

In this study, we have investigated the enhanced growth of *V. vulnificus* in media supplemented with serum. Also, we investigated the effects of physiologically important metal cations [11] on the growth of *V. vulnificus* in serum. The cations used in this study were iron, manganese, zinc, copper, calcium, and magnesium. It is well documented that the uptake of iron is associated with the pathogenicity of blood-borne microorganisms [15]. The metals manganese, zinc, and copper serve as micronutrients required in bacterial cells [4]. Calcium is involved in a variety of prokaryotic cellular processes, including cell cycle control and cell division [20], and Mg^{2+} is a cofactor in more than 300 enzymatic reactions involving energy metabolism and protein and nucleic acid synthesis [8].

A study using *Staphylococcus aureus* demonstrated that growth in serum was suitable as a model for infection in a host bloodstream [19]. Six biosynthesis genes, two surface proteins, one antimicrobial peptide, and one gene with unknown function were discovered to be induced by growth in serum [19]. The response was identical when using human or pig serum.

Possible genetic mechanisms for the rapid growth response of *V. vulnificus* were determined. The protein expression of *V. vulnificus* was compared for cells grown with or without serum. One protein overexpressed in response to serum was of the same molecular weight as a protein expressed in *Corynebacterium jeikeium* under the same conditions. This 36-kDa protein, CSIP-36, was isolated from *C. jeikeium* and partially sequenced. A degenerate oligonucleotide probe was made using this sequence to probe the *V. vulnificus* genome for the presence of the gene encoding this protein. The CSIP-36 sequence was also used to design degenerate nucleotide primers utilizing a bacterial codon usage table [13]. These primers were used for a DNA walking technique to determine the potential identity of the gene encoding the isolated protein.

Materials and Methods

Bacterial strains and microbiological media. *V. vulnificus* strain SPRC 10111 was used in this study. *V. vulnificus* was cultured in alkaline peptone water (10 g NaCl, 10 g peptone pH 8.5;) [1] or on marine agar plates (Difco, Detroit, MI, USA) [1].

Growth of *V. vulnificus* in APW vs. APW + 10% serum. In a 500-ml screw-cap Erlenmeyer flask, a 200-ml culture of *V. vulnificus* in APW was grown to an OD_{450}

of approximately 0.2. This culture was divided equally into two aliquots in 500-ml Erlenmeyer flasks. One aliquot contained 100 ml of the APW culture. The other contained 90 ml of the APW culture and 10ml of calf serum (Colorado Serum Company, Denver, CO, USA), making a 100-ml culture with 10% (vol/vol) serum. The cultures were incubated at 37°C on a rotary shaker at 170 rpm. OD_{450} readings and viable plate counts were used to monitor the growth of the serum and non-serum cultures in minimal medium. For viable plate counts, cells were serially diluted in APW to 10^{-10}, and 5 µl from each dilution were spot plated on MA plates. The number of viable colonies per ml of medium was calculated based on the number of individual colonies observed at the lowest dilution.

Use of serum for detection of *V. vulnificus* in oysters. Oyster homogenate artificially contaminated with *V. vulnificus* was pre-enriched in APW growth medium or APW medium supplemented with 10% serum. A direct plating procedure was used to determine the number of cells of *V. vulnificus* that could be detected after pre-enrichment in media with or without serum. Oyster homogenate was prepared by blending an equal weight of oyster meat and APW medium in a sterile blender jar. 1 ml of oyster homogenate was then added to 10 ml of APW pre-enrichment medium or 10 ml of APW with 10% serum and incubated at 37°C on a rotary shaker at 170 rpm. At 1-h time intervals, 100 µl from the incubated mixture was spread on T1N3 plates [1]. The plates were incubated overnight at 37°C, and colonies were transferred to Whatman no. 541 filter paper disks (Fisher, Hampton, NH, USA). 1 ml of lysis solution (0.5 M NaOH, 1.5 M NaCl) was placed in the center of a glass petri dish, and the filter paper disks were placed col-

ony side up over the solution. The petri dishes containing the wetted filters were placed in a microwave on high power for 30 s per filter to dry. Each filter was washed in 4 ml of 2 M ammonium acetate for 5 min. The ammonium acetate buffer was decanted, and the filters were washed two times in 10 ml of 1X standard saline citrate (SSC) (20X SSC is 175.4 g NaCl, 88.2 g sodium citrate in 1000 ml deionized water) solution for 2 min each time. The filters were then washed in 10 ml of 1X SSC and 20 µl of proteinase K (20 mg/ml) at 42°C for 30 min. The filters were then rinsed three times in 1X SSC for 10 min at room temperature. Filters were placed in 10 ml of hybridization buffer [0.5 g bovine serum albumin, 1 g sodiumdodecyl sulfate (SDS), 0.5 g Polyvinylpyrrolidone in 100 ml 5X SSC] for 30 min at 54°C. The hybridization buffer was removed, and 10 ml of fresh hybridization buffer were added along with 5 pmol of tlh probe (5' XAA AGC GGA TTA TGC AGA AGC ACT G 3', X = alkaline phosphatase-conjugated 5' amine-C6); the filters were hybridized for 1 h at 54°C. Filters were washed two times with 10 ml of 1X SSC/SDS (10 g SDS in 1 L 1X SSC) at 54°C, followed by five times with 10 ml of 1X SSC for 5 min at room temperature. The filters were developed in 20 ml of NBT/BCIP solution (Boehringer Manheim, Mannheim, Germany) for 2 h. Filters were washed three times in 10 ml deionized water for 10 min and were placed on absorbent paper in the dark to dry. Positive hybridization results are seen as bluish-gray or dark-brown colonies on the blot. These colonies are counted and expressed as colony-forming units.

Effect of iron (Fe^{2+}) on *V. vulnificus* growth in serum. Iron in serum was inactivated with the iron chelator 2,2-dipyridyl (Sigma, St. Louis, MO, USA). A 300-ml culture of *V. vulnificus* in APW medium supplemented with 10% (vol/vol) serum was grown

to an OD_{450} of approximately 0.2. Three aliquots of 100 ml each were separated into 500-ml screw-cap Erlenmeyer flasks. 2,2-dipyridyl was added to two of the flasks to a final concentration of 200 uM. Cultures were incubated at 37°C on a rotary shaker. Growth was monitored at an OD_{450} at various time intervals.

Effect of important divalent cations on *V. vulnificus* growth. A 700-ml culture of *V. vulnificus* in APW medium supplemented with 10% serum was grown to an OD_{450} of approximately 0.2. To chelate divalent and trivalent cations, ethylenediamine triacetic acid (EDTA) was added to the culture to a final concentration of 10 mM. The culture was dispensed into 100 ml aliquots and incubated at 37°C on a rotary shaker at 170 rpm for 2 h. The following divalent cations, Ca^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}, were added individually to separate flasks to a final concentration of 11 mM (1 mM free cation) to determine their influence on rate of growth. Incubation was at 37°C on a rotary shaker at 170 rpm, and absorbance was measured at 1 h intervals at OD_{450}.

Effect of iron (Fe^{2+}) on *V. vulnificus* growth in APW. A 200-ml culture of *V. vulnificus* in APW medium was grown to an OD_{450} of approximately 0.2. Two aliquots of 100 ml each were separated into 500-ml screw-cap Erlenmeyer flasks. Iron was added to one of the flasks to a final concentration of 1 mM. Cultures were incubated at 37°C on a rotary shaker. Growth was monitored at an OD_{450} at 1 h intervals.

Labeling of *V. vulnificus* serum-induced proteins. Cultures of *V. vulnificus* were grown to an OD_{450} of approximately 0.2 in artificial salt water minimal medium [30

ppt BIO-SEA® Marinemix (Aqua Craft®, Inc, USA) + 0.02% glucose] supplemented with 10% (vol/vol) calf serum or not supplemented, and total cellular proteins were labeled using ^{35}S-methionine (10μCi/μl; NEN Dupont, Boston, MA, USA). Following incubation, proteins were separated by sodiumdodecyl sulfate-polyacrylamide gel electrophoresis [2] and the gels dried in a gel dryer (Bio-Rad, Hercules, CA, USA). Kodak BIOMAX™ MR film was used for autoradiography, and films were developed in an AFP processor (AFP Imaging, Elmsford, NY, USA). The protein profiles of the cultures with serum were compared with each other and with the culture without serum.

Southern blot analysis. Genomic DNA was extracted and purified as described by Ausubel et al. [2]. Cells were suspended in 567 μl of TE buffer (10 mM of Tris·HCl pH 8.0, 1 mM of EDTA) with 30 μl of 10% (wt/vol) SDS and 3 μl of proteinase K (20 mg/ml; Sigma, St. Louis, MO, USA) and allowed to lyse for 1 h at 37°C. Next, 100 μl of 5 M NaCl and 80 μl of CTAB/NaCl were added and incubated for 10 min at 65°C. DNA was purified by extraction with chloroform-isoamyl alcohol (24:1), followed by extraction with phenol-chloroform-isoamyl alcohol (25:24:1). DNA was then precipitated with isopropanol, centrifuged for 5 min at 10,000*g*, washed with cold 70% (vol/vol) ethanol, and dried in a DNA SpeedVac (Savant, Holbrook, NY, USA). The dried DNA was resuspended in 25 μl of TE buffer, and the DNA concentration was measured with a Lambda II spectrophotometer (Perkin-Elmer, Wellesley, MA, USA) at a wavelength of 260 nm. *V. vulnificus* genomic DNA and human COT-1 ™ DNA (Invitrogen, Carlsbad, CA, USA) were subsequently treated with *Sau3A*I restriction endonuclease and separated in an agarose (0.9% wt/vol) gel. DNA was transferred to a Zeta-Probe™ (Bio-Rad, Hercu-

les, CA, USA) membrane by alkaline blotting using the capillary transfer method. The DNA blot was first prehybridized with 5 ml of ExpressHyb solution (Clontech, Palo Alto, CA, USA) for 30 min at 68°C and then hybridized in ExpressHyb solution at 68°C overnight with P-CSIP-36 probe labeled with BrightStar™ Psoralen-Biotin (Ambion, Austin, TX, USA). P-CSIP-36 is a degenerate probe designed from the partial amino acid sequence of a 36 kDa serum-induced protein in *C. jeikeium*. After hybridization, the membrane was washed in wash buffer (Ambion, Austin, TX, USA) and blocking buffer (Ambion, Austin, TX, USA) at room temperature as directed. Streptavidin-alkaline phosphatase and CDP-Star solutions were added to detect the labeled DNA. The membrane was blotted quickly on a piece of filter paper, wrapped in Saran wrap, and placed in an autoradiography cassette with CL-Xposure™ film (Pierce, Rockford, IL, USA) at room temperature for 5 min; the film was developed in an AFP processor (AFP Imaging, Elmsford, NY, USA).

Northern blot analysis. *V. vulnificus* was cultured in 40 ml APW medium to an OD_{450} of 0.2 in a 125-ml Erlenmeyer flask. The culture was then dispensed into two aliquots in 125-ml Erlenmeyer flasks. One aliquot contained 20 ml of the APW culture. The other contained 18 ml of the APW culture + 2 ml of calf serum, making a 20-ml culture with 10% serum. The cultures were incubated at 37°C, 170 rpm, for 4 h. Total RNA from 1.5 ml of each culture was extracted and purified using a NucleoSpin® RNA II Kit (Clontech, Palo Alto, CA, USA). Purified RNA was separated on a 1% agarose gel with formaldehyde [2] and was transferred to a Zeta-Probe™ (Bio-Rad, Hercules, CA, USA) membrane by alkaline blotting using the capillary transfer method. The RNA blot was

first prehybridized with 5 ml of ExpressHyb solution (Clontech, Palo Alto, CA, USA) for 30 min at 68°C and then hybridized in ExpressHyb solution at 68°C overnight with P-CSIP-36 probe labeled with BrightStar™ Psoralen-Biotin (Ambion, Austin, TX, USA). After hybridization, the membrane was washed two times for 5 min in wash buffer (Ambion, Austin, TX, USA) and two times for 5 min in blocking buffer, followed by one time for 30 min in blocking buffer at room temperature (Ambion, Austin, TX, USA). Streptavidin-alkaline phosphatase and CDP-Star (Ambion, Austin, TX, USA) solutions were added to detect the labeled probe. The membrane was blotted quickly on a piece of filter paper, wrapped in Saran wrap, and placed in an autoradiography cassette with CL-Xposure™ film (Pierce, Rockford, IL, USA) at room temperature for 5 min; the film was developed in an SRX-101A tabletop film processor (Konica, Wayne, NJ, USA).

Isolation and purification of *V. vulnificus* DNA. Genomic DNA was extracted and purified as described by Ausubel et al. [2]. Cells were suspended in 567 μl of TE buffer with 30 μl of 10% (wt/vol) SDS and 3 μl of proteinase K (20 mg/ml; Sigma, St. Louis, MO, USA) and allowed to lyse for 1 h at 37°C. Next, 100 μl of 5 M NaCl and 80 μl of CTAB/NaCl were added and incubated for 10 min at 65°C. DNA was purified by extraction with chloroform-isoamyl alcohol (24:1), followed by extraction with phenol-chloroform-isoamyl alcohol (25:24:1). DNA was then precipitated with isopropanol, centrifuged for 5 min at 10,000*g*, washed with cold 70% (vol/vol) ethanol, and dried in a DNA SpeedVac (Savant, Holbrook, NY, USA). The dried DNA was resuspended in 25 μl of TE buffer, and the DNA concentration was measured with a Lambda II spectrophotometer (Perkin-Elmer, Wellesley, MA, USA) at a wavelength of 260 nm.

DNA walking. The DNA walking procedure was performed using the TOPO® Walker kit (Invitrogen, Carlsbad, CA, USA). The partial amino acid sequence of CSIP-36 was converted to a degenerate nucleotide sequence using a bacterial codon usage table [13] (Table 1). Two degenerate gene specific primers, GSP1 and GSP2, were synthesized using these sequence data (Table 2). *V. vulnificus* purified genomic DNA was digested with *PstI* and dephosphorylated with calf intestinal phosphatase. Digested, dephosphorylated DNA was then extended using GSP1 in a 20-µl primer extension reaction consisting of 2 µl 10X PCR buffer, 1 µl 10-mM deoxynucleotide triphosphates, 50 ng DNA, 10 ng GSP1 primer, 2 units *Taq* polymerase, and water to 20 µl. The reaction was performed in a GeneAmp PCR System 2400 (Perkin-Elmer, Wellesley, MA, USA) with a 4 min denaturation at 94°C, followed by annealing for 1 min at 56°C and, finally, extension for 20 min at 72°C. After extension, TOPO® Linker was added to the 3'-end of the extended DNA, and PCR was performed using the LinkAmp primer and GSP2. The LinkAmp primer is complementary to the TOPO® Linker sequence and GSP2 is complementary to a specific site on the extended DNA just beyond GSP1.

Table 1. CSIP-36 partial amino acid sequence and deduced nucleotide sequence

Amino acid sequence	Deduced nucleotide sequence
APAG(CS)LGGL	gcg ccg gcg ggy wgc ttg ggy ggy ttg (y = c or t, w = t or a)

Table 2. Degenerate gene specific primers synthesized from deduced nucleotide sequence of CSIP-36

Name	Sequence	Tm value (°C)
GSP1	5'-gcg ccg gcg ggy wgc-3'	56-58
GSP2	5'-ggy wgc ttg ggy ggy ttg-3'	56-62

Detection and isolation of amplified DNA. All PCR-amplified DNAs were separated at a constant voltage of 5 V/cm in 1% (wt/vol) SeaKem agarose (FMC Bioproducts, Rockland, ME, USA) with 1X TAE (40 mM of Tris·HCl pH 8.0, 1.18 ml of acetic acid, 2 mM of Na_2EDTA per liter) [2]. The separated DNA fragments in the gel were stained with 2X 10^{-4} μg/ml of ethidium bromide and visualized on a FotoPrep I (Fotodyne, Inc., Hartland, WI, USA) ultraviolet transilluminator. The amplified DNA bands were photographed with Polaroid Type 55 film. The desired PCR product was isolated from the gel and purified using the Nucleotrap Gel Extraction Kit (Clontech, Palo Alto, CA, USA).

Cloning and sequencing of PCR product from TOPO® Walker kit. The amplified product was cloned by using pCR 4.0 and Topo TA cloning (Invitrogen). Positive transformants were selected on Luria broth agar plates supplemented with kanamycin (50 μg/ml) antibiotic, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and isopropyl-thiogalactoside as described in the kit protocol. DNA was extracted and purified by using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA). Purified DNA was subsequently treated with *Eco*RI and visualized in a 1% (wt/vol) agarose gel for analysis of the cloned fragments. A clone with the appropriately sized insert was sequenced at the University of Alabama at Birmingham core sequencing facility by using T7 and T3 primers in an ABI Prism automated DNA sequencer (Perkin-Elmer).

Analysis of sequence data. The DNA sequence data were converted to an amino acid sequence using DNASTAR™ computer software, and both sequences were com-

pared with existing sequences in the National Center for Biotechnology Information GenBank database maintained by the National Institutes of Health. Cn3D computer software was used to predict the three-dimensional structure of the amino acid sequence. This free software is available on the National Center for Biotechnology Information website. Phylogenetic analysis was performed using DNASTAR™ computer software.

Results

APW vs. APW + 10% serum. The growth rate of *V. vulnificus* in APW supplemented with 10% serum is significantly greater than growth in APW alone. The cell density of the serum-supplemented culture doubled that of the culture without serum after 2 h and is more than three times greater after 3-4 h (Fig. 1). After 2 h, a 3-log increase in viability was observed in serum-supplemented medium when compared with the same medium without serum (Fig. 2).

Use of serum for detection of *V. vulnificus* in oysters. Addition of serum to medium used for enrichment of *V. vulnificus* in oysters increases the growth rate by 2 log in 4 h. The 2-log increase in growth is maintained through 7 h (Fig. 3).

Effect of iron (Fe^{2+}) on *V. vulnificus* growth in serum. Blocking the iron available in serum with the iron chelator 2,2-dipyridyl reduces the growth rate of *V. vulnificus* to a level comparable with the growth rate in medium without serum (Fig. 4).

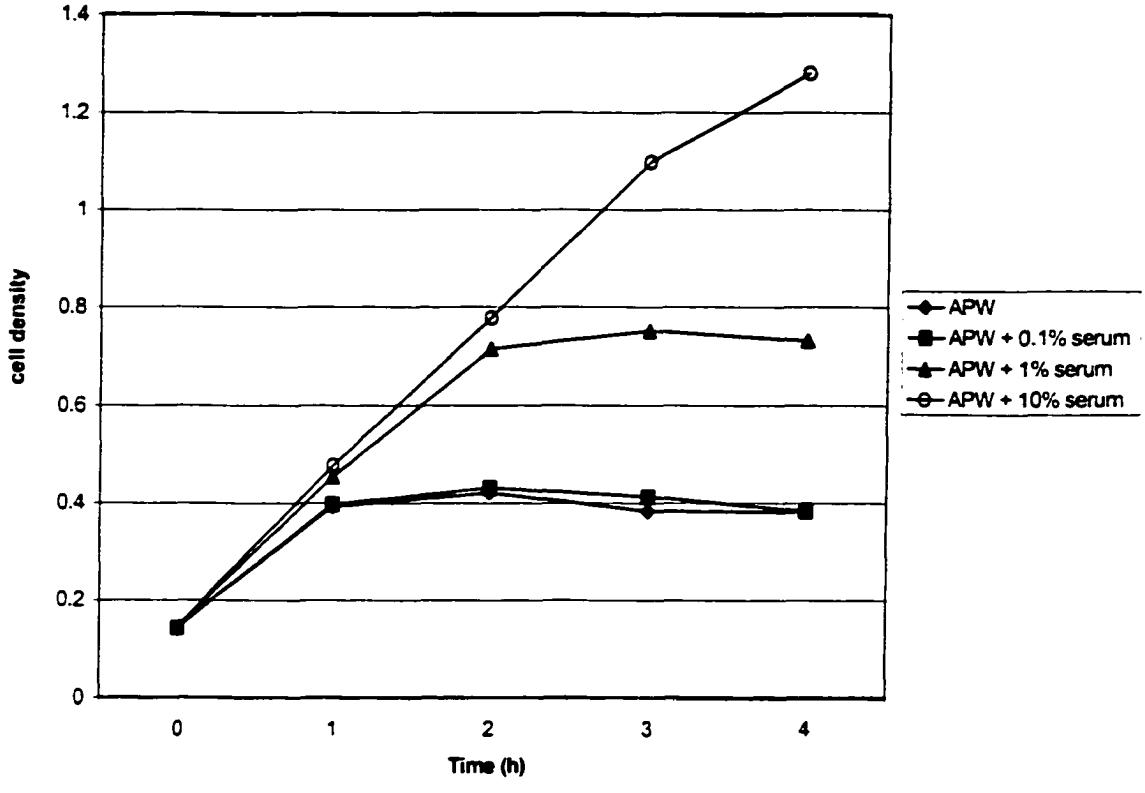

Fig. 1. *V. vulnificus* OD_{450} readings in APW media and APW media supplemented with serum. Each data point is an average of three readings. Standard error for each data point ranges from ± 0.004 to 0.010.

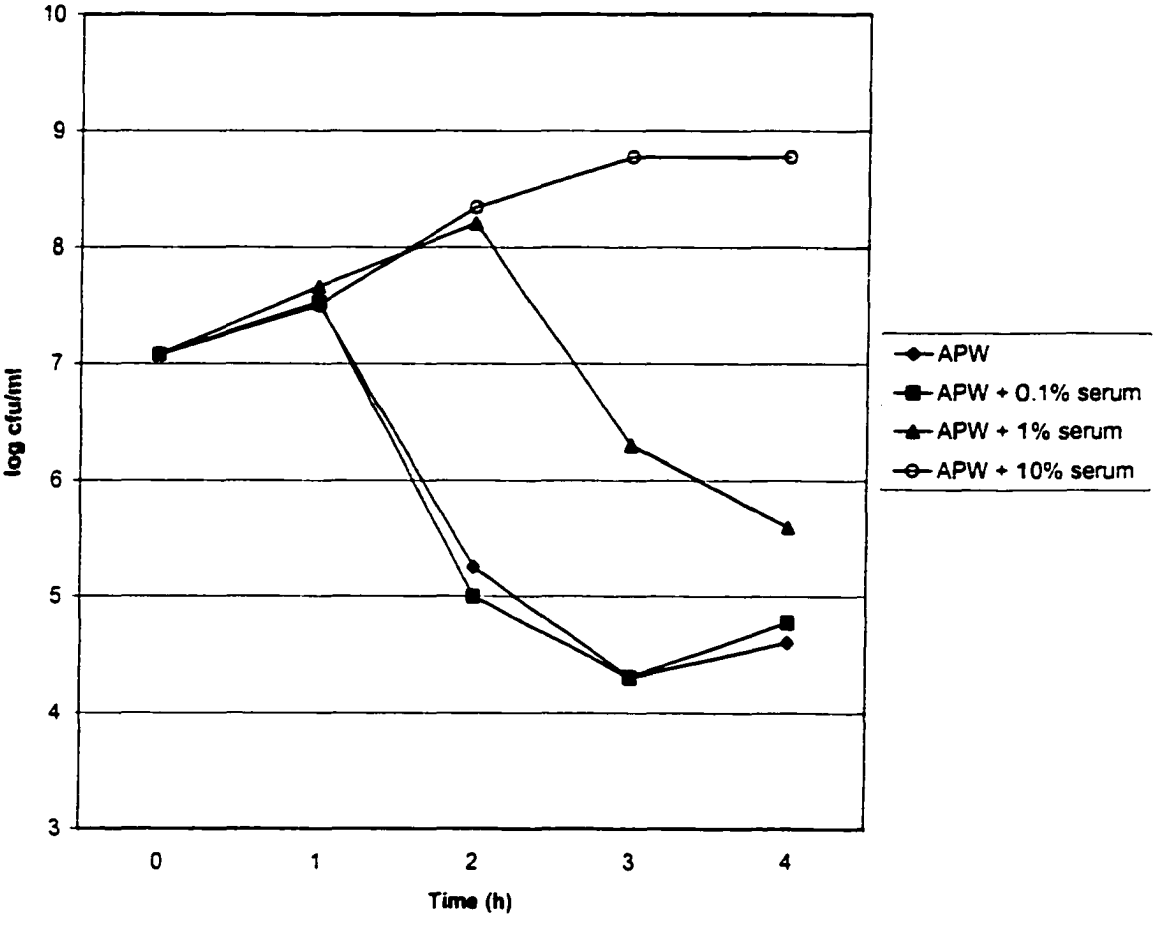

Fig. 2. *V. vulnificus* viable plate counts of growth in APW media and APW media supplemented with serum. Each data point is an average of three readings. Standard error for each data point ranges from ± 0.004 to 0.010.

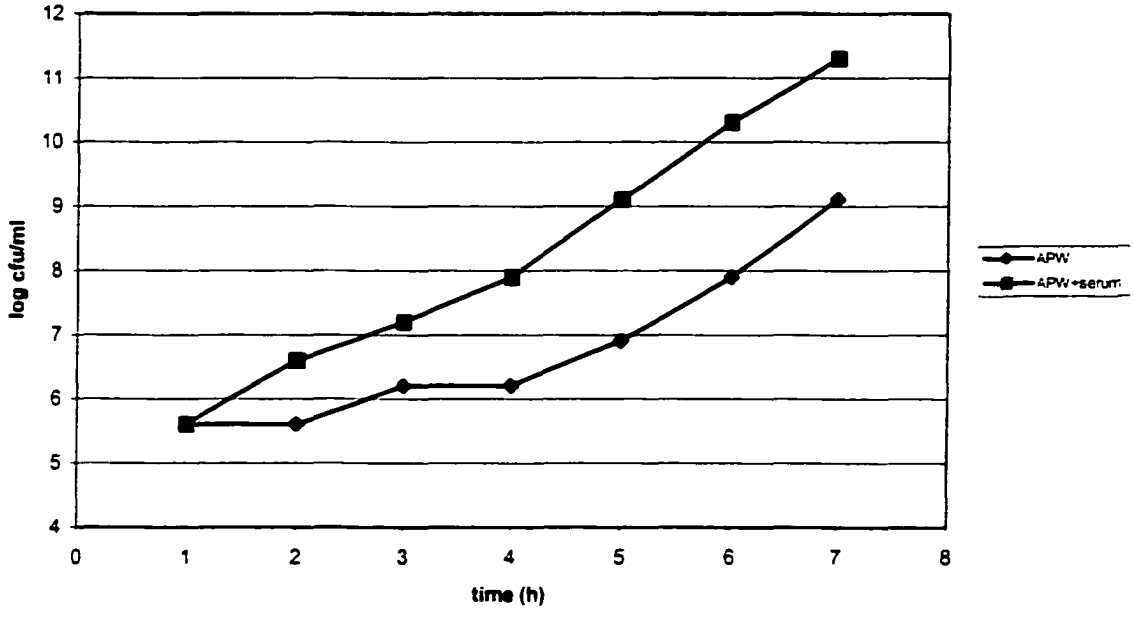

Fig. 3. *V. vulnificus* viable plate counts from artificially contaminated oysters following growth in APW pre-enrichment media supplemented with serum. Each data point is an average of three readings. Standard error for each data point ranges from ± 0.004 to 0.010.

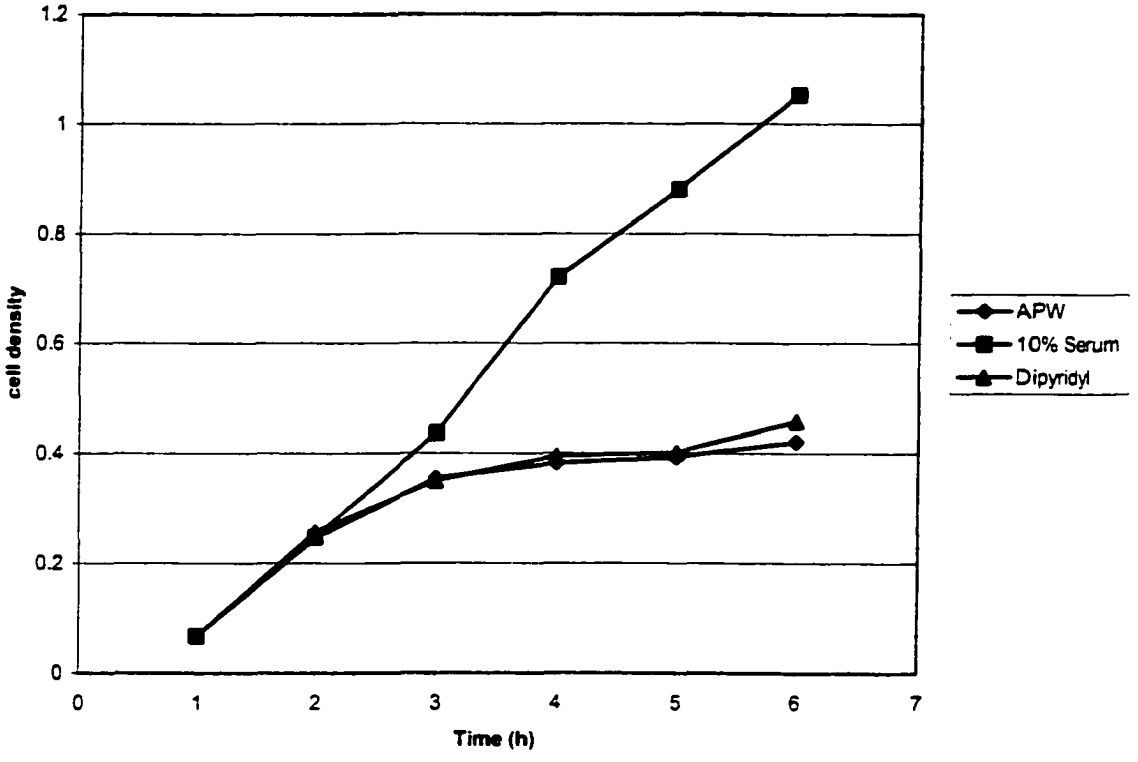

Fig. 4. Effect of the iron chelator dipyridyl on the growth of *V. vulnificus* in APW medium + 10% serum. Each data point is an average of three readings. Standard error for each data point ranges from ± 0.004 to 0.010.

Effect of nutritionally important metal cations on growth of *V. vulnificus* in 10% serum. Addition of EDTA to a culture of *V. vulnificus* in 10% serum effectively reduces the enhanced growth normally observed. Addition of excess Ca^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, or Zn^{2+} to such a culture has no effect on *V. vulnificus* growth. However, addition of Fe^{2+} to the EDTA-treated culture restores growth to a level comparable with the growth observed in the absence of EDTA (Fig. 5).

Effect of iron (Fe^{2+}) on *V. vulnificus* growth in APW. Addition of excess iron appears to have no effect on the growth rate of *V. vulnificus* grown in APW medium without serum (Fig. 6).

Serum-induced protein expression. Several proteins were differentially expressed in response to treatment with 10% serum. A protein with a molecular weight of approximately 36 kDa is clearly overexpressed in the serum-treated cultures. Comparison of the protein profiles of cultures with and without serum is found in Fig. 7.

Southern blot analysis. Positive DNA-DNA hybridization signals were noticed on the autoradiogram for *V. vulnificus* genomic DNA and the PCSIP-36 oligonucleotide probe (Fig. 8). There was no hybridization to the human COT-1™ DNA.

Northern blot analysis. A positive hybridization signal was apparent between probe PCSIP-36 and RNA isolated from *V. vulnificus* grown in medium supplemented

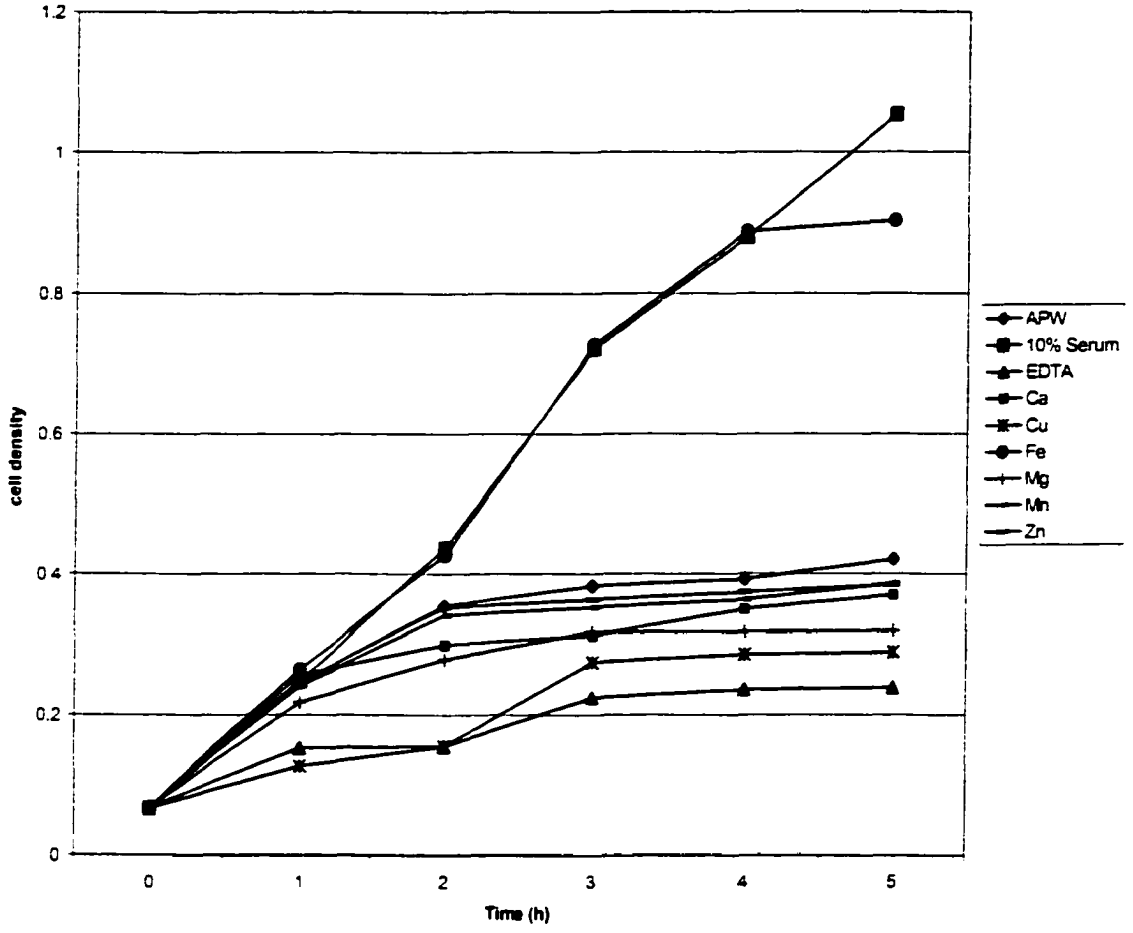

Fig. 5. Effect of metal cations on the growth of *V. vulnificus* in APW medium + 10% serum. Each data point is an average of three readings. Standard error for each data point ranges from ± 0.004 to 0.010.

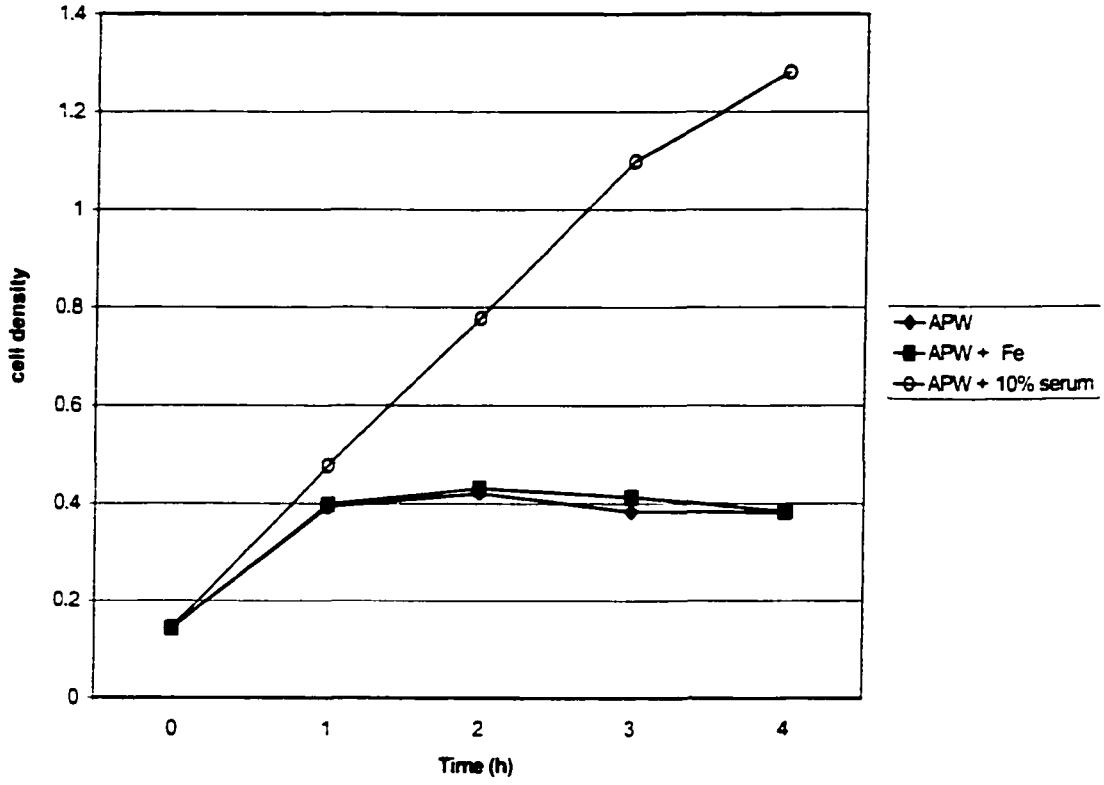

Fig. 6. Effect of iron on the growth of *V. vulnificus* in APW medium. Each data point is an average of three readings. Standard error for each data point ranges from ± 0.004 to 0.010.

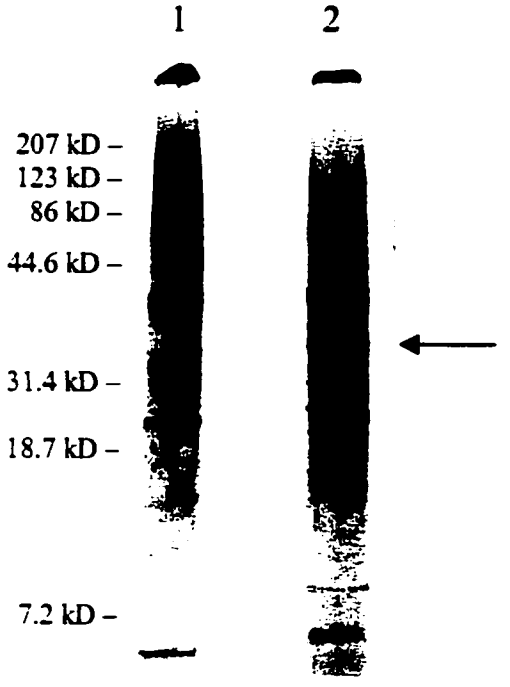

Fig. 7. Autoradiogram of ^{35}S methionine-labeled proteins in *V. vulnificus*. Lane 1, *V. vulnificus* in M9 medium; lane 2, *V. vulnificus* in M9 medium supplemented with 10% serum. An arrow indicates a differentially expressed protein with an approximate molecular weight of 36 kDa.

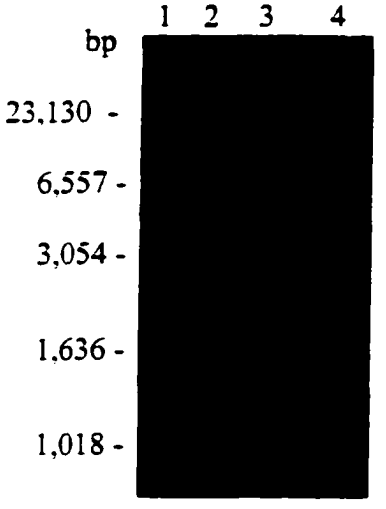

Fig. 8. Autoradiogram of Southern blot hybridization of probe PCSIP-36 to purified genomic DNA from *V. vulnificus*. Lane 1, λ DNA/*Hind*III size standard. Lane 2, 1 Kb DNA ladder. Lane 3, human COT-1™ DNA. Lane 4, *V. vulnificus* genomic DNA.

with 10% serum. There was no hybridization between the probe and RNA isolated from *V. vulnificus* grown in APW medium alone (Fig. 9).

DNA walking and analysis of DNA sequence data. A 636-bp PCR product was generated using the TOPO® Walker kit (Invitrogen) (Fig. 10). This product was isolated and purified from the gel and subsequently cloned and sequenced. The extended and amplified DNA sequence obtained using the TOPO® Walker kit (Invitrogen) was converted to an amino acid sequence using DNASTAR™ software. GenBank analysis of the deduced amino acid sequence suggests the sequence is an aminotransferase. The sequence has specific conserved domains that are consistent with other known aminotransferase proteins (Fig. 11). The predicted tertiary structure of the sequenced protein is shown in Fig. 12. This sequence was compared with the major types of aminotransferases and was determined to be most closely related to type I and type II (Fig. 13).

Discussion

The growth rate of *V. vulnificus* in medium supplemented with serum is significantly more rapid than the rate at which this microorganism grows when cultured without serum. Thus, se- rum could possibly be used to reduce the time necessary for *V. vulnificus* detection in shellfish. The current method suggested by the Food and Drug Administration for the detection of *V. vulnificus* in shellfish involves a pre-enrichment step in growth media for 5-6 h [9]. It was determined that cells grown in enriched medium supplemented with serum for 2 h had a higher cell density than cells grown in the same media without serum for 5 h. If serum was added to the pre-enrichment media, the time re-

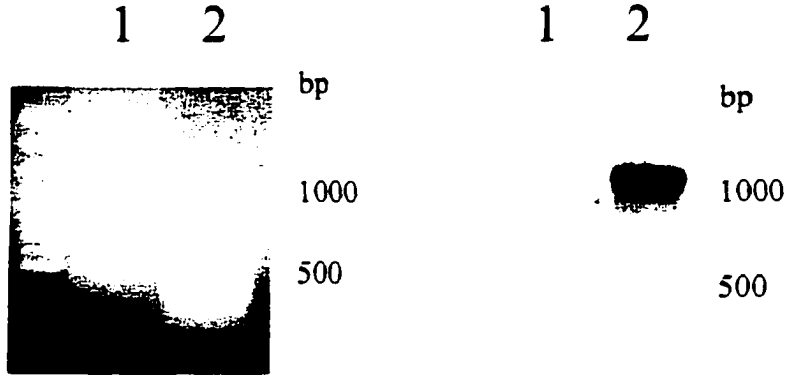

Fig. 9. Northern blot analysis using probe PCSIP-36 and *V. vulnificus* mRNA. Lane 1, RNA from *V. vulnificus* grown in APW. Lane 2, RNA from *V. vulnificus* grown in APW + 10% serum.

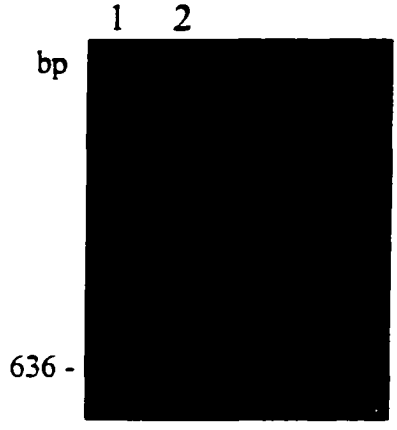

Fig. 10. Results from DNA walking procedure. Lane 1, Clone-Sizer DNA ladder. Lane 2, 636-bp PCR product generated using the TOPO® Walker kit.

*GSLGGL*PDEQTFPIELMKPTLEKLSEMPQVFQYGATA
GYAPLLNFLKTYMSLPETHMAMACTGSQQGLDLIAR
AYINPGDTVVMEAPSYLGAMQVFGLVSANIVTVSQT
EAGPNLDELEACFKQHSPKMFYAVPDFHNPTGVCWS
LETRKQVAKLCIEHKVAFIEDAPYRELRFXVKRCRW
FLISARKIRLXFVRFQRSXXXVYALVSYP

Fig. 11. Deduced amino acid sequence of the putative aminotransferase in *V. vulnificus*. Italics indicate the location of GSP2. Conserved domains that are consistent with all known type I and II aminotransferase proteins are indicated by underlined colored text. Red text indicates identical amino acid residues. Maroon text indicates a conserved substitution with one other amino acid residue. Purple text indicates a conserved substitution with two other amino acid residues. Blue text indicates a conserved substitution with three other amino acid residues.

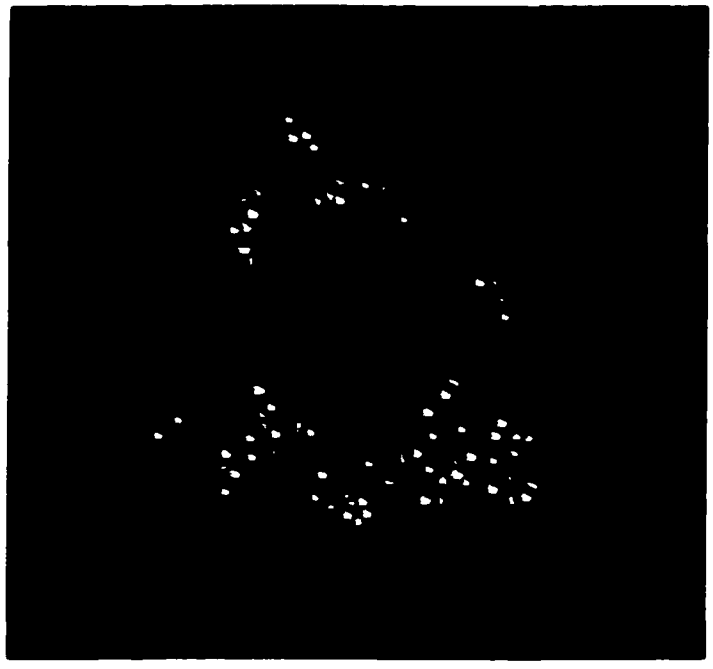

Fig. 12. Predicted three-dimensional structure of the putative aminotransferase from *V. vulnificus*. The amino acid residues shown in red are identical to all known types I and II aminotransferases. Maroon text indicates a conserved substitution with one other amino acid residue. Purple text indicates a conserved substitution with two other amino acid residues. Blue text indicates a conserved substitution with three other amino acid residues.

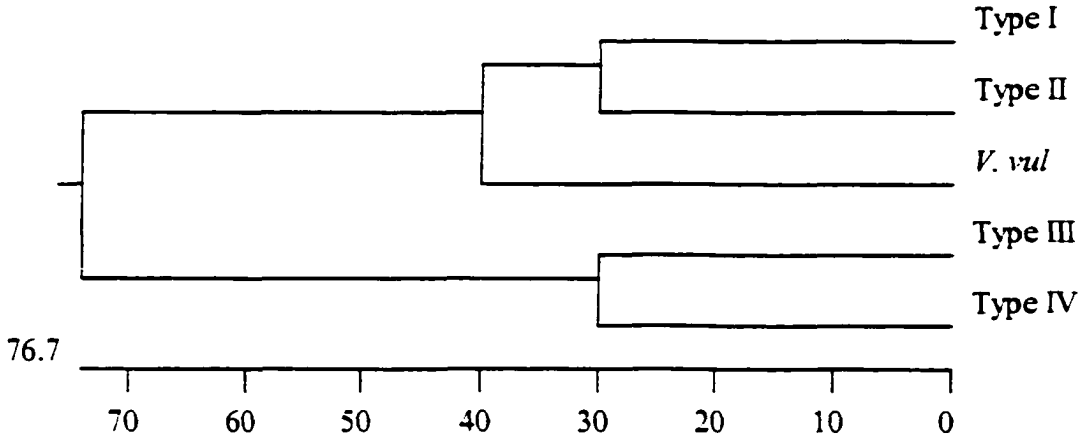

Fig. 13. Phylogenetic tree of the major types of aminotransferase proteins and the putative aminotransferase from *V. vulnificus*. Relatedness was determined using Clustal method with weighted residue weight table.

quired to culture a detectable amount of cells could be reduced by 3-4 h. The use of serum in the rapid detection of *V. vulnificus* could lead to fewer incidences of illness and death as a result of this pathogen's consumption in shellfish. Quicker detection would lead to quicker reporting and would reduce the chances of contaminated oysters being consumed.

Fe^{2+} is necessary for the rapid growth response of *V. vulnificus* in serum. *V. vulnificus* is able to sequester iron from the host blood stream using the siderophore vulnibactin [7]. It is known that Fe^{2+} plays a role in the virulence of *V. vulnificus* in blood-borne infections [10, 18], and we have found that Fe^{2+} is essential for rapid growth of *V. vulnificus* in serum. To survive and grow in a host's bloodstream, a microorganism needs to acquire nutrients and have a resistance to the host's immune system. Most nutrients are freely available in the bloodstream, but not iron [15].

Several proteins are differentially expressed in *V. vulnificus* in response to serum, suggesting there are serum-inducible genes in this pathogen that are likely involved in the rapid growth response. One of these proteins, CSIP-36, is of the same molecular weight as a protein expressed in *C. jeikeium* following serum treatment; therefore, it is likely that a similar gene is expressed in response to serum in these two pathogens and, possibly, in other bacteria. Sequence data from CSIP-36 were used to design an oligonucleotide DNA probe, PCSIP-36. DNA-DNA hybridization of probe PCSIP-36 to *V. vulnificus* genomic DNA provides further evidence for the presence of a similar serum-inducible gene in these microorganisms.

The deduced nucleotide sequence of CSIP-36 was used for DNA walking in an attempt to identify the gene encoding this protein. Based on sequence analysis, this protein,

which is overexpressed in response to serum exposure, is a putative aminotransferase. Aminotransferases are involved in the biosynthesis of most of the amino acids. There are four major classes of aminotransferases identified in bacteria: types I, II, III, and IV. Phylogenetic analysis reveals that the putative aminotransferase gene from *V. vulnificus* is most closely related to types I and II. *V. vulnificus* grows rapidly when exposed to serum; such rapid growth would involve the biosynthesis of amino acids.

Bacteria employ several methods to help resist a host's natural defenses. Some bacteria produce and secrete bioactive compounds that disturb the immune response by blocking important protein-protein interactions involved in the complement cascade. For example, *Streptomyces lavendulae* produces a cyclic peptide called complestatin that antagonizes the formation of the C4b, 2b complex in the complement cascade [5]. Genes involved in the biosynthesis and regulation of complestatin were characterized, and it was determined an aminotransferase is involved in the biosynthesis of this anticomplement protein. It is possible the aminotransferase discovered in *V. vulnificus* could be involved in the synthesis of a similar type of bioactive compound. Further study is necessary to determine whether the serum-induced aminotransferase in *V. vulnificus* has a similar function. It is possible that the putative aminotransferase in *V. vulnificus* plays a role in its growth in blood or, perhaps, in helping to protect this pathogen from the host's immune response.

Acknowledgments

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CONCLUSIONS

Septicemia is a systemic disease in which microorganisms rapidly multiply in the blood. This serious medical condition affects thousands of people a year. Surprisingly, there is little information about the response of bacteria when they come into contact with blood. Only a few studies have been done on the effects of blood or blood serum on bacteria. In this study, we found *C. jeikeium* and *V. vulnificus* display an enhanced growth rate when exposed to serum-supplemented media. The enhanced growth rate of *V. vulnificus* and *C. jeikeium* during exposure to serum could possibly indicate how these pathogens respond upon exposure to a host's bloodstream. The ability to grow rapidly in serum is a likely factor in causing septicemia. In addition, the enhanced growth rate of *V. vulnificus* in medium supplemented with serum could be used to reduce the time necessary for *V. vulnificus* detection in shellfish. The current method suggested by the Food and Drug Administration for the detection of *V. vulnificus* in shellfish involves a pre-enrichment step in growth media for 5-6 h [20]. It was determined that cells grown in enriched media supplemented with serum for 2 h had a higher cell density than cells grown in the same media without serum for 5 h. If serum was added to the pre-enrichment media, the time required to culture a detectable amount of cells could be reduced by 2-3 h. The use of serum in the rapid detection of *V. vulnificus* could lead to fewer incidences of illness and death as a result of this pathogen's consumption in shellfish.

It was determined that Fe^{2+} is necessary for the rapid growth response of *V. vulnificus* and that Mg^{2+} is necessary for the rapid growth response of *C. jeikeium* in serum. To

survive and grow in a host's bloodstream, a microorganism needs to acquire nutrients and have a resistance to the host's immune system. Most nutrients are freely available in the bloodstream except for iron [56]. It is known that Fe^{2+} plays a role in the virulence of *V. vulnificus* in blood-borne infections [42, 63], and we have found that Fe^{2+} is an essential factor for rapid growth of *V. vulnificus* in serum. Mg^{2+} is essential for the growth and maintenance of cells and is the regulatory signal for the *phoP/phoQ* two-component signal transduction system. The *phoP/phoQ* system has been shown to regulate over 40 genes in *S. typhimurium*, including genes required for virulence and survival in a host [23]. The virulence factors for *C. jeikeium* and how this pathogen resists host defenses remain unknown. It is possible that there is an Mg^{2+}-dependent system involved in infection and persistence in the host bloodstream in *C. jeikeium* that is similar to that found in *S. typhimurium*.

It could appear that increased growth rate is simply a result of added nutrients from serum. However, not all bacteria grow rapidly in serum. For instance, in *V. parahaemolyticus*, growth is actually slightly inhibited in serum-supplemented medium. *V. parahaemolyticus* causes disease in humans but, unlike *V. vulnificus* and *C. jeikeium*, has not been implicated in causing septicemia. Not all human pathogens are able to infect the blood, and there may be a correlation between the ability of these pathogens to cause septicemia and their ability to respond to serum. It is possible that common features in blood-borne pathogens have evolved, allowing these microorganisms to proliferate in the presence of serum and, thus, cause septicemia. The serum-responsive aminotransferase we discovered could be one of these significant evolutionary features,

because the aminotransferase was found in both of these diverse microorganisms. It is possible a similar protein is expressed in response to serum in other blood-borne bacteria.

Aminotransferases are involved in the biosynthesis of most of the amino acids. In a general transamination reaction, any amino acid and and keto acid exchange the amino group. Aminotransferases require the coenzyme pyridoxal-5'-phosphate (PLP) for transaminase reactions. PLP is a versatile enzyme cofactor that can be utilized by various enzymes to catalyze many distinct chemical reactions. Reactions catalyzed by PLP-requiring enzymes include transamination, decarboxylation, and general cleavage reactions [46]. Because aminotransferase enzymes catalyze diverse reactions, it is not surprising that the greatest structural similarities involve residues that participate in PLP binding rather than residues that participate in substrate binding. Four major aminotransferases have been identified in *E. coli*: types I, II, III, and IV. These aminotransferases are each involved in the synthesis of three to six different amino acids. There is some redundancy in the amino acids synthesized by the different types of aminotransferases. For instance, phenylalanine can be synthesized by three of the four types of aminotransferase enzymes. Phylogenetic analysis reveals that the putative aminotransferase gene from *V. vulnificus* is most closely related to type I and type II bacterial aminotransferases. This aminotransferase, which is expressed during serum exposure, has not been previously identified; other than specific conserved domains, it has unique protein and DNA sequences. This feature is common in bacterial aminotransferases, which share little similarity in their overall DNA and protein sequences. The gene encoding the CSIP-36 protein is predicted to be between 1 and 1.2

kb based on Northern analysis. This is consistent with the typical size of bacterial type I and type II aminotransferase genes, which range from 1 to 1.4 kb.

The results of this pioneering study provide several directions for continued research in this largely unknown area of bacterial response to serum. Future research will include the study of *V. vulnificus* and *C. jeikeium* in human serum. The growth rate and protein expression of these blood-borne pathogens in human serum should be compared with the results obtained using calf serum. We observed that several proteins are overexpressed in *V. vulnificus* and *C. jeikeium* when exposed to serum and have identified one of these proteins as a novel aminotransferase. Two-dimensional gel electrophoresis provides better separation of cellular proteins and would likely reveal more proteins differentially expressed in serum; therefore, this could allow identification of other genes involved in the response of *V. vulnificus* and *C. jeikeium* to serum. The novel aminotransferase overexpressed in *V. vulnificus* and *C. jeikeium* in serum-supplemented medium can be further characterized, by cloning and sequencing the entire open reading frame and regulatory regions of this gene. Identification of the regulatory region of this gene could provide information on how this gene is turned on in response to serum. Cloning the aminotransferase gene would allow study of its function. The cloned gene could be expressed in a host bacterial cell, and the protein product could be harvested and used for antibody production. This antibody would be used for Western blot analysis to study the quantitative expression of CSIP-36. Mutagenesis studies can determine the function of the *csip36* gene by creation of a knockout mutant strain, by using a chloramphenicol gene (*cm*) cassette to disrupt the *csip36* gene on the *V. vulnificus* chromosome. The *csip36* knockout mutant strain of *C. jeikeium* would then be compared

with the wild-type strain for growth in medium supplemented with serum. Further study of blood-borne bacteria in serum should provide helpful insights into the mechanisms of virulence and infection by these pathogens.

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