Isolation, characterization and possible biocontrol application of Bdellovibrionaceae (BD) isolated from NZ sources

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Abstract

Bdellovibrionaceae (BD) are unique, predatory, endoparasitic, Gram-negative bacteria. As the world's smallest living hunter they prey on other Gram-negative bacteria giving them potential as biological control agents. Prior to this study, however, there were no reports of BD in New Zealand. The overall aim of this research was to isolate BD from New Zealand sources, characterise them and investigate their potential role as a biological control agent. The history, characteristics, life cycle and mechanism of predation of this organism are reviewed and the possibility of the industrial applications of BD, are discussed.

In this study, a halophilic species of BD was isolated from fourteen coastal sea water sites around New Zealand. Thirteen isolates were characterised using proven characterisation techniques including general, microscopic and molecular techniques. It was found that the isolates were taxonomically identical or very closely related to each other and belong to the genus *Bacteriovorax*.

The predation pattern of BD isolates was examined against a group of Gram negative bacteria in solid and liquid media. The predation patterns and efficiencies of the different BD isolates were similar, which confirms that the BD isolates are closely related, are selective in their predation, and prey on some Gram-negative bacteria but not all.

The rapid loss of culture viability of BD is well known, but no studies have been reported to date on the survival of pure cultures of BD at different temperatures. The survival rate of BD in dense suspensions at different temperatures without host bacteria was investigated and it was observed that pure BD cultures can be stored with minimal reduction in numbers at temperatures ranging from 4°C to 20°C. However, significant reductions in numbers were observed at -18°C, 30°C and 37°C after 13 to 16 days.

The effects of the 13 New Zealand BD isolates on the growth of a population of *Photobacterium phosphoreum* were examined to select the best isolate for *in vitro* application. All of the isolates tested had considerable reduction effect against *P. phosphoreum*. Some isolates were more

effective than others, despite their taxonomic similarity to each other. The isolate OT2 was selected for further studies based on these results.

The *in vitro* efficacy of BD was assessed against late exponential cultures of a seafood spoilage bacterium, *P. phosphoreum*, originally isolated from Cod fillets from Denmark. Log_{10} reductions of *P. phosphoreum* and some other Gram–negative bacteria ranged from 4.5 to 4.8 after 9 h of incubation at 25°C. BD was effective in reducing the numbers of *P. phosphoreum* at pH 5.5 to 8.5 and salinity 0.9 to 4.5% (w/v). A significant interaction was observed between the prey and predator concentrations and nutrient concentration. Prey concentrations were observed to be the most vital factor in predation and the most favourable predation conditions were at a prey concentration of ~8 log_{10} colony forming units (CFU)/mL, together with a predator concentration of 3 – 7 log_{10} plaque forming units (PFU)/mL and a prey : predator ratio of >5.0. The thresholds of the prey and predator concentrations for predation were observed to be 3.7 log_{10} CFU/mL and 3.9 log_{10} PFU/mL, respectively. The trials carried out in this study focused on the efficiency of BD on a pure culture of one organism, *P. phosphoreum* and not on mixed cultures of Gram-negative spoilage bacteria, the normal condition observed in saltwater fish. There has been very little research in this field and the results of these trials suggest further investigation into the effect of BD on mixed cultures of Gram-negative spoilage organisms is warranted. Since only one isolate of BD (OT2) was examined against only one spoilage bacterium (*P. phosphoreum*) in liquid medium, the evidence of these findings must be restricted to these particular conditions. Future studies, using a range of BD isolates against a mixture of spoilage and pathogenic organisms in solid medium are warranted.

The biopreservation capability of BD in extending the shelf life of king salmon was evaluated. A significant effect was observed at 20°C but not at 10°C. At 20°C the shelf life was extended through extension of the lag phase of growth of the prey bacteria and a reduction in total numbers attained. Sensory evaluation of the salmon product being tested confirmed that the shelf life was extended. However, at 10°C there was no reduction in prey organisms, which suggested that the strain of BD used is ineffective at refrigeration temperatures.

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Chapter 1

General Introduction

1.1 General review of literature

1.1.1 Early history of Bdellovibrionaceae

The unique, predatory, endoparasitic, Gram-negative bacterial family Bdellovibrionaceae (BD) are the world's smallest living hunters: they prey upon other Gram-negative bacteria, which makes them an interesting family of organism. In 1962, while attempting to isolate bacteriophage from soil samples, Stolp and Petzold accidentally noted a number of odd plaques. Instead of taking just hours to appear, they took several days to develop and continued to grow for more than a week. They termed this bacterium *Bdellovibrio bacteriovorus* as a single genus and species (Stolp and Starr, 1963). The name *Bdellovibrio* comes from the Latin, '*bdella,*' which means leach-like, and *vibrio*, which means curved bacteria. The morphology, physiology and developmental cycle of BD have been studied in great detail (Stolp and Starr, 1963; Shilo, 1969; Abram and Davis, 1970; Starr and Seidler, 1971). However, knowledge of their ecology and taxonomic classification is limited and often controversial.

1.1.2 Morphology of Bdellovibrionaceae

BD was suggested by Stolp and Starr (1963) to be in the order Pseudomonadales, which are considerably narrower than ordinary bacteria, but the organism is now classified in the order of Bdellovibrionales (Williams *et al.*, 2003). Their electron microscopic observations revealed that this group of bacteria are small, curved, clearly vibrio-shaped, about 0.2 μm in length and 0.35 μm in width, possessing a long, unusually thick, single, sheathed polar flagellum approximately 50 nm in length.

BD colonies do not have any distinctive morphological features and thus are usually characterised by microscopic examination. Several electron microscopic studies have

described their structural properties, intraperiplasmic invasion and features of their flagellum (Seidler and Starr, 1968; Abram and Davis, 1970; Thomashow and Rittenberg, 1985). Seidler and Starr (1968) described the *Bdellovibrio* flagellum as about 28 nm in thickness and uniform in width, consisting of an inner core (13 nm) and a surrounding sheath (7.5 nm) originating from the cell wall and being continuous with the core. The flagellar sheath has been observed by various investigators to be a continuous extension of the outer membrane of the cell wall (Abram and Shilo, 1967; Thomashow and Rittenberg, 1985) and the composition is similar to that of the outer membrane. The flagellum of BD exhibits two unique morphologies: either a distinctive damping wave with typical decreasing amplitude and wavelength from the cell outward, or they appear relaxed with no regular waves (Abram and Shilo, 1967). The entire flagellum is lost during the attacking phase after its attachment to a susceptible host.

BD exhibit a typical Gram-negative morphology and consist of an outer membrane surrounding a thin peptidoglycan layer. The chemical compositions of both the outer membrane and peptidoglycan are similar to those of most of the Gram-negative bacteria except for the presence of sphingophosphonolipids (SPNLs) in their cell envelope. These comprise a large proportion of the membrane lipids (Steiner *et al.*, 1973) but their functions are not known (Williams *et al.*, 2003). Steiner *et al.* (1973) reported the presence of three SPNLs in *B. stolpii* strain Uki2. Jayasimhulu *et al.* (2007) confirmed the presence of 18 molecular species of *B. stolpii* Uki2 SPNLs, which were phosphonyl in nature.

Starr and Baigent (1966) examined the internal structure of BD by transmission electron microscopy (TEM). They found that the cell wall consists of two electron dense layers separated by a middle, lighter layer, whereas the cell wall of typical Gram-negative bacteria consists of three electron dense layers interspersed with two lighter layers. Abram and Davis (1970) studied the structural properties of the cell wall using electron microscopy and confirmed the earlier report of Abram and Shilo (1967) that the presence of several unique organelles may have a bearing on its parasitic nature, and provided additional criteria for their identification. The pole, which attaches to the prey cell, has a

characteristic shape. Electron microscopic examination of some negatively-stained specimens has revealed several rigid, spike-like filaments, 45-55 Å in diameter and up to 0.8 μm in length arising from this pole. When present, these filaments are always an extension of the attachment pole, and it is thought that they may play a role in the attachment and penetration of the prey cell.

BD have a well defined nucleoplasm, surrounded by densely packed granules, which are presumably ribosomes (Starr and Baigent, 1966; Burnham *et al.*, 1968). These workers observed the presence of mesosomes in the anterior region of the organism but they did not characterise the functional significance of their presence. *Bdellovibrio* sp. strain W is not only capable of the typical alternation between an attack and growth phase, but can also form a third, resistant, multilayered resting stage within the bdelloplast, termed the bdellocyst (Burger *et al.*, 1968; Hoeniger *et al.*, 1972; Tudor and Conti, 1977). These investigators demonstrated increased resistance of these bdellocysts to unfavourable conditions, such as high temperature, desiccation and disruption.

1.1.3 Physiology of Bdellovibrionaceae

BD are mesophilic organisms with an optimal growth temperature of 20 to 30°C. Although some marine strains have been found to grow at temperatures as low as 6°C, other isolates grow at up to 37°C (Richardson, 1990). Some strains of BD can be converted to be host-independent (HI), or facultatively predacious forms, by growing them in a medium enriched with cell extracts (Seidler and Starr, 1969).

Little is known about the energy metabolism of BD. Hespell (1976) indicated that BD have a strict respiratory type of metabolism with oxygen as the terminal electron acceptor. They possess an electron transport chain with typical cytochrome components, and a functional citric acid cycle. The generation of adenosine triphosphate (ATP) by BD is accomplished mainly by oxidative phosphorylation during electron transport. They are obligatory aerobic organisms and oxygen is required for their motility, for attachment to their host, for the growth of the host independent (HI) strains, and for intraperiplasmic

development (Varon and Shilo, 1968; Seidler and Starr, 1969). Simpson and Robinson (1968) observed that azide and cyanide inhibited endogenous respiration of *B. bacteriovorus* strain 6-5-S, which contains cytochromes, enzymes of the tricarboxylic acid cycle, reduced nicotinamide adenine dinucleotide oxidase and low levels of some glycolytic enzymes. They suggested that strain 6-5-S obtains energy by oxidative phosphorylation during electron transport and by substrate-level phosphorylations of the glycolytic system.

Rittenberg and Shilo (1970) studied the early host damage in the infection cycle of BD and found that the respiratory apparatus of the host organism was non-functional shortly after the attack and growth of BD. They suggested that BD must generate their own energy during intraperiplasmic growth. Hespell *et al.* (1973) studied the oxidation rates, respiratory quotients (RQ) and the energy substrates for intraperiplasmic growth and showed that amino acids, derived from protein breakdown, served as a major energy source during intraperiplasmic growth of *Bdellovibrio* on its host. They observed that glutamate and synthetic and natural amino acid mixtures were oxidised by suspensions of free BD cells and the oxidation of these compounds largely inhibited the endogenous respiration of BD cell material, which prolonged the viability of free BD cells. They also found that the RQ of the BD cells changed from a value characteristic of the oxidation of glutamate or of a balanced amino acid mixture very shortly after the BD attack on its host. They further demonstrated that glutamate or a mixture of amino acids in the external environment contributed to the carbon dioxide produced by the BD cells growing intraperiplasmically.

1.1.4 Life cycle of Bdellovibrionaceae

Early researchers when observing their distinctive lifestyles sometimes described BD as either 'parasites' due to their close relationship with host bacteria or as 'predators' feeding on another living organism (Ruby, 1992). Due to the nature of their propagation, BD have a biphasic life cycle. They have a non-growing motile predatory phase called the 'attack phase' and a nonmotile intracellular reproductive phase called the 'growth

phase' or 'multiplication phase'. Different stages of the predatory developmental cycle of BD are shown in Fig. 1.1. The attack phase cells are small vibroid to rod-shaped, flagellated, do not exhibit chromosome replication or cell proliferation (Ruby, 1992). In this phase, the cells swim at high speed (capable of covering distances exceeding 160 µm/s) using a single sheathed polar flagellum with a characteristic dampened filament waveform (Thomashow and Rittenberg, 1985). Rendulic *et al.* (2004) and Lambert *et al.* (2006 a) detected six clusters of motility and flagellar synthesis genes together with six copies of flagellin genes (*fliC*) at four independent loci in both *B. bacteriovorus* 109J and *B. bacteriovorus* HD100. Lambert *et al.* (2006 b) reported that only one flagellar filament protein (flagellin) was necessary for flagellar assembly, motility and efficient predation while the other flagellins play a minor role in the structure of the flagellar filament. The mobility of BD cells enables it to collide with prey bacteria randomly and the use of chemotaxis for locating the prey cells varies between BD isolates. A group of researchers (Straley and Conti, 1974; LaMarre *et al.*, 1977; Straley and Conti, 1977; Straley *et al.*, 1979) studied the chemotaxis assay system of BD and concluded that at least some strains of BD locate their prey cells by means of chemotaxis. Straley *et al.* (1979) observed that *B. bacteriovorus* strain Uki2 exhibits a chemoattraction to soluble nutrients such as amino acids and other organic compounds, which probably increases the chances of a collision with prey (Williams *et al.*, 2003). Lambert *et al.* (2003) observed that disruption of the gene that encodes a single methyl-accepting chemotactic protein (MCP) decreased the predation efficiency of *B. bacteriovorus* 109J. Lambert *et al.* (2006 b) reviewed the works related to chemoattraction and suggested the bias motility of BD towards prey-rich areas possibly the biofilms. Recently, Chauhan and Williams (2006) investigated the chemotactic response of directly concentrated aquatic environmental samples against yeast extract, casamino acid, dextrose, succinate, pyruvate and concentrated cells of *V. parahaemolyticus*. They observed that the aquatic bacteria, which they identified as *Pseudoalteromonas* spp., *Marinomonas* spp., *Bdellovibrio* spp. and *Bacteriovorax* spp. showed strong chemoattraction to only yeast extract and casamino acid.

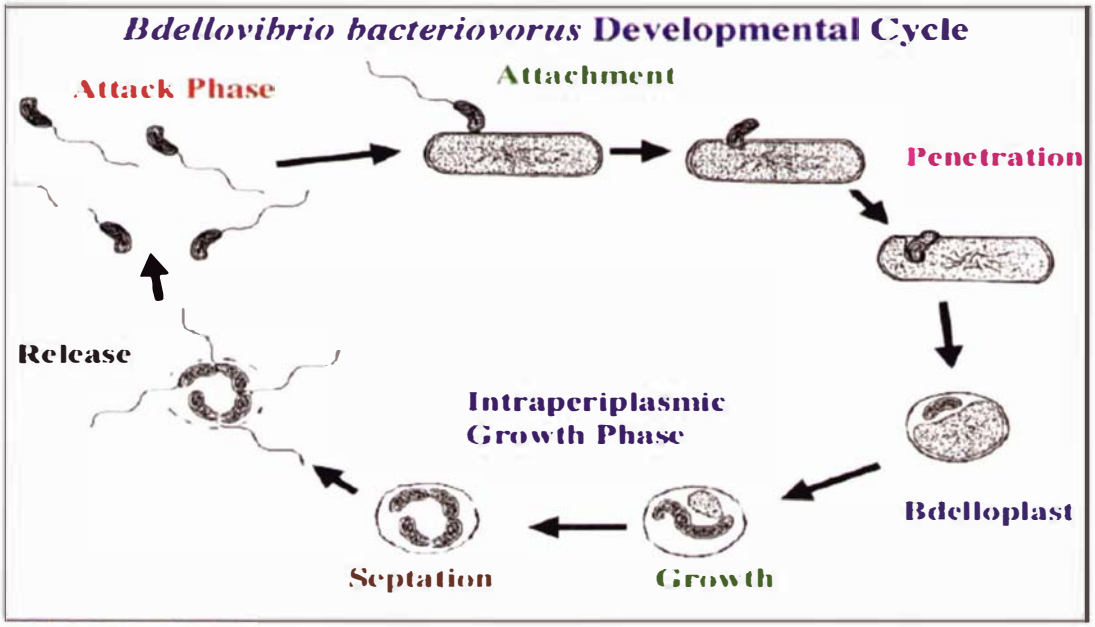


Fig. 1.1 The predatory developmental cycle of Bdellovibrionaceae (BD) (from Saint Joseph's University web page, 2006).

Growth phase cells are intracellular, filamentous and immobile, can initiate chromosome replication and increase in size as single, elongating, spiral cells with an unsepted cytoplasm (Ruby, 1992). The growth or multiplication phase of the life cycle of the bacterium begins when BD has collided with a prey cell. Following the initial collision, two stages of attachment take place; BD cells remain reversibly and non-specifically attached to the prey for a short recognition period, which is followed by an irreversible and specific attachment via the pole opposite the flagellum. Rendulic *et al.* (2004) found multiple candidate genes for adhesion in the genome and they suggested active adhesion by multiple pili genes besides passive protein-protein and LPS-LPS interactions between outer membrane components. Schwudke *et al.* (2005) confirmed increased expression of the host-interaction locus (hit locus) and putative pilin genes (*flp1*) during the attack phase, when the bacterium penetrates the outer membrane and the peptidoglycan layer of the prey cell, generating a small opening, which is subsequently resealed. It has been shown that a variety of enzymatic activities, degradative and biosynthetic modifications occur at this time (Ruby, 1992). Thomashow and Rittenberg (1978a,b,c) detected several enzymes including a glycanase, a peptidase, an N-deacetylase, an acylase directed against the prey peptidoglycan, a LPSase, and an enzyme that removes the Braun's lipoprotein.

Rendulic *et al.* (2004) described the candidate genes for this enzyme, which include serine-, cysteine-, aspartate- and metal-dependent proteases. Once within the host, BD loses its flagellum and resides in the periplasmic space of the prey cell. The prey cell loses its rigidity and is converted into a spherical structure, consisting of the inactive invaded cell and the developing BD. This is termed the 'bdelloplast'. The periplasmic compartment is apparently no longer distinctly separate from the cytoplasm or extracellular environments (Cover and Rittenberg, 1984; Crothers and Robinson, 1971). The characteristic types and amounts of macromolecular components of the periplasm, change, with some being lost to the exterior and others becoming available to the BD. The invading cell then elongates unidirectionally until it has increased in length approximately 20 times. The extension occurs at the non-flagellated end of the BD, the flagellated end having been converted into a holdfast type structure. The elongated spiral-shaped cell then undergoes concurrent multiple septations and fragments into multiple unit length cells. The bdelloplast then lyses and the progeny BD are released to carry out the attack phase of their life cycle.

The life cycle of BD has been studied by phase contrast microscopy, electron microscopy and atomic force microscopy (Seidler and Starr, 1969; Varon and Shilo, 1968; Varon and Shilo, 1969a; Núñez *et al.*, 2003). Varon and Shilo (1969a) investigated the interaction of BD cells and host bacteria and found that under suitable conditions (inoculum size, initial host concentration, time of incubation, media) the attachment of young and active BD cells to the host cells is a rapid process, which was completed within a few minutes after host and parasite were mixed. They observed that the growth rate of BD was not significantly affected by the inoculum size or by the initial host concentration. The final yield was also unaffected by the inoculum size but was markedly influenced by the initial concentration of the host cell in the medium. They detected a linear relationship between the host concentration and the BD yield and found the highest yields obtained under these conditions were upto 7.5×10^{10} PFU / mL. They observed that the number of PFU of BD cells growing under standard environmental conditions increased rapidly after a two to four hour latent period, which they indicated was similar to bacteriophage growth.

Núñez *et al.* (2003) investigated the life cycle of *B. bacteriovorus* 109J using atomic force microscopy. They studied the predator-prey communities grown on filters at hydrated air-solid interfaces, with repeated cycles of hunting, invasion, growth and lysis. They obtained evidence for multiple invasions per prey cell as well as significant heterogeneity in the morphology of BD.

Rendulic *et al.* (2004) studied the life cycle of BD from a genomic perspective. They reported the complete genome sequence of *B. bacteriovorus* strain HD100, which can grow only in the presence of prey and found 3,782,950 base pairs (bp) on a single circular chromosome encoding 3564 proteins. Surprisingly, they did not detect any evidence of recent gene transfer from its prey.

1.1.5 Mechanisms of predation

To enter the periplasmic region of a substrate or prey cell, a BD must pass through the substrate cell's outer membrane and peptidoglycan layer. Two mechanisms have been proposed to explain the formation of this pore, vis. mechanical drilling (Starr and Baigent, 1966) or enzymatic digestion of the cell wall (Engelking and Seidler, 1974; Fackrell and Robinson, 1973; Huang and Starr, 1973). After penetration, the BD and the substrate cell are converted into a bdelloplast. Prey cell DNA, RNA and proteins are then degraded in a highly regulated fashion (Rittenberg and Shilo, 1970; Matin and Rittenberg, 1972; Rittenberg and Thomashow, 1979). At this point, BD initiates DNA replication as it elongates into a multi-cell filament. When the prey cell's components are exhausted, the filament fragments into daughter cells, each of which synthesizes a new flagellum. A lytic enzyme is then produced which ruptures the bdelloplast and releases the attack phase cells.

Electron microscopic studies have suggested that to accomplish the invasion, an entry pore is created in the substrate cell's wall layers through which the BD passes (Abram *et al.*, 1974; Burnham *et al.*, 1968; Starr and Baigent, 1966). Thomashow and Rittenberg (1978b) suggested that BD could mechanically drill its way through the outer membrane

which is composed of phospholipids, protein and lipopolysaccharide (LPS) assembled in a three dimensional structure by non-covalent interactions. To pass through the peptidoglycan layer, however, covalent bonds must be ruptured, which is unlikely to be accomplished mechanically by the drilling action of BD. Although there are exceptions, including *B. bacteriovorus* 109J (Thomashow and Rittenberg, 1978c), the peptidoglycan layer of most bacterial cells is responsible for maintaining the cell's osmotic stability in hypotonic environments. When the integrity of this layer is disrupted, the cytoplasmic membrane of the cell ruptures and the cell lyses. Therefore, BD must break enough covalent bonds in the peptidoglycan layer of the substrate cell to make a pore through which it can pass, but it must also limit the number of bonds broken so that the substrate cell does not lyse prematurely.

Thomashow and Rittenberg (1978c; 1978d) demonstrated a number of enzymes such as (i) a glycanase, (ii) a peptidase, (iii) an N-deacetylase, (iv) an acylase, (v) an activity removing Braun lipoprotein, possibly a protease, and (vi) a final lytic activity enzyme directed against the modified peptidoglycan, that solubilise and modify the peptidoglycan layer of *Escherichia coli* when it serves as the substrate organism for BD intraperiplasmic growth. They also reported that an enzyme(s) cleaves glucosamine from the LPS of the *E. coli*.

Thomashow and Rittenberg (1978a; 1978b; 1978c; 1978d) mentioned that glycanase and LPSase were active only during BD penetration, whereas the peptidase was active throughout most of the BD growth phase. They proposed that glycanase and LPSase might be responsible for making the entry pore in the *E. coli*'s peptidoglycan and LPS layers, respectively. The glycanase and/or the peptidase could be responsible for permitting conversion of the substrate cell to a spherical shape. They considered the N-deacetylase enzyme as a stabilizing enzyme, also active early in the intraperiplasmic growth cycle. They believed that the N-deacetylase stabilizes the bdelloplast structure by bringing the potentially lytic glycanase activity to a halt by substrate modification soon after BD penetration is complete.

Once the BD penetrates into the periplasmic space of the substrate organism and stabilizes the bdelloplast structure, it grows and produces progeny BD. Then, with completion of growth, BD synthesizes a new enzyme(s) that completely solubilises the remaining peptidoglycan (Thomashow and Rittenberg, 1978b) and the progeny BD are released.

Paracrystalline protein surface arrays (S-layers) are present in many Gram-positive and Gram-negative bacteria and archaebacteria (Sleytr and Messner, 1983) and these may prevent attack by predators such as bacteriophages or BD spp. The S-layer protein is a major component of the cell and can constitute up to 10% of the total cellular protein. It covers the entire cell and is non-covalently associated with the underlying components of the cell envelope. Most of the susceptible prey cells for BD do not possess S-layers. Huang and Starr (1973) reported that some organisms containing S-layers might be susceptible to predation, but only when naked. Buckmire (1971) observed that *Aquaspirillum serpens* VHA was resistant to predation by *B. bacteriovorus* 6-5-S, but the cells were susceptible to predation when the S-layer protein was removed from intact cells by guanidine hydrochloride treatment. Koval and Hynes (1991) studied the effect of S-layers on predation by BD isolates and confirmed that cells possessing complete S-layers were protected and those with incomplete S-layers are eventually parasitized when exposed to competent BD strains.

1.1.6 Cell-associated Bdellovibrionaceae products

1.1.6.1 Lipopolysaccharide

BD growing intraperiplasmically synthesise nucleic acids, proteins and lipids from monomeric units derived from homologous macromolecules of the substrate cell (Rittenberg and Thomashow, 1979). In contrast, components of the substrate cells' peptidoglycan are not utilized by BD (Rittenberg and Thomashow, 1979). These observations raised the question as to whether BD utilizes substrate cell lipopolysaccharide (LPS) components as precursors for the synthesis of its own LPS that might be required for structural integrity of the bdelloplast.

Nelson and Rittenberg (1981) obtained data that clearly showed that BD derive a large proportion of the lipid A portion of their LPS from lipid A components of the substrate cell. They determined the composition of BD lipopolysaccharide (LPS) for cells grown axenically and intraperiplasmically on *E. coli* or *Pseudomonas putida*. The LPS of axenically grown BD contained glucose and fucosamine as the only detectable neutral sugar and amino sugar, and nonadecenoic acid as the predominant fatty acid. Additional fatty acids, heptose, ketodeoxyoctoic acid, and phosphate were also detected. The data show that LPS from BD grown intraperiplasmically contained components characteristic of both axenically grown BD and the substrate cells. Substrate cell-derived LPS fatty acids made up the majority of the BD LPS fatty acids and were present in about the same proportions as in the substrate cell LPS. Glucosamine derived from *E. coli* LPS amounted to about one-third of the hexosamine residues in intraperiplasmically-grown BD LPS. However, galactose, characteristic of the *E. coli* outer core O antigen, was not detected in the BD LPS, suggesting that only the lipid A components of the substrate cell were incorporated. Schwudke *et al.* (2003) detected the neutral lipid A from *B. bacteriovorus* HD100 which had a unique structure, suggesting the *de novo* synthesis of lipopolysaccharide by the predator.

Thomashow and Rittenberg (1978c) found that bdelloplast peptidoglycan was resistant to lysozyme digestion because of the presence of muramic acid and glucosamine, which are not N-acetylated. They suggested that the molecule responsible for hydrophobic interactions in the initial stages of BD development was not a protein, but might be a long chain fatty acid. Thomashow and Rittenberg (1978d) observed that the peptidoglycan of *E. coli* becomes acylated with the long chain fatty acids, palmitic acid (60%) and oleic acid (20%), during the initial stages of intraperiplasmic growth of BD.

1.1.6.2 Nucleic acid

The nucleic acid metabolism of BD during intraperiplasmic growth includes the degradation of the substrate organism's nucleic acids (Hespell *et al.*, 1975; Hespell, 1978; Matin and Rittenberg, 1972). These processes appear to be highly regulated. The degradation of the DNA and RNA of the substrate organism into large fragments is apparently completed prior to the initiation of bulk synthesis of BD nucleic acids. BD preferentially utilizes the degraded nucleic acid fragments as precursors for its own DNA and RNA synthesis (Hespell *et al.*, 1975; Hespell, 1978; Matin and Rittenberg, 1972). Matin and Rittenberg (1972) observed that the DNA of a substrate organism was rapidly and completely degraded into pieces within 45 to 60 min of a 3 to 4 hour growth cycle, and was retained in the bdelloplast. The BD then initiated its own DNA synthesis. The authors suggested that the rate of breakdown of the DNA fragments to soluble pieces and the rates of BD uptake and polymerization of these DNA products were similar. At the completion of BD development in a cell of normal composition, about 30% of the initial DNA of the substrate organism is solubilised and the remaining 70% is incorporated into BD DNA.

The highly regulated nature of DNA degradation suggests that BD enzymes are responsible for this process. However, the possibility that the DNAses of the substrate cell function exclusively or in concert with BD enzymes for DNA breakdown must also be considered. Rosson and Rittenberg (1979) presented data on DNAse activity and the kinetics of substrate cell DNA breakdown during intraperiplasmic growth of BD on

normal and heat-treated *E. coli* and concluded that the regulated degradation of the DNA of the substrate organism during development of BD is a consequence of sequential synthesis and activity of the DNAses of the BD.

1.1.6.3 Enzymes

BD produces a variety of enzymes targeted against the prey cell envelope when it parasitizes a suitable host. Both Fackrell and Robinson (1973) and Huang and Starr (1973) detected muramic acid hydrolase and two lytic enzymes (one lysozyme-like and the other a peptidase) in the culture fluid of BD. Thomashow and Rittenberg (1978b; 1978c) studied the intraperiplasmic growth of *B. bacteriovorus* 109J in an *E. coli* prey cell and detected four enzyme activities (glycanase, peptidase, N-deacetylase and protease), which were actively involved in solubilising the peptidoglycan layer of the substrate cell and ultimately making an entry pore into the host cell.

1.1.6.4 Membrane proteins

Tudor and Karp (1994) detected a protein in the cytoplasmic membrane of BD-infected prey cells which shows similarity to the outer membrane protein OmpF. They did not observe this protein in preparations of non-invaded *E. coli*, but it appeared within minutes of attack and persisted throughout the intraperiplasmic developmental cycle. It is believed that BD either implants an outer membrane protein of its own into the prey cell's cytoplasmic membrane, or translocates an outer membrane protein belonging to the prey cell into the cytoplasmic membrane. This would result in the instant and complete collapse of the membrane potential, which would kill the cell, and its cytoplasm would be allowed to leak into the periplasm to feed the growing BD (Diedrich *et al.*, 1984; Talley *et al.*, 1987). Later, McCann *et al.* (1998) observed over 30 polypeptides at various times in a synchronously growing culture of *B. bacteriovorus* strain 109J, using two dimensional gel analysis. The polypeptides fall into nine categories: attack phase specific or one of eight different kinetic groups. Beck *et al.* (2004) revealed that major OMPs (OmpF, OmpC and one other protein of unknown function) of *B. bacteriovorus* strains were not homologous to the respective OMPs of prey sequences, suggesting that intact

outer membrane proteins are not incorporated directly from prey cells (Lambert *et al.*, 2006 b).

1.1.7 Taxonomy of Bdellovibrionaceae

In the eighth edition of Bergey's Manual® of Determinative Bacteriology, Burnham and Robinson (1974) described BD in the category of 'spiral and curved bacteria'. BD were regarded as members of the family Spirillaceae, representing a genus (*Bdellovibrio*) of uncertain affiliation. In addition to the original species, *Bdellovibrio bacteriovorus* (Stolp and Starr, 1963), Seidler *et al.*, (1972) established two additional species, *Bdellovibrio starrii* and *Bdellovibrio stolpii*, later classified in the genus *Bacteriovorax*.

In Volume One of Bergey's Manual® of Systematic Bacteriology, Burnham and Conti (1984) proposed an additional species, '*Bdellovibrio* sp. (marine strains)', under the single genus *Bdellovibrio* based on the diversity of physiological and biochemical characteristics. Although they described the genetic diversity of *B. stolpii* and *B. starii* from the *B. bacteriovorus* group, they suggested the necessity of a taxonomic separation within the genus *Bdellovibrio*. However, despite this, they recommended maintaining the previous taxonomy perhaps due to lacking the number of reports needed to propose the new taxa.

In Volume Two of the second edition of Bergey's Manual® of Systematic Bacteriology, Williams *et al.* (2003) placed BD in the family of Bdellovibrionaceae under the order of Bdellovibrionales. They divided the family into four genera, *Bdellovibrio*, *Bacterivorax*, *Micavibrio* and *Vampirovibrio*. The genera *Bdellovibrio* and *Bacterivorax* are differentiated from *Micavibrio* and *Vampirovibrio* by their unique intraperiplasmic predatory capability and the presence of a sheathed flagellum; on the contrary, *Micavibrio* and *Vampirovibrio* are exoparasitic on other bacteria and contain single, sheathless flagella.

The genus *Bdellovibrio* consists of a single species, *B. bacteriovorus* 243^{AL} (Stolp and Starr 1963), isolated from soil, and is differentiated from the genus *Bacteriovorax* based on its 16S rDNA (ribosomal deoxyribonucleic acid) sequencing pattern, genetic fingerprinting, antibiotic sensitivity pattern and host range characterisation (Williams *et al.*, 2003). Recently, the genus *Bacterivorax* was reclassified based on phylogenetic analysis of 16S rRNA (ribosomal ribonucleic acid) gene sequencing into four named species: *Bacteriovorax stolpii* [(previously called *Bdellovibrio stolpii*) Seidler *et al.*, 1972; Baer *et al.*, 2000], isolated from terrestrial or non-halophilic environments; *Bacteriovorax starii* [(previously called *Bdellovibrio starii*) (Seidler *et al.*, 1972; Baer *et al.*, 2000] isolated from soil in California, USA; *Bacteriovorax marinus* (Schoeffield *et al.*, 1991; Baer *et al.*, 2004), isolated from ocean waters, US Virgin Island, and *Bacteriovorax litoralis* (Schoeffield *et al.*, 1991; Baer *et al.*, 2004), isolated from crab gill, Chesapeake Bay, USA. Davidov and Jurkevitch (2004) proposed to rename *Bacteriovorax starii* as *Peredibacter starii* under a new genus, *Peredibacter*.

BD can be divided into two groups based on the requirement for sodium chloride. The halophilic BD require at least 0.58%(w/v) NaCl to prey on bacteria in culture (Reichelt and Baumann, 1974), while the terrestrial isolates are inhibited by 0.85%(w/v) or higher concentrations of salt (Varon and Shilo, 1968). Some other methods that have been used to further characterize BD include serology (Kramer and Westergaard, 1977; Schelling and Conti, 1983), 16S rDNA sequencing pattern (Baer *et al.*, 2000), molecular fingerprinting techniques such as pulsed field gel electrophoresis (Williams *et al.*, 2003), ribotyping (Jurkevitch *et al.*, 2000; Schwudke *et al.*, 2001), genome size (Seidler *et al.*, 1972), guanine-plus-cytosine content (Scherff *et al.*, 1966; Seidler and Starr, 1969; Seidler *et al.*, 1972), DNA/DNA and DNA/RNA hybridization (Seidler and Starr, 1969), host susceptibility and optimum salinity or temperature requirements (Marbach *et al.*, 1976; Taylor *et al.*, 1974), deoxyribonucleic acid ribosomal binding sites, enzyme migration patterns, differences in cytochrome spectra, sensitivity to antibiotics (Seidler and Starr, 1969) and susceptibility to phage lysis (Althauser *et al.*, 1972).

Biochemical tests are not reliable for identification purposes since wild type isolates must be grown in dual culture which creates difficulty in distinguishing between reactions caused by prey and predator enzymes, thus making it difficult to develop a traditional taxonomic methodology (Ruby, 1992). Most biochemical tests cannot be used for BD unless the host-independent (HI) strains are available. However, the utilization of HI derivatives of BD for taxonomic studies is inconvenient because of the intrinsic difficulty involved in the isolation of HI derivatives, the slow and irregular growth in the media used for biochemical tests, and the limited spectrum of useful and available biochemical tests (Torrella *et al.*, 1978).

BD lack a substantial amount of the metabolic machinery typical in a eubacterium, such as enzymes used in carbohydrate degradation and in the synthesis of nucleic acid precursors (Seidler and Starr, 1969; Reichelt and Baumann, 1974). Nevertheless, Seidler and Starr (1969) used a few conventional tests (oxidase, catalase, gelatin hydrolysis) and the cytochrome spectra to characterise new BD isolates. The systematic entities at the species level in BD rely on analysis of the molecular nature of the nucleic acids (Seidler and Mandel, 1971). Schelling and Conti (1983) suggested the use of serotyping techniques in the study of the taxonomy of BD. Host-range spectra have also been suggested as useful in identification (Seidler and Mandel, 1971; Stolp and Starr, 1963; Taylor *et al.*, 1974; Piñeiro *et al.*, 2004).

BD serve as a model system for studying intracellular parasitism, although a thorough knowledge of the molecular diversity is a necessary prerequisite in achieving any unifying concepts (Seidler *et al.*, 1972). Seidler *et al.* (1969) studied the guanine plus cytosine (G + C) content and relative association of the DNA of 11 isolates of host-dependent (HD) BD and 18 HI derivatives using thermal denaturation curves and buoyant densities in CsCl. They found that most HD and HI cultures of BD have 50.4 ± 0.9 moles% G + C in their DNA. They also observed that two BD isolates of uncertain nomenclatural status contain DNA of about 43 moles% G + C.

Seidler *et al.* (1972) continued with more extensive comprehensive studies of BD nucleic acids and enzymes. They proposed nomenclative designations for two new species of *Bdellovibrio* to be *Bdellovibrio stolpii* Uki2 and *Bdellovibrio starii* A3.12 based on the electrophoretic migration distances of selected enzymes and the moles% G + C content of the DNA. Using starch gel electrophoresis, they observed a high degree of similarity in the malic and isocitric dehydrogenases and furamases of all the BD strains tested, except the two new proposed species. In the latter, notable differences were also detected in the moles% G + C contents of the DNA, about 43% as in the previous study (Seidler *et al.*, 1969).

Torrella *et al.* (1978) studied a variety of BD cultures in terms of DNA analysis (G + C mol%, genome size, DNA hybridisation), cytochrome spectra, and host range. They found a broad range of moles% G + C content ranging from 37 to 51 moles% G + C, and considerable differences in genome sizes. This is in contrast to the result of Seidler *et al.* (1972). DNA hybridisation also revealed a high level of heterogeneity.

The comparison of ribosomal DNA sequences has become a powerful evolutionary tool for determining phylogenetic relationships (Weisburg *et al.*, 1991). Woese (1987) proposed that rDNA sequences provide a useful and stable 'molecular clock' for phylogenetic analysis. These sequences are found in all organisms and can be sequenced directly through application of PCR (Saiki *et al.*, 1988).

By using analysis of partial 16S sequencing, Donze *et al.* (1991) showed that the level of similarity between *B. bacteriovorus* strains 114, Ox9-2, Ox9-3, 6-5-S and 109 D was greater than 97%. Baer *et al.* (2000) investigated whether, by complete 16S rDNA sequencing of three BD species (*B. bacteriovorus* 100^T, *B. stolpii* Uki2^T and *B. starrii* A3.12^T), it was possible to clarify their taxonomic relationship with respect to other related taxa within the delta division of the proteobacteria. They observed that the level of similarity between some BD strains (*B. bacteriovorus* 100^T, *B. bacteriovorus* 109J and *B. bacteriovorus* E) was greater than 99.5%. In contrast, they found that the similarity between *B. bacteriovorus* 100^T and *B. stolpii* Uki2^T was 81.7%, and that between *B.*

starrii A3.12^T and *B. bacteriovorus* 100^T was 81.2%. However, the percentage similarity level between *B. stolpii* Uki2^T and *B. starrii* A3.12^T was significantly higher (90.0%). They further observed that the similarity levels between *B. bacteriovorus* 100^T and other members of the delta division were: *Desulfovibrio desulfuricans*, 81.3%; *Myxococcus xanthus*, 82.3%; *Geobacter metallireducens*, 83.8%; *Desulfonile tiedjei*, 84.2%; *Desulfuromonas acetoxidans*, 83.5% and *Desulfuromusa kysingii*, 83.4%. On the basis of these data, they proposed to reclassify *B. stolpii* Uki2^T and *B. starrii* A3.12^T into the new genus, *Bacteriovorax* gen. Nov. as *Bacteriovorax stolpii* comb. Nov. and *Bacteriovorax starrii* comb. Nov., respectively.

Jurkevitch *et al.* (2000) studied thirty BD strains isolated from soil and rhizospheres and performed 16S rRNA sequencing of three soil- and three root-associated isolates by amplifying 830-bp fragments of the 16S rRNA genes using a BD-specific primer combination. They supported the reclassification proposal of Baer *et al.* (2000) and reported that one soil isolate belonged to the *Bdellovibrio stolpii-Bdellovibrio starrii* clade, while all the other isolates clustered with *Bdellovibrio bacteriovorus,* and hence, formed two distantly related, heterogenous groups.

Schwudke *et al.* (2001) performed taxonomic studies of 13 strains of BD, obtained from the gut of animals, using 16S rRNA analysis and ribotyping. They confirmed the distant relationship between the *Bdellovibrio* cluster and the *Bacteriovorax* cluster as observed by Baer *et al.* (2000). Snyder *et al.* (2002) assessed the genetic diversity of *Bdellovibrio*-and-like organisms (BALO) by sequencing the 16S rRNA gene. They designed experiments to amplify target predator genes while avoiding prey cell DNA amplification using four species-specific reverse primers with a universal *Bdellovibrio* forward primer. The 16S rDNA sequence revealed 25 of the 26 BALO isolates clustered into two groups. All members of the BD isolated from fresh water or terrestrial sources belonged to one group, supported by 100% bootstrap analysis, and the genetic distance between these isolates was less than 12%. They found that the other group, supported by 94% bootstrap analysis, included *Bacteriovorax starrii*, *Bacteriovorax stolpii* and the salt water isolates. They observed that the salt water isolates form a subgroup (83% by bootstrap) and differ

within the subgroup by less than 11%. They concluded that the salt water isolates might have arisen from *Bacteriovorax* progenitors.

Baer *et al.* (2004) studied the marine isolates AQ, SJ^T and JS5^T isolated by Schoeffield *et al.* (1991). They characterized the isolates by phylogenetic analysis of 16S rRNA gene sequencing, DNA-DNA similarity results, DNA G + C contents and phenotypic properties. They observed that the above isolates clustered in a separate clade from *Bdellovibrio bacteriovorus* 100^T, but the isolate SJ^T showed slightly higher similarity to *Bacteriovorax stolpii* Uki2^T and *Bacteriovorax starii* A3.12^T (7.7 and 3.5% respectively), while the DNA-DNA similarities between JS5^T and the *Bacteriovorax* species were slightly lower (4.9 and 3.1% respectively). Phenotypically, they observed that the lack of trypsin activity distinguished the isolate JS5^T from SJ^T and AQ. Based on these data, they reclassified the marine isolates SJ^T (=ATCC BAA-682^T =DSM 15412^T) and AQ as *Bacteriovorus marinus* sp. nov. and isolate JS5^T (=ATCC BAA-684^T =DSM 15409^T) as *Bacteriovorax litoralis* sp. nov. Davidov and Jurkevitch (2004) proposed to place *Bacteriovorax starii* into a new genus, *Peredibacter* and rename *Bacteriovorax starii* as *Peredibacter starii* based on extensive diversity and large distance in phylogenetic analysis of the *bdellovibrio*-and-like organisms

Schwartz and Cantor (1984) developed pulsed field gel electrophoresis (PFGE), a molecular fingerprinting technique that is a modified technique for restriction endonuclease analysis of the chromosome (REAC) that has a greater resolving power for strain analysis. The main principle of PFGE is to separate the DNA fragments by alternating the electric field between pairs of electrodes. In this way the fragments are forced to re-orient and move at different speeds by changing the field direction. The large fragments are more slowly re-oriented than smaller fragments, and thus different patterns of DNA fragments are generated. Williams *et al.* (2003) reviewed a single unpublished work of PFGE where the fingerprinting patterns were compared for five HI strains of BD, two *Bacteriovorax* species, one terrestrial BD isolate, and two halophilic BD-like organisms using restriction enzymes *Asc*I, *Fse*I, *Sfi*I and *Not*I. They reported that the

restriction patterns produced for the *B. bacteriovorus* were similar, but there were ± 2-3 band differences between the strains.

Ribotyping has been one of the more commonly used molecular fingerprinting techniques. This technique involves the restriction digestion of chromosomal DNA followed by southern blotting with rRNA probes in order to generate DNA banding patterns, which allow subtype differentiation of bacterial isolates beyond the species and subspecies levels. A southern blot analysis was performed by Jurkevitch *et al.* (2000) on four newly isolated soil BD strains and a reference strain (*B. bacteriovorus* 109J) using restriction enzyme *Sau*IIIA. They observed a heterologous, cross-hybridizing sequence in strains BEP2, BRP4 and reference strain 109J, which were distantly related to strains TRA2 and SRP1. Schwudke *et al.* (2001) compared the ribotypic patterns of four reference strains using restriction enzymes *Sma*I or *Eco*RV and found that the ribotyping pattern of *B. bacteriovorus* HD114 was different from that of the reference strains HD100, HI 100 and HD 127.

Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR – DGGE) of ribosomal DNA, perhaps the most commonly used culture-independent molecular fingerprinting technique, was introduced into microbial ecology by Muyzer *et al.* (1993). It is based on the unique migration properties of amplified bacterial 16S rDNA fragments in a denaturing gel, which can be used to profile species in a mixed bacterial community. Davidov *et al.* (2006) compared the detection of predatory bacteria from a soil community using culture-dependent and culture-independent (PCR-DGGE) methods. They detected nine operational taxonomic units (OTUs) of *Bdellovibrio*-and-like organisms (BALOs) using culture-dependent techniques, whereas nineteen OTUs of BALOs were detected using PCR-DGGE fingerprinting technique.

1.1.8 Isolation of Bdellovibrionaceae

The procedures for the isolation of BD are intimately connected with their nutritional requirements, growth characteristics, choice of lawn-forming bacterium for wildtype BD or complex nutrient media for HI BD, and some physical separation from other plaque-forming microorganisms (Stolp, 1981; Ruby 1992). The plating of a mixture of BD with host bacteria results in the formation of individual plaques or confluent lysis similar to bacteriophages. In contrast to phage plaques, which develop within one day, the lytic zones caused by BD increase in size for several days. In liquid culture, lysis of host bacteria is accompanied by a decrease of the optical density (Stolp, 1981).

1.1.8.1 Nutritional requirements

Although initially BD was described as an ectoparasitic and bacteriolytic organism that attacks Gram-negative bacteria, HI strains were subsequently isolated (Stolp and Starr, 1963; Shilo and Bruff, 1965; Seidler and Starr, 1969). The isolation of BD in axenic cultures was a step towards a better understanding of the inter-relationship between parasite and host. However, although Stolp and Starr (1963) isolated HI strains, this phenomenon appeared to involve the loss of predacity and motility. Shilo and Bruff (1965) successfully isolated and enriched HI BD in artificial media (nutrient broth with some vitamins and minerals) in the absence of host organisms without the loss of predacity and motility.

Seidler and Starr (1969) developed a reliable method for the isolation of HI strains of BD from 16 different HD cultures. They grew streptomycin-resistant HD cultures in streptomycin-susceptible host cells and transferred them to a selection medium (enriched nutrient broth with casamino acids and yeast extract) containing antibiotics. They compared the morphology and biochemical characteristics of the HD and HI strains and found them almost identical except that the HI strains were less motile in host-free

medium, while the HD cultures were actively motile during growth in the presence of host cells.

Several authors have demonstrated the existence of a growth factor for HI derivatives of *B. bacteriovorus* 109 Davis (Shilo and Bruff, 1965; Ishiguro, 1973). Shilo and Bruff (1965) observed that a minimum of 10^4 organisms per plate was required to obtain isolated colony formation on solid media of HI derivatives of *B. stolpii* A3.12 strains. Ishiguro (1973) failed to isolate *B. bacteriovorus* strain 109 Davis on host-free media from concentrated suspensions of HD cultures. However, he isolated this strain after adding a growth factor, which he found essential for its growth. He mentioned that this factor might be necessary only for the initiation of growth of washed stationary phase cells rather than exponential phase cells from small inocula transferred to fresh medium. This growth factor was also present in cell-free extracts of *E. coli* and a variety of other microorganisms including HD and HI derivatives of strain 109 Davis. They characterised the growth factor as heat stable, non-dialyzable and present in both soluble and particulate fractions of extracts.

The strains isolated from marine environments have been found to require NaCl or seawater for growth (Mitchell *et al.*, 1967; Taylor *et al.*, 1974; Williams *et al.*, 1980). In contrast, Varon and Shilo (1968) have reported that motility of the terrestrial isolates was inhibited by as little as 15 mM (0.088%) NaCl. Taylor *et al.* (1974) observed that the minimum requirement for sodium ion of marine BD was 75 to 100 mM (0.44 – 0.58%) for plaque formation, while the optimum was 125 – 150 mM (0.73 – 0.88%), and this could not be replaced by potassium ions.

1.1.8.2 Choice of lawn-forming organism for cultivation of Bdellovibrionaceae

BD express some specificity for their host organism (Taylor *et al.*, 1974; Schoeffield and Williams, 1990). Taylor *et al.* (1974) tested 42 different host bacteria and found that between 17 – 32 species were parasitized by BD. Although they observed some terrestrial Gram-negative bacteria to be parasitized by marine BD, most vigorous plaque formation

occurred on lawns of marine bacteria. Schoeffield and Williams (1990) suggested that the selection of *Vibrio parahaemolyticus* as the lawn-forming bacterium maximised the efficiency of detection of BD in some estuarine water samples. In contrast, the apparent usefulness of *Aquaspirillum serpens* MW5 as a 'universal host' was more recently disputed (Ruby, 1992).

The question of the minimum host abundance required for the normal growth of BD has been of some debate. It is clear that a large number of prey is required and without a suitable concentration the predators are susceptible to starvation and death, although the minimum threshold number is unknown. Previous studies have suggested that large numbers of host bacteria (> 10^4 /mL) are required for the survival of BD (Hespell *et al.*, 1974; Varon and Zeigler, 1978; Varon *et al.*, 1984). Hespell *et al.* (1974) calculated that a minimum density of 1.5 x 10^5 host cells /mL was required by BD to have a 50% survival rate over a 10-h period. However, in 1978, Varon and Zeigler, applying a mathematical model based on certain assumptions of the ecology and behaviour of BD, calculated that a minimum density of 10^6 host cells /mL would be needed for the survival of the organisms and 10^7 cells /mL would be required for population growth. Later, Varon *et al.* (1984) reported that in the laboratory environment, survival of BD was possible with concentrations of prey as low as 2.5 x10^4 cells /mL. The question arises, whether, if prey concentrations drop below 10^2 to 10^3 cells /mL, BD also develops normally at that decreased level or not. Rice *et al.* (1998) recovered a mean number of 3.9 x 10^3 CFU /mL of BD-susceptible bacteria from water samples, which was less than the numbers estimated to sustain a population of the predators (Varon and Zeigler, 1978; Varon *et al.*, 1984), which indicate that BD can be maintained in lower prey concentration as well.

From the above, it is clear that the prey range of seawater BD is mainly the marine bacteria. *V. parahaemolyticus* is observed to be the universal and most efficient host for seawater BD. It is also noted that prey concentration is one of the most important factors for the survival of BD, although, the threshold of prey bacteria is disputed.

1.1.8.3 Separation of Bdellovibrionaceae from host cells

In nature, BD are usually found in low abundance and are often mixed with bacteriophages, myxobacteria, protozoa and other plaque forming organisms (Ruby, 1992). Starr and Stolp (1976) described a selective filtration procedure, where they passed the sample through a series of decreasing pore size membrane filters (1.2, 0.8 and 0.45 μm) to eliminate large bacterial predators and protozoa. They also noted that the relatively slow rate of formation of BD plaques differentiates them from plaques formed by bacteriophages. Others used low speed centrifugation (800 x g for 5 min) to remove large unwanted particles, followed by high speed centrifugation (27,000 x g for 20 min) which efficiently concentrated the BD cells in the pellet and eliminated bacteriophages (Varon and Shilo, 1970; Jurkevitch *et al.* 2000). The pellet was then suspended in buffer and passed through a 1.2 μm-pore size Nucleopore filter. Williams *et al.* (1980; 1982); used a 0.45 μm filter to separate the BD from larger organisms.

1.1.8.4 Association of Bdellovibrionaceae with surfaces

Many microbial species, including BD, rely heavily upon submerged surfaces, such as oyster shells, for their habitat (Williams *et al.*, 1995). The role of surfaces and surface association may be critical to the survival of BD in the aquatic environment, which may provide some protection and nutrition, and aid in the survivability of BD when environmental conditions become unfavourable (Williams *et al.*, 1995; Markelova and Gariev, 2005). Oysters naturally have a rough surface due to the presence of chitin, and this provides BD and other bacteria with shelter from extreme conditions such as harsh seasonal changes and the agitation incurred from currents. BD may also obtain large numbers of prey organisms from bacteria associated with these surfaces.

Williams *et al.* (1995) studied the association of BD with surfaces in the aquatic environment. They investigated the time at which the predators become a member of the developing microbial surface community on submerged surfaces, monitored the effects of the type of surface and examined the persistence of the predators on natural surfaces (shells), metals (commonly used in marine engineering applications e.g. piping and

cooling systems) and surfaces with smooth and rough topography such as glass slides, steel and titanium. They observed BD to be very rapidly associated with surfaces submerged in natural waters and that the numbers of the predators associated with surface biofilms increased with the time of submersion. Oyster shell yielded the greatest number recovered at all sample intervals except those at 24 hours, at which time greater numbers were recovered from steel. After 48 hours they observed a 3.5 $\log_{10}$ increase on the oyster shell, and 2-3 $\log_{10}$ more BD on steel and titanium than on glass surfaces. Markelova and Gariev (2005) evaluated the negative effect of ecologically hazardous, common, environmental pollutants such as heavy metals and cadmium on *B. bacteriovorus* 100 NCID 9529 in agar medium. They observed the aggregation of BD cells around agar particles supporting the concept of BD being surface-associated in nature.

1.1.8.5 Temperature

BD have been observed to be mesophilic, normally growing in the range of 25-35°C (Varon and Shilo, 1969 a). The optimal conditions for growth are between 28 and 30°C and poor growth has been observed above 37°C and below 10°C. Williams *et al.* (1995) found that the association of BD with biotic (oyster shell) or abiotic (metals, glass slide, steel, titanium) surfaces was influenced by environmental conditions such as temperature and found that decreased temperatures caused decreased associations. At 26°C, they recovered BD on surfaces within hours following submersion, whereas at lower temperature, the rate of association of BD was greatly reduced. Following 1 week submersion at 6°C, BD were not detected on glass surfaces and the mean number recovered from oyster shell surface was less than 1/cm^2.

1.1.8.6 Other considerations

BD are obligate aerobes, though they seem to have a preference for low oxygen environments (microaerophilic), which may reflect an adaptation to intracellular growth (Seidler *et al.*, 1969). Recent findings have shown that bacteria with paracrystalline protein surface layers (S-layers) may have increased resistance to attack by BD, indicating BD may require prey without this layer in order to survive (Koval and Hynes,

1991). Some evidence is available showing that for *Escherichia* and *Salmonella*, only certain serogroups are susceptible to attack, indicating a requirement for certain receptor sites on the prey wall (Fratamico and Whiting, 1995). Other studies have shown that increased attachment can occur on prey with O-specific side chains and prey which also have a complete R-core, indicating that the lipopolysaccharides may be involved in the attachment receptor (Varon and Shilo, 1969b).

1.1.9 Distribution of Bdellovibrionaceae in the natural environment

BD are widely distributed in nature, and have been isolated from soil (Stolp and Starr, 1963; Klein and Casida, 1967; Keya and Alexander, 1975), rice paddies (Uematsu, 1980), rhizosphere of plants (Jurkevitch *et al.*, 2000), sewage (Dias and Bhat, 1965; Burger *et al.*, 1968; Staples and Fry, 1973), river water and sediments (Fry and Staples, 1976) and man-made water systems (Richardson, 1990) and marine environments (Mitchell *et al.*, 1967; Marbach *et al.*, 1976; Miyamoto and Kuroda, 1975; Taylor *et al.*, 1974; Williams *et al.*, 1980; 1982; Williams and Falkler, 1984).

Stolp and Petzold (1962) isolated the first representative of BD from a soil filtrate in Berlin. Subsequently, a comprehensive study was made in 1963 by Stolp and Starr (1963) where they isolated 11 strains of BD by filtration of California soils and they observed that the lytic activity on Gram-negative bacteria varied between the strains; some were able to attack a broad spectrum of host bacteria, others had a limited host range. Klein and Casida (1967) detected BD from 21 out of 23 soil samples in the eastern and central United States and observed that soil-derived BD were parasitic on all *E. coli* tested (25 serogroups). They evaluated various procedures for the enumeration of BD parasitising *E. coli* and found that direct plating and "dilution to extinction" procedures produced similar results, although they preferred the former because it requires less manipulation and shorter incubation times. They also tested different soil diluents for the enumeration of soil BD and found that enumeration in sterile tap water was the same as yeast extract-peptone broth.

Parker and Grove (1970) were the first workers both to isolate BD strains parasitizing *Rhizobium meliloti*, *R. trifolii*, *Agrobacterium tumefaciens* and *A. radiobacter* and to isolate BD from Australian soils. They isolated BD from the Perth metropolitan area and nearby agricultural areas and obtained 2 x 10^0 to 1.2 x 10^2 BD PFU / g dry soil. They failed to detect any from 8 samples of sandy soil from newly developed pasture lands.

Keya and Alexander (1975) determined whether particulate components or the humus fraction of soil or soil suspensions might affect the growth of BD on *Rhizobium*. They observed clay minerals had a marked influence on the interactions between *Rhizobium* K 04S and a BD strain. A clay layer surrounding the rhizobia might protect them from invasion by BD and retard BD attachment to, or penetration of, the larger organism. Within 22 to 65 hours, the rhizobial numbers were reduced from 3 x 10^9 per mL to 2 x 10^7 per mL in a 4% montmorillonite suspension, from 1 x 10^9 per mL to 1 x 10^7 per mL in a 4% kaolinite suspension and from 1 x 10^9 per mL to 4 x 10^6 per mL in a vermiculite suspension. In contrast, the host cell density fell to 2 x 10^5 per mL in the absence of clay material.

Uematsu (1980) isolated up to 10^3 PFU / g of BD from rice paddies in Japan by the filtration method using *Xanthomonas oryzae* as the host organism. Jurkevitch *et al.* (2000) isolated thirty new strains of BD by centrifugation and filtration methods from soil, rhizosphere and roots of bean and tomato plants grown in the soil of an experimental farm of the Hebrew University of Jerusalem. The detected concentrations of BD from soil and rhizosphere of plants were 3 x 10^2 to 6 x 10^3 and 2.8 x 10^2 to 2.3 x 10^4 PFU / g respectively. They observed that the soil strains of BD were characteristically different from the rhizosphere strains in host range, 16S rDNA sequence and phylogenetic tree analysis.

Dias and Bhat (1965) isolated BD from raw sewage and activated sludge, and concluded that the BD populations were not affected by activated sludge treatment. Fry and Staples (1974) studied the occurrence and role of BD in a polluted river and found that BD had no controlling influence on the autopurification of river water and no influence on the

activated sludge process. Burger *et al.* (1968) and Hoeniger *et al.* (1972) isolated a very different strain of BD, designated as *Bdellovibrio* sp. strain W, from a sewage drain in Freiburg, Germany. This strain produced the unique resting stages called bdellocysts only in the presence of the host *Rhodospirillum rubrum*. Fry and Staples (1976) studied the distribution of BD in sewage treatment works, polluted and fresh river water and sediments. They examined the relative abundance of BD in inflow, filter-effluent and final-effluent of nine different sewage treatment works in South Wales. They detected BD in all the treatment works, but the number increased with increasing inflow to the effluent site, which they suggested was probably due to the growth of BD in percolating filters. They also surveyed the BD in 19 rivers in South Wales and elsewhere and detected very low numbers (0 to 3 PFU / mL) of BD from unpolluted rivers, whereas grossly polluted rivers had higher numbers (18 to 51 PFU / mL). They observed similar results from the polluted river sediments compared with those of unpolluted rivers and concluded that sewage effluent was probably the major source of BD in those rivers.

River water is not an ideal environment for BD / host interaction because the water flow is too rapid, the host population is too low and the ambient river temperature is below optimum (Fry and Staples, 1974). In man-made water systems such as hot water plumbing, calorifiers, condensers and cooling towers these conditions might be different. Richardson (1990) investigated the incidence of BD in man-made water systems and examined their coexistence with legionellas. BD and *Legionella pneumophilia* were respectively isolated from 57.8% and 9.5% of 135 water samples obtained from 81 separate sources.

The recovery of marine BD from various marine environments such as seawater (Shilo, 1966; Mitchell *et al.*, 1967; Taylor *et al.*, 1974; Miyamoto and Kuroda, 1975; Sutton and Besant, 1994; Pan *et al.*, 1997), estuaries (Williams *et al.*, 1980), a brackish tidal pond and saltwater aquarium (Schoeffield and Williams, 1990), gills of blue crab (Kelley and Williams, 1992), oyster shells in brackish water (Kelley *et al.*, 1997), coastal seawater and aquaculture farms (Pan *et al.*, 1997), aquatic plant surfaces (Williams *et al.*, 1995)

and high saline environments (Piñeiro *et al.*, 2004) have all suggested that the organism might be ubiquitous in marine environments.

The isolation of BD from seawater was first reported by Shilo (1966) during his study on the effect of predatory bacteria in the ecosystem. He recovered 4 x 10^4 to 5 x 10^4 BD PFU / L of seawater samples from the coast of Israel and suggested that BD may reduce the microbial abundance in the natural ecosystems. Mitchell *et al.* (1967) isolated BD strains during their investigations of the lethal effect of seawater on *E. coli*. They attempted to explain the role of native microflora in the disappearance of *E. coli* from seawater but they could not confirm or disprove the speculation of biological interaction between the organisms.

Taylor *et al.* (1974) isolated and enumerated marine BD from the coastal waters of Oahu, Hawaii. They isolated thirteen strains of BD which were able to parasitise between 17 – 32 species of marine and terrestrial bacteria, and they observed that the marine hosts were better hosts than terrestrial organisms. Off the coast of Oahu they recovered a lower number of BD (1.2 x 10^2 to 1.9 x 10^2 PFU / L of seawater samples) than from the Mediterranean Sea, and they suggested that this was probably due to a lower quantity of organic matter in the Oahu seawater. Miyamoto and Kuroda (1975) recovered 2 x 10^4 to 1.5 x 10^6 PFU / L of BD plaques from Osaka Bay, Japan using the host bacterium *V. parahaemolyticus*.

Williams *et al.* (1980) studied the incidence of marine BD at selected sampling sites in Chesapeake Bay during the months of June 1978 and 1979. Greater numbers of BD were recovered from estuaries than from ocean or seawater. They detected the highest numbers of BD at the mouths of the Potomac and Partuxent rivers (>10^2 PFU / mL) and very few (<1 PFU /mL) from the sampling stations north of the Choptank river or at the southernmost location in the bay at Cape Henry. They did not detect any apparent relationship between the number of BD isolated from the sampling stations and the environmental parameters measured, although they recovered the highest number of BD from salt (NaCl) concentrations of 0.9%-1.8% (w/v).

Williams *et al.* (1982) studied the seasonal distribution of BD through an annual cycle from July 1979 to June 1980 at the mouth of the Patuxent River in Chesapeake Bay, and observed a statistically significant seasonal variation in the number of BD recovered. BD were observed to decrease in number, often to undetectable levels, during the colder months as compared with the warmer months, and they detected the highest number of BD in the spring (1.8×10^4 to 3.1×10^4 PFU / L of seawater).

Schoeffield and Williams (1990) recovered 2.4×10^4 to 1.7×10^5 PFU / L of BD from an aquarium and 1.4×10^4 to 4.9×10^4 PFU / L from a brackish tidal pond in the USA using *V. parahaemolyticus* as host bacterium. Pan *et al.* (1997) surveyed the occurrence of BD around the coastal areas and aquaculture ponds of Taiwan during summer and winter and recovered the highest number of BD during summer in estuaries. Piñeiro *et al.* (2004), using *V. parahaemolyticus* as host, recovered six BD isolates from the Great Salt Lake, Utah, where the salinity ranges from 4 to 17% (w/v). Sutton and Besant (1994) reported the isolation of BD from the Great Barrier Reef, Australia. They compared the BD populations in three tropical marine habitats (a sandy beach, a mangrove and a fringing coral reef) over a 12 month period and recovered the highest numbers of BD from the mangrove habitat (3.7×10^4 PFU / L) and the lowest from the coral reef (9.5×10^3 PFU / L). They recovered the highest number of BD in summer, and the lowest in winter.

Kelley and Williams (1992) tested the occurrence of BD in the blue crab. They tested the gill tissues of 17 blue crabs from natural waters and 11 from the supermarket. The numbers of BD from natural water crab samples were substantially greater than from the supermarket samples, which established blue crabs as a natural reservoir. Previous investigators failed to recover BD from the intestinal contents of fish, crabs or mussels (Westergaard and Kramer, 1977), which suggested that specific factors favourable to colonisation by BD may be operative in the crab gills.

The most complete studies of the ecology of BD are those reported by Williams and his co-workers, who examined the temporal and spatial distribution of these bacteria in

estuarine waters. Williams and Falkler (1984) studied the distribution of BD from five depths in the vertical water column of the Miles River every 4 hours over a 24 hour period. They also measured the salinity, temperature, turbidity and dissolved oxygen (DO). They recovered BD at concentrations of 0.3 to 9.3 PFU / mL from the five depths of water column, but they did not detect any significant difference over the 0.5 m to 13 m column depth, suggesting uniformity of the distribution of BD at the various depths. They observed that the water temperature (23.2°C – 24.7°C) and salinity measurements (12.3 – 16.5%) increased with depth whereas the turbidity was relatively uniform. Although they found uniform distribution of BD in the water column, the DO measurements of the water column varied widely from 5.16 to 6.45 mg / L at 0.5 m, to zero at 13 m depth.

Williams (1988) studied the occurrence and distribution of BD in estuarine sediment over an annual cycle and revealed for the first time, the nature of the association of BD with sediment in the aquatic environment. He observed that the mean number of BD recovered per mL of sediment in each month of the 12 month period was always greater than the number recovered from the same volume of water. He monitored the BD from three depths of the sediment and did not find any significant difference. He observed that the number of BD recovered from sediment was more uniformly distributed during the warm months and recovered the greatest number of BD from the uppermost sediment during July – August (temp. 26.5°C) and the lowest number during winter (December to April). He also compared the number of BD in the top and bottom water samples and recovered more BD from the top water samples.

Ibragimov (1980) first reported the occurrence of BD in animals. He isolated BD from some domestic animals, such as 62 BD isolates from the faeces of 100 cows, 58 from 68 horses, 22 from 40 tested pigs and 17 BD isolates from 40 tested ducks. He failed to detect any BD isolates from the stools of 297 healthy people or from white mice, frogs or fish.

From the above findings, it is noted that BD are widely distributed in the natural environment. In marine environment, BD have been found to be quite abundant in almost

all habitats. Estuaries are observed to be the better place for isolation of BD. The concentration of the recovery of BD correlated with the abundance of nutrition or water pollution. Among the environmental parameters, salinity and seasonal variation show some effect on the rate of isolation, and the best recovery of BD was in summer months and at salinities of 0.9 to 1.8% (w/v). The distribution of BD is uniform in different depths of the water column and sediment. In ocean water, the recovery of BD has ranged from 1.2×10^2 to 1.7×10^5 PFU/L.

1.1.10 Biocontrol applications of Bdellovibrionaceae

The use of BD for reducing bacterial populations has been attempted by some investigators with some success. Rendulic *et al.* (2004) identified many novel antimicrobial enzymes (~250) that have lytic or antimicrobial capabilities, and they speculated that live BD might also be used as a unique type of living antibiotic. Their laboratory experiments revealed no evidence of gene transfer between BD and its host, while the inability of BD to invade mammalian cells has suggested that the application of BD to human and animals would be harmless. Hobley *et al.* (2006) observed a reduction of $\log_{10}$ 3.0 CFU/mL of the population of *E. coli* S17-1 by *B. bacteriovorus* HD100 both in the presence and absence of live bacterial decoy cells of *Bacillus subtilis* nonsporulating strain 671. However, a lag time between 3 and 7 h during predation was noticed when administered in the presence of decoy cells. They speculated on possible therapeutic applications of BD in wound treatment and suggested that BD could eliminate the Gram-negative infection even if a wound contained both species of bacteria at concentrations above the wound infection threshold. Recent observations have produced much speculation regarding the potential of BD to be highly effective over a range of biodefensive applications, particularly where biofilm formation is of critical concern. Atomic force microscopy observations by Núñez *et al.* (2005) demonstrated that *B. bacteriovorus* 109J can prevent the formation of a simple bacterial biofilm and destroy an established biofilm formed by *E. coli* ZK1056 in dilute nutrient conditions (DNB medium). However, although BD could attack and reproduce, they could not eradicate the prey bacteria in a richer medium [PYE (Peptone-Yeast Extract) and LB (Luria broth)].

Phase contrast and epifluorescence microscopy observations by Kadouri and O'Toole (2005) showed that BD can reduce the biomass of both *E. coli* and *Pseudomonas fluorescens* biofilms. Takaaki *et al.* (2000) patented the mass pure culture of BD in Japan and Takaaki (1994) also patented BD as a therapeutic and prophylactic agent for *Vibrio* infectious diseases. Some researchers have suggested BD as a potential biopreservation agent to extend the shelf life of food (Jackson and Whiting, 1992; Fratamico and Whiting, 1995). Epton *et al.* (1989) reported considerable variability and controversial success in the use of BD to control soft rot and black leg of potato caused by *Erwinia carotovora*. Uematsu (1980) observed an efficient reduction of *Xanthomonas oryzae* populations from rice paddy field water by administrating *Bdellovibrio* isolates, although they obtained mixed results against *E. carotovora* subsp. *carotovora* in soil. Scherff (1973) investigated three BD strains for the control of bacterial blight of soybean caused by *P. glycinea* and observed that the blight was inhibited completely by *B. bacteriovorus* isolate Bd-17, moderately by isolate Bd-19 and not at all by Bd-10. In animal models, BD has been successfully used to treat *Shigella flexneri*-induced keratoconjunctivitis in rabbits (Nakamura, 1972).

1.2 Objectives of this study

The economy of New Zealand depends heavily on the export of food and food products. New Zealand exports ~NZ$1,300 million of fish and fish portions in each year. The majority of the fish and fish portions are exported frozen and only 4.6% are exported as fresh fish (Fletcher *et al.*, 2002). Overseas markets are more interested in fresh fish, rather than in paying a lower price for frozen fish, but there are a number of factors limiting the export of fresh fish, particularly its short natural shelf life. Internationally, food biopreservation is being considered as a new option for controlling bacterial spoilage as a replacement for food additives and aggressive heat processes. It has been one of the dominant topics for microbiological research for a decade but commercial applications are still pending.

As detailed in Chapter 1.1, Bdellovibrionaceae are organisms that prey on other Gram negative bacteria. Although, BD was accidentally isolated by Stolp and Petzold in 1962 from soil, subsequently it has been found that BD are widely distributed in nature, in environments such as soil, marine environments, sewage, river water and sediments and man-made water systems. There are indications that they are associated with surface biofilms in aquatic environments. BD strains have been shown to be able to prey on a range of Gram-negative bacteria, including *Escherichia*, *Aeromonas*, *Salmonella*, *Shigella*, *Pseudomonas*, *Photobacterium* and *Vibrio*. Although BD are less selective than bacteriophages in their prey selection, with each strain preying on a range of Gram-negative species, they are by no means generalists and the prey range of bacteria that are susceptible to specific BD strains is variable. In addition, it has also been observed that BD can protect themselves from environmental damage and pollutants by the formation of bdelloplast clusters and cell aggregation, which supports the concept of a surface-associated existence for BD in nature (Markelova and Gariev, 2005). Based on these properties, recent research efforts have been directed at selecting particular strains for special applications.

Many researchers consider BD to be novel probiotics or alternatives to antibiotics. Recent successful research on the sequencing of the entire genome of one isolate has unveiled the entire life cycle and found many novel antimicrobial enzymes, which could be useful in designing alternative antibiotics (Rendulic *et al.*, 2004). There are reports claiming that BD is a probiotic agent against some Gram-negative bacteria, can reduce the biomass of both *E. coli* and *P. fluorescens* biofilms, control food-borne bacteria, control bacteria in sewage, and control diseases in plants and animals, and some patents have been filed in these areas (Takaaki *et al.*, 2000; Takaaki, 1994). However, these applications have not been commercialised.

Increased understanding of the unique predatory mechanisms of BD could create a new and potentially fertile field to control harmful Gram negative bacteria. To pursue this, there is a need to isolate and characterise BD in New Zealand, and to investigate the potential to apply BD commercially as a probiotic or an alternative to antibiotics. To date, there have been no published reports on the isolation of BD in New Zealand.

The hypothesis of the current study was that Bdellovibrionaceae are present in New Zealand marine environments and that the organism has biopreservation potential in the seafood industry by controlling other Gram-negative bacteria.

Hence the aims of the current work were to:

- Isolate Bdellovibrionaceae from New Zealand sources.
- Characterise New Zealand isolates using general and molecular techniques.
- Screen the BD isolates from New Zealand sources for their ability to reduce the numbers of some Gram-negative spoilage and pathogenic bacteria and select the most effective for more detailed study.
- Investigate the biopreservation potentiality of BD in extending the shelf life of King Salmon.

Chapter 2

General methodologies

2.1 Growth of host organism (*Vibrio parahaemolyticus*) for routine cultivation of BD

The host *V. parahaemolyticus* (ATCC 43996) was obtained from the culture collection of Seafood & Marine Extracts, New Zealand Institute for Crop & Food Research Limited, Auckland, New Zealand. This culture was originally isolated from a food poisoning incident in England and was supplied by ESR, Porirua, Wellington. The organism was stored on glass beads in glycerol broth at –80°C. A single glass bead of *V. parahaemolyticus* was picked from the –80°C freezer stock and dropped into 10 mL of sea water yeast extract (SWYE) broth (Appendix 1A) and incubated at 30°C for 1 day. After incubation, the absorbance of the culture was measured at a wavelength of 610 nm using a spectrophotometer (Genova MK3 uv/visible spectrophotometer, Jenway Ltd, England). The culture was streaked onto a SWYE agar plate (Appendix 1B) and incubated overnight at 30°C. Colony purity was verified by Gram stain. A single colony was inoculated into 5 mL of SWYE broth and incubated for 24 h at 30°C for standard growth curve preparation.

A standard growth curve was prepared by taking spectrophotometric readings at 610 nm at 0 h, 4, 8, 12 and 24 h of incubation at 30°C. A lawn of *V. parahaemolyticus* was prepared by streaking a single colony onto a SWYE plate and incubating at 30°C for 24 h. After incubation, the lawn was harvested from the surface of the agar plate by flooding with 9 mL of sterile 70% artificial sea water (ASW, Instant Ocean; Aquarium system; USA) (Appendix 1.1) and suspending the bacteria using disposable sterile 'hockey sticks'. The suspension was then adjusted to contain approximately 10^9 CFU /mL (McFarland Standard 5 or OD of 0.85 at 610 nm). The concentration of the organism was verified using 10-fold serial dilutions and plating onto SWYE agar plates in triplicate.

2.2 Isolation, selection, purification and cultivation of BD

The isolation, purification and cultivation techniques used follow methodologies used by Williams *et al.* (1980; 1982), Williams and Falkler (1984) and Piñeiro *et al.* (2004) with some modifications. Volumes of 250 mL of water samples were sequentially filtered through 1.2, 0.8, 0.45 and 0.2 µm syringe filters (Sartorius Minisart non-pyrogenic, hydrophilic syringe filter, Vivascience, Hannover, Germany). A portion of the sample (1 mL) was taken from each of the filtrates for direct plating. Serial 10-fold dilutions were made from 10^{-2} to 10^{-4} in 70% ASW. The dilutions were well mixed by vortexing. One mL of the dilution was added to tubes containing 3.5 mL of Polypeptone 20 top agar [(Pp 20; 0.7% agar) (Appendix 1.C)] tempered to 45°C and 0.5 mL of *V. parahaemolyticus* (10^9 CFU/mL). The contents were gently mixed and overlaid onto Polypeptone 20 bottom agar [(Pp 20; 1.5% agar) (Appendix 1.D)] in 85 mm diameter standard non-vented petri dishes (Labserv, BioLab) in triplicate. After the top agar had solidified, the plates were incubated at 25°C for one week to check for the presence of BD-like plaques. Five mL of the unfiltered samples and each of the filtrates were enriched separately in flasks containing 50 mL of 70% ASW and 1 mL of *V. parahaemolyticus* (approximately 10^8 – 10^9 CFU/mL). The initial turbidity of the dual culture was measured by spectrophotometer (OD of c.a. 0.300 at 610 nm). The flask was incubated aerobically at 25°C on an orbital shaker (Lab line SHK A2000 orbital shaker, Barnstead International, USA) at 130 rpm until turbidity reduced by approximately 0.2 OD units. The low turbidity was verified by spectrophotometric readings (OD of 0.055 to 0.110). If this level was not reached or if incubation was continued beyond this point, poor recovery of BD was achieved.

To obtain a pure plaque of BD, the dual culture was passed through a range of syringe filters as previously described. To ensure that all the prey cells were removed in the purification process, 100 µl of the filtered culture was plated on SWYE and incubated overnight at 30°C to check for the presence of any viable host cells. The BD culture was then plated onto Pp20 medium with the double agar overlay technique as described previously. Randomly selected plaques were examined by Gram stain reaction and phase

contrast microscopy to confirm the presence of Gram negative coccobacillus and small, highly motile cells characteristic of the family Bdellovibrionaceae. To ensure a pure culture of each of the BD isolates, the purification process was repeated at least two times. At least 5 representative BD plaques from each isolate were stored at –80°C for long term storage (Appendix 2A).

2.3 Enumeration of BD

For enumeration of BD, aliquots were aseptically taken from each flask or tube. Serial 10-fold dilutions were made from 10^{-2} to 10^{-9} in 70% ASW. Portions of the dilution (1 mL) were added to tubes containing 3.5 mL of Pp 20 top agar and 0.5 mL of host organism (*V. parahaemolyticus*; 10^9 CFU/mL). Contents of the tubes were gently mixed and overlaid onto Pp 20 bottom agar in triplicate as described in Section 2.2. After the top agar had solidified, the plates were incubated at 25°C in the orbital shaker (130 rpm) for one week to check for the presence of BD-like plaques. Plates showing approximately 30-300 plaques were selected for counting and plaques were counted using a manual colony counter (Stuart colony counter, Bibby Sterilin Ltd., UK). The PFU were calculated by multiplying plaque counts by the dilution factor and the results expressed as $\log_{10}$ PFU/mL.

2.4 Preparation of pure BD pellet for characterisation study

A single, characteristic BD plaque was inoculated into a sterile 250 mL Erlenmeyer flask containing 50 mL of 70% sterile ASW and 1 mL of 10^9 host cells (*V. parahaemolyticus*). BD was propagated and purified as described in Section 2.2. The pure BD culture was concentrated into a pellet by centrifugation at 18,500 x g (fixed-angle rotor Type F-34-6-38, Eppendorf centrifuge 5810 R, Eppendorf AG-22331, Hamburg, Germany) for 40 min. The purification was verified as described in Section 2.2.

2.5 Preparation of large-scale BD inocula

BD was propagated as described in Section 2.2. After the turbidity cleared and the spectrophotometric reading dropped to 0.1, the contents of the dual culture flask were transferred into a 500 mL sterile Erlenmeyer flask containing 250 mL of 70% sterile ASW and 5 mL of host cells (*V. parahaemolyticus*). The flasks were incubated at 25°C in the orbital shaker described previously (130 rpm) for 24 h. The dual culture was centrifuged at 14,500 x g (fixed-angle rotor Type F-34-6-38, Eppendorf centrifuge 5810 R, Eppendorf AG-22331, Hamburg, Germany) for 30 min. The pellet was resuspended in 80 mL of 70% ASW, 2 mL of the host (*V. parahaemolyticus*) was added and the flasks were incubated at 25°C on the orbital shaker (130 rpm) for 24 h. The culture was then passed through a range of syringe filters (0.8 µm to 0.45 to 0.2 µm) to get pure BD culture. The purity of the culture was verified as previously described (Section 2.2). The filtered culture was centrifuged at 14,500 x g for 30 min. The pellet was resuspended with an appropriate amount of 70% ASW so that the culture contained approximately 10^{10} PFU /mL of BD (adjusted by McFarland standard 5). The count of plaque forming unit of BD (PFU/mL) was confirmed by plating BD culture onto polypeptone 20 medium (Pp20) with the double agar technique as previously described (Section 2.2).

2.6 Preparation of the challenge inocula of *P. phosphoreum* and other spoilage and pathogenic bacteria

P. phosphoreum (NCIMB 13477) was obtained from the Microbiology Laboratory culture collection, Seafood & Marine Extracts section, Institute for Crop & Food Research, Auckland, New Zealand. This culture was originally supplied by Paw Dalgaard, Denmark, isolated from MAP cod fillets (cod from Denmark). Other spoilage and pathogenic organisms used in this study are listed in Appendix 2.B. Similar methodologies were used as described in Section 2.1 for preparation of the challenge inocula of *P. phosphoreum* and other spoilage and pathogenic bacteria.

2.7 Enumeration of *P. phosphoreum* and other bacteria

For enumeration of *P. phosphoreum* and the other spoilage and pathogenic bacteria (Appendix 2.B), aliquots (5 - 10 mL) were aseptically taken from each flask. Serial 10-fold dilutions were made from 10^{-2} to 10^{-9} in 70% ASW. The dilutions were well mixed by vortexing. Portions of the dilution (0.1 mL) were spread onto Sea Water Yeast Extract (SWYE) agar plates in triplicate and incubated at 30°C for 24 – 48 h. Plates showing approximately 30-300 colonies were selected for counting and colonies were counted using a manual colony counter (Stuart colony counter, Bibby Sterilin Ltd., UK). The CFU were calculated by multiplying colony counts by dilution factors and the results expressed as $\log_{10}$ CFU/mL.

Chapter 3

Isolation and characterisation of BD isolates

3.1 Introduction

Bdellovibrionaceae are ubiquitous and widespread in many habitats (Stolp, 1981), including marine environments (Shilo, 1966). The most comprehensive ecological studies of marine BD have been done in the USA (Taylor *et al.*, 1974; Williams *et al.*, 1980; 1982; Williams and Falkler, 1984; Williams, 1988; Schoeffield and Williams, 1990; Kelley and Williams, 1992; Williams *et al.*, 1995; Kelley *et al.*, 1997; Piñeiro *et al.*, 2004) and only a few isolation and enumeration studies have been reported outside the USA (Marbach *et al.*, 1976; Miyamoto and Kuroda, 1975; Sutton and Besant, 1994; Pan *et al.*, 1997; Song *et al.*, 2005). Although marine BD have been isolated under various environmental conditions, they have never previously been isolated from New Zealand environments and the extent of the diversity of these predator bacteria is unknown. Therefore, this study is aimed to report the isolation, characterisation and diversity of marine BD from the North Island of New Zealand. Preliminary studies, over a period of six months, attempted to isolate BD from fifty samples collected from a range of environments: freshwater, seawater, fish gut, chicken gut and cattle faeces. However, no BD were detected probably in part due to the the techniques used. At this point, new methodology was adopted following a period of five months work in Professor Henry N Williams' laboratory at the University of Maryland, Baltimore, USA.

Because BD are usually found in nature in rather low abundance (Klein and Casida, 1967; Fry and Staple, 1974; Williams, 1988) and often are mixed with bacteriophages, myxobacteria, protozoa, and other plaque forming organisms, scientists depend mostly on specific enrichment techniques. To date, the basic approach to isolation of BD from natural samples has been dependent on the degree of apparent clearing of water samples estimated "by eye" (Williams *et al.*, 1980; 1982; Piñeiro et al., 2004). This method does not allow recovery of the maximum number of BD plaques, particularly the "by eye" estimation of the degree of clearing of the samples after incubation may result in either all

the host being lysed or few of the predators entering the host at the time of filtration. This raises the question of the correct time to separate BD from the host which is one of the critical steps in isolation. Preliminary work found that routine absorbance measurement techniques were useful to determine when best to separate BD from the host during the isolation and enumeration of BD in natural samples. The best time to separate BD from the host was found to be when a decrease in absorbance of about 0.2 OD units from the initial level of 0.3 units to 0.1 had occurred. Good recovery of BD was obtained by this technique but if this level of reduction was not achieved or if incubation with host was continued beyond this point, poor recovery of BD was obtained. This method (Section 2.2) was used for the attempted isolation and enumeration of BD from all the samples described in this thesis.

In the present study, *V. parahaemolyticus* was chosen as host bacterium for the recovery of BD isolates because of its proven ability as the most efficient host (Schoeffield and Williams, 1990).

The phenotypic characterisation of BD is limited to just a few techniques as most of the laboratory-based common traditional tests cannot be used due to the inability of BD to grow in pure culture. A few researchers have attempted some conventional tests (oxidase, catalase, gelatin hydrolysis and cytochrome spectra) to characterise BD isolates (Seidler and Starr, 1969), but others observed them to be insufficient and questionable for taxonomic differentiation (Seidler and Mandel 1971; Burnham and Robinson, 1974).

More recently, API ZYM enzymic reactions and antibiotic sensitivity profiles, which do not require growth of the organisms, have been successfully used by Baer *et al.* (2004) to differentiate the marine and fresh water BD isolates. However, phylogenetic analysis of 16S rRNA gene sequencing, ribotyping, and DNA-DNA hybridization have been proved to be the most useful techniques (Baer *et al.*, 2000; Jurkevitch *et al.*, 2000; Baer *et al.*, 2004), and these were reviewed in Chapter 1. In contrast, the use of pulsed field gel electrophoresis for discriminating BD isolates has been reported in the literature only as a single review of an unpublished work (Williams *et al.*, 2003).

The currently used characterization techniques based on common morphology, microscopy and molecular techniques such as 16S rDNA sequencing and DNA – DNA hybridization, which revealed sufficient diversity only among the genus, do not take into consideration species or strain diversity which may exist in these organisms. In this study the proven phenotypic techniques were chosen to identify and characterise BD isolates and differentiate the isolates from other bdellovibrio like organisms (BALO): techniques such as plaque morphology, API ZYM enzymic reactions and antibiogram profiling, fluorescence and electron microscopy and molecular techniques such as 16S rDNA sequencing and phylogenetic analysis. DNA fingerprinting techniques such as pulsed field gel electrophoresis (PFGE), as well as or in conjunction with, 16S rRNA gene sequencing and SDS-PAGE were also applied as potentially important tools to understand the diversity of New Zealand marine BD isolates.

Various microscopic techniques, including phase contrast and epifluorescence microscopy (Kadouri and O'Toole, 2005), electron microscopy (Abram and Davis, 1970; Burnham and Robinson, 1974; Kadouri and O'Toole, 2005), atomic force microscopy (Núñez *et al.*, 2003; 2005) and confocal laser scanning microscopy (Macedo *et al.*, 2005) have been successfully used by researchers for the structural and topological study of BD during the various stages of its life cycle.

In the present study, fluorescence microscopy using 'Molecular Probes' Live/Dead® *Bac*Light™ bacterial viability kits (Molecular Probes, Eugene, Oregon, USA) were used to determine the viability, purity and concentrations of BD cells as well as the presence of bdelloplasts and host cells. The kits provide a novel two-colour fluorescence assay of bacterial viability that has proven useful for a diverse array of bacterial genera including BD and its host cells (Kadouri and O'Toole, 2005; Macedo *et al.*, 2005). *Bac*Light™ is comprised of two dyes, SYTO®-9 dye, 3.34 mM (Component A) and propidium iodide (PI), 20 mM (Component B). SYTO-9 is a green nucleic acid stain that can penetrate both intact and damaged membranes when used alone. PI is a red fluorescent dye that penetrates only damaged membranes. In an appropriate mixture of SYTO-9 and PI,

bacteria with intact cell membranes fluoresce green, whereas bacteria with damaged membranes fluoresce red. In electron microscopy, negatively stained specimens were examined for the structural and topological studies of BD and its host cell.

3.2 Materials and methods

3.2.1 Isolation of BD from New Zealand seawater

The isolation, propagation and cultivation techniques used follow methodologies described by Williams *et al.* (1980; 1982), Williams and Falkler (1984) and Piñeiro *et al.* (2004) with some minor modifications. Twenty water samples were collected from five different sampling sites around Auckland (Princes Wharf, Kelly Tarlton's, Mission Bay, Cox's Bay and South Piha) in the winter (July and August). Water samples were collected once from fourteen different coastal sites on the east and west coasts of the North Island of New Zealand (Table 3.2) in the spring months of 2004 (September to November). Air temperature, water temperature and weather conditions were recorded during the sample collection. Salinity was recorded during sample collection using a handheld refractometer (Atago Hand Refractometer, USA). Two water samples were collected in sterile bottles (500 mL – 1L) at each sampling site. The bottles were submersed to a depth of 500 – 800 mm below the surface, 2 – 20 m from the shore and transported to the laboratory immediately after collection or kept in ice until processing. Typical well-isolated BD plaques were selected, purified, propagated and cultivated as described in Section 2.2. The turbidity of the dual cultures of BD and host cells was recorded as described in Section 2.2. Plaque purity was verified by Gram stain, phase contrast microscopy and on SWYE agar as described in Section 2.2.

Four single, well-isolated characteristic plaques were removed from each plate and inoculated into an enrichment culture and incubated until the suspension was clear. The process of filtering and inoculation of the filtrate onto Pp20 agar, and the enrichment steps, were repeated at least three times to ensure a pure culture of each of the isolates. At

least 5 representative BD samples from each isolate were stored at –80°C for long term storage (Appendix 2.A).

3.2.2 Characterisation of BD by fluorescence microscopy

The following cell suspensions were used in this study: early lag phase cells of strain OT2 and its host cells, late exponential cells of pure OT2 and late exponential cells of host *V. parahaemolyticus*. The late exponential pure cultures of OT2 were grown in a medium of 50 mL of 70% ASW and 1 mL of host (*V. parahaemolyticus*), incubated in an orbital shaker (130 rpm) at 25°C for 2 days. OT2 was purified, propagated, and pure pellets were prepared as described in Sections 2.2 and 2.4. The early lag phase cells of OT2 and its host (*V. parahaemolyticus*) were prepared by transferring 5 mL of freshly grown pure OT2 cells (as described in Section 2.2) into a medium of 50 mL of 70% ASW and 1 mL of host (*V. parahaemolyticus*), and incubated in an orbital shaker (130 rpm) at 25°C for 6 h. The dual culture was concentrated into a pellet by centrifugation at 18,500 x g (fixed-angle rotor Type F-34-6-38, Eppendorf centrifuge 5810 R, Eppendorf AG-22331, Hamburg, Germany) for 30 min. The suspension of host cells was prepared as described in Section 2.1. A dense suspension of the dual culture pellet was prepared with approximately 5-10 mL of 0.85% NaCl and standardised to a turbidity of McFarland standard 5 – 6 and spectrophotometric reading of 0.85 OD at a wavelength of 610 nm.

A dye mixture containing 500 µL component A (SYTO®-9) and 500 µL component B (propidium iodide) from 'Molecular Probes' Live/Dead® *Bac*Light™ bacterial viability kit was mixed thoroughly and 3 µL of the dye mixture was added to 1 mL of each suspension of the test samples and kept at room temperature in the dark for 15 min. The stained bacterial suspension (ca 5 µL) was trapped between a slide and an 18-mm square coverslip. The slides were observed using a Carl Zeiss® fluorescence microscope (Carl Zeiss, Germany) using EX G365, BS FT 395, and EM LP 420 filters and photographed using a Axiocam MRc 5 (D) high resolution camera (ZEI0450-354, Axiocam, Carl Zeiss, Germany).

3.2.3 Characterisation of BD by transmission electron microscopy

Two cell suspensions were used in this study: early lag phase cells of a dual culture containing OT2 and its host cells and late exponential cells of pure OT2. These were prepared as described in Section 3.2.2. A dense suspension of each pellet was prepared using 5-10 mL of 0.85% NaCl to achieve a standardised turbidity as described in Section 3.2.2.

Bacterial suspensions were pipetted in small drops onto parafilm and formvar carbon-coated grids (400 mesh) were inverted onto the drops for 2-3 min. The grids were dried with filter paper and then inverted onto approximately 10 µL of 1% phosphotungstic acid for a further 1 min. The grids were then viewed using a JEOL JEM-1200 EX II transmission electron microscope at an accelerating voltage of 80 kV and the images captured on cut film.

3.2.4 Characterisation of BD isolates using enzymic reactions

The API ZYM (bioMe'rieux) identification test involves the inoculation of a dense suspension of organisms to rehydrate the enzymatic substrates. The metabolic end products produced during the incubation period are detected through colour reactions revealed by the addition of enzymes. These reactions are read according to the table supplied with the kit. Thirteen BD isolates were used in this study (Appendix 2D). BD pellets were prepared as described in Section 2.4. A dense suspension of the pellets was prepared with 1.5 – 2mL of distilled water and standardised as per Section 3.2.2 and the instructions in the kit manual. Five mL of distilled water was distributed into the honey-combed wells of each incubation box prepared with the tray and lid to create a humid atmosphere. Sixty five µL of each BD dense suspension was dispensed into each cupule Fourteen API ZYM strips (for 13 BD isolates and a negative control) that contained 20 cupules in each strip were each placed in an incubation box. The inoculated strips were then placed into the incubation box for 4.5 h at 37°C.

After incubation, 1 drop each of ZYM A and ZYM B reagents were added to each cupule and held for 5 min for colour development. The reactions were recorded on the result sheets according to the table supplied with the kit and the value ranged from 0-5 based on the development of the colour. The strength of reactions was based on the colour intensity and values of 0, 1 and 2 corresponded to negative reactions (insufficiently intense colour indicating insufficient reaction). Values of 5 corresponded to maximum reaction while 3 or 4 were intermediate reactions depending on the level of intensity. Values of 3, 4 and 5 were considered as positive reactions.

3.2.5 Characterisation of BD isolates by antibiotic sensitivity

Antibiogram profiles of 13 BD isolates (Appendix 2D) were generated against 29 antibiotics using the double-agar overlay technique described by Baer *et al.* (2004). Pure cultures of the BD isolates were grown in a medium of 50 mL of 70% ASW and 1 mL of host *Vibrio parahaemolyticus* incubated in an orbital shaker (130 rpm) at 25°C for 2 days. The BD cultures were then purified as described in Section 2.2. The BD cultures were concentrated by centrifugation at 20,800 x g for 40 min. The concentrated BD cultures were re-suspended in an appropriate amount of the supernatant and adjusted to a final concentration of approximately 10^7 PFU/mL. A lawn of *V. parahaemolyticus* was prepared by streaking a single, pure colony onto a SWYE plate and incubating at 30°C for 24 h. The lawns were harvested in 3 mL of 70% artificial sea water (ASW). To prepare lawns on Pp20 double agar overlay plates, 1 mL suspension of *V. parahaemolyticus* was added to 3 mL of molten Pp20 top agar and overlaid onto Pp20 bottom agar plates and allowed to harden. The plates were incubated at 30°C for 1-2 days. The lawn-forming Pp20 plates were divided into 8 quadrants, and an antibiotic disc was placed on each. Ten µL of each BD isolate was dropped on the top of each disc. Plates were incubated uninverted at 30°C for 24 h to allow the BD spots to absorb into the top agar. The plates were then inverted and incubated for a further 48 h. Resistance to the antibiotics was observed by the growth of BD isolates to the edge of the discs, as indicated by clearing or lysis of the prey lawn. A sensitive reaction to antibiotics was recorded in the absence of a clearing zone on and around the discs. Three replicate trials

were performed and the results were recorded as the average of the results obtained from these experiments.

3.2.6 Characterisation of BD isolates using 16S rDNA sequencing

3.2.6.1 Bacterial strains, culture medium and growth conditions

Thirteen BD isolates were used in this study (Appendix 2D). The pure cultures were grown in a medium of 50 mL of 70% ASW with 1 mL of host *V. parahaemolyticus* incubated in an orbital shaker (130 rpm) at 25°C for 2 days. The cultures were purified, propagated, and pure pellets prepared as described in Sections 2.2 and 2.4.

3.2.6.2 Genomic DNA extraction from BD isolates

Genomic DNA was extracted from each of the isolates using a Dneasy Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. BD pellets were resuspended in 190 µL of ATL buffer. Proteinase K (20-µL) was added, mixed by vortexing and the suspension incubated for 1 h at 55°C. AL buffer (200 µL) was added to each tube and the samples were incubated for 10 min at 70°C then thoroughly mixed. The sample was made up to 200 µL with ethanol (96-100%) and thoroughly mixed. The mixture was transferred into the Dneasy Mini spin column and placed in a 2 mL collection tube. The mixture was then centrifuged at 6,000 x g (fixed-angle rotor Type F-45-30-11, Eppendorf centrifuge 5810 R, Eppendorf AG-22331, Hamburg, Germany) for 1 min and the flow-through and collection tubes were discarded. The Dneasy Mini spin column was placed in a new 2 mL collection tube followed by the addition of 500 µL of buffer AW1. The column was centrifuged for 1 min at 6,000 x g and the flow-through and collection tubes were discarded. The column was placed into a new 2 mL collection tube, and 500 µL of buffer AW2 was added. The column was centrifuged for 3 min at 20,800 x g to dry the Dneasy membrane. The Dneasy Mini spin column was carefully removed and placed in a clean 1.5 mL microcentrifuge tube and 100 µL of buffer AE was pipetted directly onto the Dneasy membrane. The column was incubated at room

temperature for 1 min and then centrifuged at 6,000 x g (8,000 rpm) to elute the DNA. The elution was repeated once more as described above. The DNA sample was then stored at –20°C and thawed at room temperature as required.

3.2.6.3 Determination of DNA concentration, yield and purity

DNA yield was measured by determining the absorbance of the elute at 260 nm wavelength. The purity of the DNA was calculated by the ratio of the absorbance at 260nm and 280nm, which provided an estimate of the purity with respect to contaminants that absorb UV light, such as protein. Pure DNA has an A_{260}/A_{280} ratio of 1.8-2.0. DNA concentrations were measured using spectrophotometric readings at 260 nm (Genova MK3 uv/visible spectrophotometer, Jenway Ltd, England).

3.2.6.4 Amplification of 16S rDNA by polymerase chain reaction (PCR)

In PCR, initial problems were encountered with the sensitivity and specificity of the reactions and modifications to optimise the protocol were required. Following optimisation, a protocol was chosen for the two primer sets. To generate a complete 16S rDNA sequence, primers U16a and U16b were used (Table 3.1). For generating a partial 16S rDNA sequence, *Bdellovibrio*-specific reverse primer 842R and a forward primer 63F (Table 3.1) were selected based on the work of Jurkevitch *et al.* (2000):

Table 3.1 List of primers used for Partial and complete 16S rDNA sequencing.

Primer No.	Primer name	Sequence	Length (bases)	GC (%)
1	U16a	5′-AGAGTTTGATCCTGGCTC-3′	18	50
2	U16b	5′-TACCGYTACCTTGTTACGACTT-3	22	43
3	16F357	5′-ACTCCTACGGGAGGCAGCAG-3	20	65
4	16R1087	5′-CTCGTTGCGGGACTTAACCC-3	20	60
5	842R	5′-CGWCACTGAAGGGGTCAA-3	18	55
6	63F	5′-CAGGCCTAACACATGCAAGTC-3	21	52
7	1492R	5′-GGTTACCTTACGACTT-3	16	42
8	27F	5′-AGAGTTTGATCCTGGCTCAG-3	20	50

The sensitivity and specificity of the PCR are affected by magnesium ion concentration, denaturation, annealing and extension times, annealing temperature and the number of amplification cycles. Therefore, preliminary test reactions were carried out using primers 5 and 6 to determine the optimum conditions.

Based on an assessment of the PCR products, the magnesium ion concentration in the reaction was held at 2.0 mM $MgCl_2$, while 3 µg of each DNA template was found to be the most appropriate amount in a PCR master mix. Hence, the PCR mix contained 5 µL of 10 x PCR buffer (Invitrogen, Carlsbad, USA), 2.0 µL of 2.0 mM $MgCl_2$ (Invitrogen), 1.0 µL each of reverse and forward primers, 0.5 µL of *Taq* DNA polymerase (Invitrogen), 3 µL of DNA template and the appropriate amount of double distilled water to make the total volume up to 50 µL.

Amplifications were performed in 0.5 mL PCR tubes in a programmable Eppendorf thermal cycler (Mastercycler gradient, Hamburg, Germany). To optimise the denaturation, annealing and extension times and the number of amplification cycles, the following parameters were tested. Initial denaturation at 94°C: 4 – 8 min in increments of 1 min followed by 30, 35, 38 cycles of denaturation at 94°C for 25 s or 30 s; annealing at 60°C for 30 s or 42 s and extension at 72°C for 10 or 13 s. The final extension of 1 cycle of 72°C was for 7 min. After assessing all the parameters, the following temperature profiles and amplification cycles were determined as the best for NZ-BD isolates: initial denaturation of 1 cycle at 94°C for 7 min followed by 35 cycles of denaturation at 94°C for 25 s, annealing at 60°C for 42 s and extension at 72°C for 13 s and one cycle of extension at 72°C for 7 min. After completion of the amplification cycles the tubes were cooled to 4°C to stop the reaction. After amplification, the PCR products were analysed by gel electrophoresis and the remaining samples were stored at –20°C.

3.2.6.5 Gel electrophoresis of PCR products

Standard electrophoresis protocols were used for all BD isolates using a Gel Electrophoresis device (Bio-Rad, Hercules, CA, USA). PCR products (8 µL) were mixed with 2 µL of gel loading dye (Appendix 1.L). The reactions were loaded into the wells of a 1% agarose gel prepared as 1 g agarose (Invitrogen, Carlsbad, USA) in 100 mL of 0.5 x TAE buffer along with 1 kb plus DNA ladder (Invitrogen, Carlsbad, USA) and a negative control reaction (no DNA added). Electrophoresis was conducted at 100 volts in an electrophoresis chamber for 90 min.

3.2.6.6 Staining and photographing of the gel

After electrophoresis, the gels were stained in fresh aqueous ethidium bromide (80 µL in 1 L distilled H_2O) for 20 min. The gels were then de-stained in water for 30 min, and photographed using a Kodak Gel Documentation System (Kodak, New Haven, USA).

3.2.6.7 Purification of PCR products and DNA sequencing

The PCR products were purified using a QIAquick® PCR purification kit (Qiagen, Hilden, Germany) following the instruction manual of the manufacturer. Each PCR product (40 µL) was mixed with 200 µL of Buffer PB (supplied). The mixed sample was transferred into a QIAquick spin column and placed in a 2 mL collection tube and centrifuged at 16,100 x g in a microcentrifuge (Eppendorf centrifuge 5415 D, Eppendorf AG- 22331, Hamburg, Germany) for 1 min. The flow-through was discarded and the QIAquick column was placed back into the same tube. PE buffer (0.75 mL, supplied) was added and centrifuged for 1 min. The flow-through was discarded and the column was placed back into the same tube and centrifuged for an additional 1 min. The Qiaquick column was placed in a clean 1.5 mL micro centrifuge tube and 50 µL of buffer EB (supplied) was added to the centre of the Qiaquick membrane. The column was

centrifuged for 1 min and the purified eluted DNA was stored at –20°C until thawed at room temperature as required.

3.2.6.8 Sequencing of the PCR product

Sequencing was carried out by the Allan Wilson Centre Genome Service (AWCGS, Massey University, Albany) using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kits in a Gene Amp PCR system 9700 machine, and analysed on an ABI PRISM 377 DNA sequencer. For complete 16S rDNA sequencing of the DNA fragment, primers U16a, U16b and two additional primers 16F357 and 16R1087 (Table 3.1) were used. Partial 16S rDNA sequencing of the DNA fragment was performed using primers 842R, 63F and two additional primers 1492R and 27F (Table 3.1).

3.2.6.9 Assembling of sequences and similarity searches in Genbank

The 16S rDNA sequences for each of the BD isolates were assembled, overlapping sequences were pieced together and the contigs were generated using Sequencer version 3. The obtained contigs of each of the isolates were subjected to similarity searches against the NCBI database using the Basic Local Alignment Search Tool (BLAST) programme (Altschul *et al.*, 1997), available on the internet (http://www.ncbi.nlm.nih.gov/BLAST/).

3.2.7 Characterisation of BD isolates using SDS-PAGE

Thirteen BD isolates (Appendix 2D) were used in this study. BD cultures were purified, propagated, and pure pellets were prepared as described in Sections 2.2 and 2.4. The pellets were stored at –20°C until use. Samples of frozen bacterial culture pellets were thawed on ice. One-mL of HEPES-based extraction buffer (Ryan *et al.*, 1998) was added to each of the pellets and the total soluble proteins were then extracted manually using disposable micropestles (Kontes) for 30 s. Extracts were centrifuged at 10,000 x g for 15 min (fixed-angle rotor Type F-45-30-11, Eppendorf centrifuge 5810 R, Eppendorf AG-22331, Hamburg, Germany) and the supernatant was assayed for protein concentration (Bradford, 1976) to standardise protein loadings on the gels. The BioRad microplate method and BioRad protein assay concentrate were used. Thirty ug of protein from each extract was loaded onto 10% SDS-PAGE gels. Five uL of Precision Dual Colour molecular weight prestained standards (BioRad Cat # 161-0374) were also applied. The molecular weight standards contained ten purified recombinant proteins, including three reference bands from 10 to 250 KD. The gels were run for 0.2 h at 30V, 90mA, followed by 1.50 h at 150V, 90mA. Gels were stained by placing them into a fixative solution (50% methanol, 7% acetic acid) for 30 min with gentle agitation, and then onto Sypro Ruby stain (Molecular Probes) overnight, with agitation. The stain was removed and replaced with 10% methanol, 7% acetic acid for 30 min and then replaced with distilled water for 10 min. The Sypro stained gels were digitally scanned on a Typhoon 9400 scanner (Amersham). After Sypro staining, samples were stained with a modified Neuhoff colloidal Coomassie stain (Neuhoff et al., 1988) (17% ammonium sulphate, 3% phosphoric acid, 34% methanol, 0.1% Coomassie G-250) for 24 h. The stain was removed and replaced with three washes of distilled water for 2 h each, and the gel was then dried on glass plates at room temperature under cellophane and stored.

3.2.8 Characterisation of BD isolates using pulsed-field gel electrophoresis

This PFGE method used follows that described by Fenwick (1997) with some minor exceptions.

3.2.8.1 Preparation of pellets from BD isolates

15 BD isolates (Appendix 2E) were used in this study. Isolates SP-enr and MCB-enr are the enrichment cultures of SP and MCB respectively, which were not studied further in the current research. BD isolates were purified, propagated, and pure pellets were prepared as described in Sections 2.2 and 2.4. The pellets were stored at –20°C.

3.2.8.2 Preparation of DNA plugs

The pellet was resuspended in 2 mL of cell suspension buffer (100 mM Tris-HCl, 100 mM EDTA). The turbidity was adjusted to OD 0.48 – 0.52 at 610nm. Cell suspension (400 µL) was mixed with 20 µL proteinase K (20 mg /mL) and 400 µL of 1% seakem gold agarose (Pulsed-field certified, Bio-Rad) + 1% SDS (equilibrated to 55°C). The components were mixed by gently pipetting up and down and 75 µL aliquots of the agarose-cell suspension were dispensed immediately into plug moulds and left to solidify for 15 min at 4°C.

The agarose plugs were placed into 1 mL of lysis buffer in Eppendorf tubes (50 mM EDTA pH 8.0, 50 mM Tris-HCl pH 8.0, 1% sodium lauroyl sarcosine, 0.1% proteinase K) and incubated in a water-bath at 56°C overnight. The plugs were washed eight times in 15 mL of TE buffer pH 8.0 (10 mM Tris-HCl, 1 mM EDTA) with vigorous shaking on an orbital shaker (Lab line SHK A2000 orbital shaker, Barnstead International, USA) at 180 rpm for 15 min. Finally the plugs were transferred into an Eppendorf tube containing 1 mL of TE buffer and stored at 4°C until required.

3.2.8.3 Restriction endonuclease digestion of DNA embedded in agarose plugs

*Sma*I restriction enzyme was selected based on the cutting ability and GC content of the DNA. A 2 mm strip of DNA plug was excised using a sterile scalpel blade and placed in an Eppendorf tube containing 100 µL of cutting buffer (10 µL *Sma*I buffer, 0.8 µl 100 mg/mL BSA, 30 units *Sma*I (1.5 µL of 20U/µL) and 88.5 µL of sterile MQ water) and equilibrated on ice for 10 – 15 min. The plugs were then incubated at 37°C for 2 h.

3.2.8.4 Pulsed-field gel electrophoresis of digested DNA

Standard electrophoresis protocols were used for all isolates using a Contour Clamped Homogeneous electric field (CHEF) electrophoresis system (CHEF Mapper, Bio-Rad Laboratories, Richmond, California, USA).

The DNA plug slices were loaded into the wells of a 1% seakem gold agarose gel [1.0 g agarose in 100 mL of 0.5 x TBE buffer (1 M TRIS base, 1 M HBO3, 2 mM di-Na^+ EDTA pH 8.0)]. The gel was equilibrated in the electrophoresis chamber with the buffer (0.5 x TBE) circulating for 2 h. Lambda marker (New England Biolabs Inc., USA) was also run in the first, middle and last run of each gel to provide DNA size standards. The wells were filled with 1% agarose and the gel was left at room temperature for 10 min to allow the agarose to solidify.

Two litres of 0.5 x TBE buffer were poured into the electrophoresis chamber. The water chiller and pumps were turned on to equilibrate the buffer to 14°C and the gel was placed in the chamber. Electrophoresis was conducted under the following conditions: 6.0 V/cm; switch times - initial 3 s, final 50 s; ramp 79%; duration of run – 22 h.

3.2.8.5 Staining, photography and interpretation of pulsotype patterns

After electrophoresis, the gels were stained in fresh aqueous ethidium bromide (80 µL of 1 µg/mL) for 20 min. The gels were then de-stained in water for 30 min and photographed on GelDoc 2000. The photographic images of the gels were analysed based on the degree of dissimilarity of patterns of bands between isolates using the criteria set up by Tenover *et al.* (1995) and Fenwick (1997):

Identical - all bands are in similar position;

Closely related - differ by one to three bands;

Possibly related - four to six band differences;

Unrelated - six or more band differences.

3.2.8.6 Dendrogram of the pulsotypes of BD isolates

A dendrogram of the pulsotypes of the 15 BD isolates was prepared using Diversity database software version 2 (Bio-Rad).

3.3 Results

3.3.1 Isolation of BD from New Zealand seawater

Table 3.2 and Fig 3.1 summarize the sampling site locations, and the weather conditions, air temperature and salinity that prevailed during the sampling periods. The surface salinity ranged from 3.5 – 3.7% except in Cox's Bay where the salinity was lower due to heavy rainfall prior to sample collection.

BD isolates were isolated from all samples collected in the spring months of 2004. No BD isolates were recovered from the 20 samples collected in the winter months of 2004. Well-isolated BD-like plaques were directly isolated from 14 collection points, while enrichment techniques were needed for the isolation of BD plaques from OT-enr and TB-enr samples (Table 3.3). The concentration of the BD isolates in the samples after sequential filtrations of 1.2, 0.8, 0.45 and 0.2 μm filters, plaque morphology, plaque purity on Gram stain reaction, phase contrast microscopy and on SWYE agar plates and turbidity reduction of the dual cultures are summarized in Table 3.3. The concentration of the characteristic BD plaques varied between isolates (6 – 40 PFU/mL). The plaque morphologies of the BD isolates were observed as well-isolated BD-like plaques without any centre. The BD cells of 13 of the isolates were observed as pure, Gram negative coccobacillus on Gram stain slides and small (0.1 - 0.3 μm), highly motile, curved, rod shaped without any contaminating cells in phase contrast microscopy. However, the purity verification of the three BD isolates AH, KT and MB on Gram stain slides and phase contrast microscopy showed a mixture of different shaped cells and on SWYE agar showed the growth of cells, which could not be purified even after three consecutive purification steps. Of sixteen initial BD isolates, 13 (MCB, MNA, MNZ, OT1, OT2, OT3, OT4, OT5, OT-enr, SP, TB1, TB2 and TB-enr) were selected for further studies on the basis of the above mentioned criteria. Fig. 3.2 and 3.3 show the enlarged photographic image of the plaques of OT2 after 48 h and 72 h of incubation at 25°C. The size of the plaques increased dramatically from 2.0 to 8.0 mm in diameter over this period.

Table 3.2 Fourteen sampling sites of the North Island of New Zealand from where seawater samples were collected for BD isolation.

Isolate	Sample site	Location	Date	Weather	Air temp.	Water temp.	Salinity
AH	Princes Wharf, Auckland	S 36° 50' E 174° 46'	16-09-04	Sunny	18.6°C	18.0°C	3.5%
KT	Kelly Tarlton's, Auckland	S 36° 51' E 174° 48'	16-09-04	Sunny	19.1°C	18.4°C	3.5%
MB	Mission Bay, Auckland	S 36° 51' E 174° 49'	17-09-04	Sunny	18.8°C	18.2°C	3.5%
MCB	Cox's Bay, Auckland	S 36° 51' E 174° 43'	14-09-04	Windy and rainy	17.7°C	17.8°C	2.3%
MNA	Napier	S 39° 29' E 176° 55'	19-10-04	Sunny	22.8°C	21.2°C	3.5%
MNZ1	Ferry terminal, Auckland	S 36° 50' E 174° 47'	04-04-04	Sunny	18.9°C	19.1°C	3.5%
OT1	One Tree Point, Whangarei Harbour	S 35° 46' E 174° 27'	18-09-04	Sunny	18.6°C	15.8°C	3.5%
OT2	One Tree Point, Whangarei Harbour	S 35° 48' E 174° 27'	18-09-04	Sunny	18.6°C	15.8°C	3.5%
OT3	One Tree Point, Whangarei Harbour	S 35° 47' E 174° 27'	06-10-04	Cloudy and windy	18.1°C	17.8°C	3.5%
OT4	One Tree Point, Whangarei Harbour	S 35° 47' E 174° 28'	06-10-04	Sunny	18.1°C	17.8°C	3.5%
OT5	One Tree Point, Whangarei Harbour	S 35° 49' E 174° 27'	17-10-04	Cloudy and windy	18.6°C	16.8°C	3.5%
OT-enr	One Tree Point, Whangarei Harbour	S 35° 49' E 174° 27'	17-10-04	Cloudy and windy	18.6°C	16.8°C	3.5%
SP	South Piha, Waitakere, Auckland	S 36° 57' E 174° 27'	24-11-04	Sunny	19.6°C	16.8°C	3.7%
TB1	Moturiki Island, Tauranga	S 37° 38' E 176° 11'	27-09-04	Sunny	20.8°C	19.1°C	3.5%
TB2	Pilot Bay, Tauranga	S 37° 37' E 176° 11'	27-09-04	Sunny	20.8°C	20.3°C	3.5%
TB-enr	Moturiki Island, Tauranga	S 37° 38' E 176° 11'	27-09-04	Sunny	20.8°C	19.1°C	3.5%

Fig. 3.1 Map of the North Island of New Zealand showing the locations of sampling sites from where seawater samples were collected for BD isolation.

Table 3.3: Plaque morphology, purity, turbidity reduction and concentration of BD cells in sea water samples after dual culturing of suspected samples with *V. parahaemolyticus*.

Isolate	Plaque morphology[a]	BD concentration (PFU/mL)[b]	Gram reaction[c]	Purity on phase contrast microscopy [d]	Purity on SWYE agar[e]	Turbidity change[f]
AH	+	8	-	-	-	0.19
KT	+	12	-	-	-	0.21
MB	+	6	-	-	-	0.18
MCB	+	36	-	+	+	0.20
MNA	+	18	-	+	+	0.24
MNZ1	+	28	-	+	+	0.25
OT1	+	24	-	+	+	0.26
OT2	+	32	-	+	+	0.25
OT3	+	28	-	+	+	0.22
OT4	+	36	-	+	+	0.24
OT5	+	32	-	+	+	0.22
OT-enr	+	no growth	-	+	+	0.15
SP	+	40	-	+	+	0.17
TB1	+	16	-	+	+	0.22
TB2	+	12	-	+	+	0.24
TB-enr	+	no growth	-	+	+	0.16

[a] *Plaque morphology + means well-isolated BD-like plaque without any centre*

[b] *BD concentrations means BD plaques enumerated from the dual culture after sequential syringe filtration of 1.2, 0.8, 0.45 and 0.2 µm syringe filters. Enumeration (plaque forming units per mL) was on Pp20 double agar plate with added host (*V. parahaemolyticus, *10^9 CFU/mL)*

[c] *Gram reaction '–' means Gram negative coccobacillus cells*

[d] *Purity on phase contrast microscopy '–' means host cells (*V. parahaemolyticus*) still present and '+' means small, highly motile, curved, rod shaped without any sign of host cells (*V. parahaemolyticus*).*

[e] *Purity on SWYE agar was tested by sequential filtration of suspected BD samples, plating 100 µl on SWYE agar and incubating overnight at 30°C. '+' means pure BD cultures (no growth of host cells,* V. parahaemolyticus, *on SWYE agar plate;) '–' means presence of host cells,* V. parahaemolyticus, *on SWYE agar plate.*

[f] *Turbidity change means initial turbidity measured by spectrophotometer at 610 nm of the dual cultures of BD and host cells (*V. parahaemolyticus*) minus final turbidity of the same cultures after incubating aerobically at 25°C in 70% ASW on an orbital shaker.*

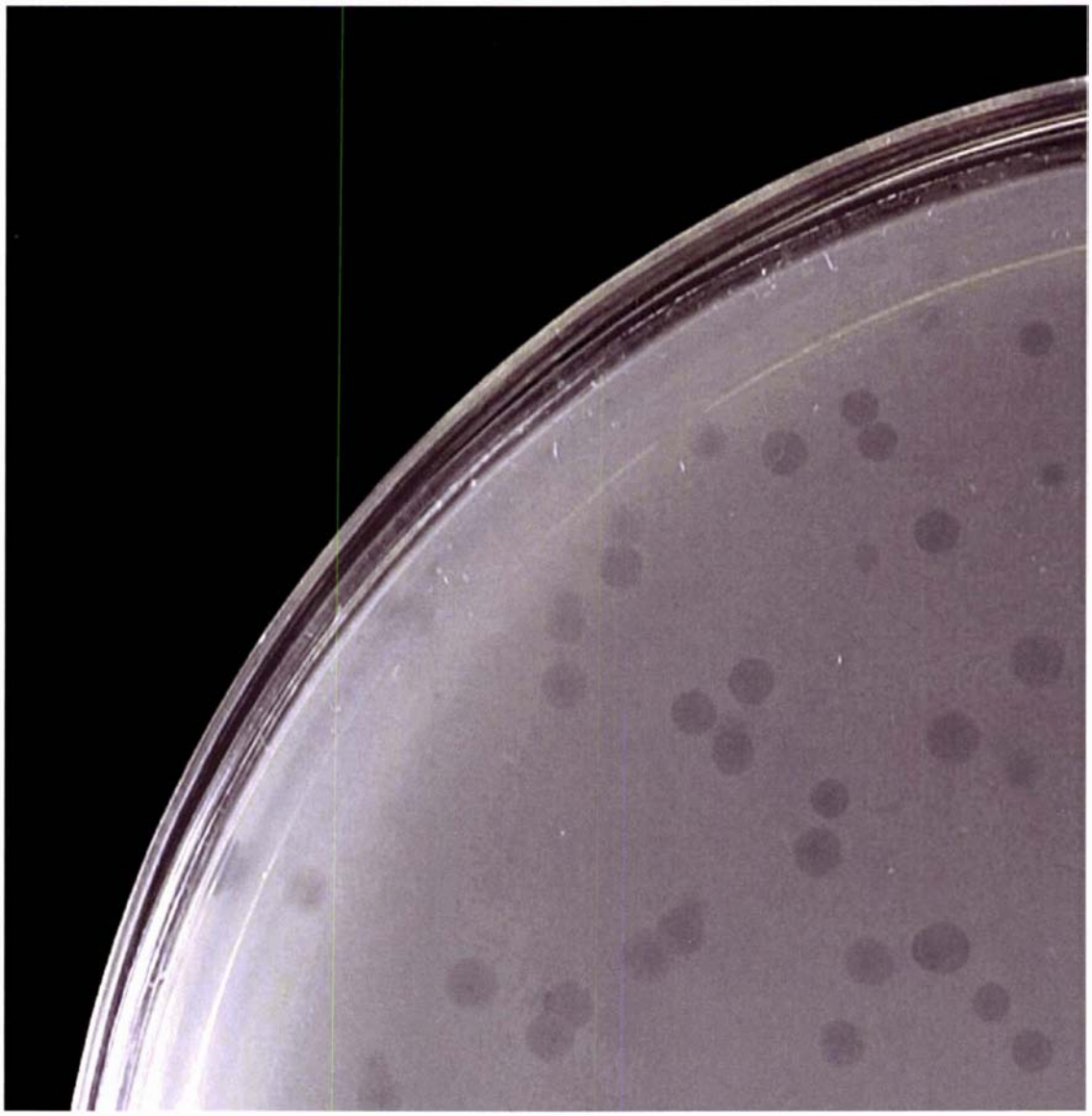


Fig. 3.2 Enlarged photographic image of the plaques of isolate OT2 (~2.0 mm diameter) grown in the lawn of host, *V. parahaemolyticus*, after 48 h incubation at 25°C on Pp20 agar plate.

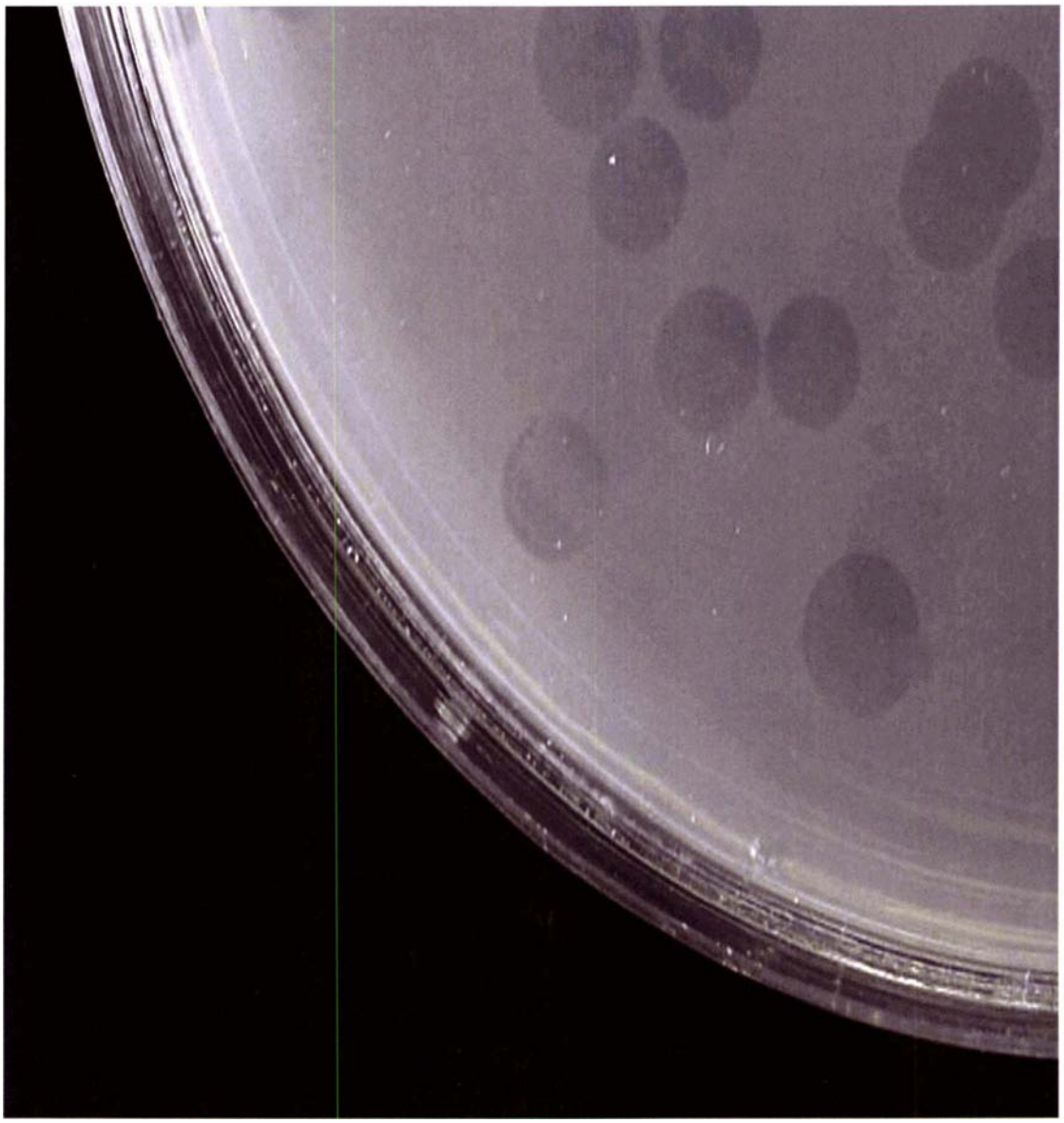


Fig. 3.3 Enlarged photographic image of the plaques of isolate OT2 (~8.0 mm diameter) grown in the lawn of host, *V. parahaemolyticus*, after 72 h incubation at 25°C on Pp20 agar plate.

3.3.2 Characterisation of BD by fluorescence microscopy

Fig. 3.4 shows the fluorescence microscopy images of mixture of early lag phase cells of strain OT2, late exponential cells of host *V. parahaemolyticus*, and a spherical structure consisting of the inactive host cell and BD (bdelloplast). In addition, irregular 'short or long spiral' shaped organisms, presumed to be host-independent BD were seen in some, but not all, fields. In contrast, small, highly motile, late exponential cells of pure OT2 without any host cells were observed in Fig. 3.5, which confirms that BD cells can be separated from their host by sequential filtration through 1.2, 0.8, 0.45 and 0.2 µm syringe filters. In Fig. 3.4, the small green stained cells (8 – 10 in number) are the BD cells and large green stained cells are the host cells. The numbers of BD cells in Fig. 3.4 were only a few because of the incubation time of the cultures (6 h). Why some of the host cells took up the red stain prior to the infection with BD is not clear and needs further investigation. Possibly, it is due to the mechanism of the assay which does not actually test live/dead cells but whether the bacterial membrane is breached. When BD is attacking the host, the host membrane is probably damaged sufficiently to allow the propidium iodide (PI) to penetrate even though the host is not actually dead but will continue to metabolise while the BD multiplies within it.

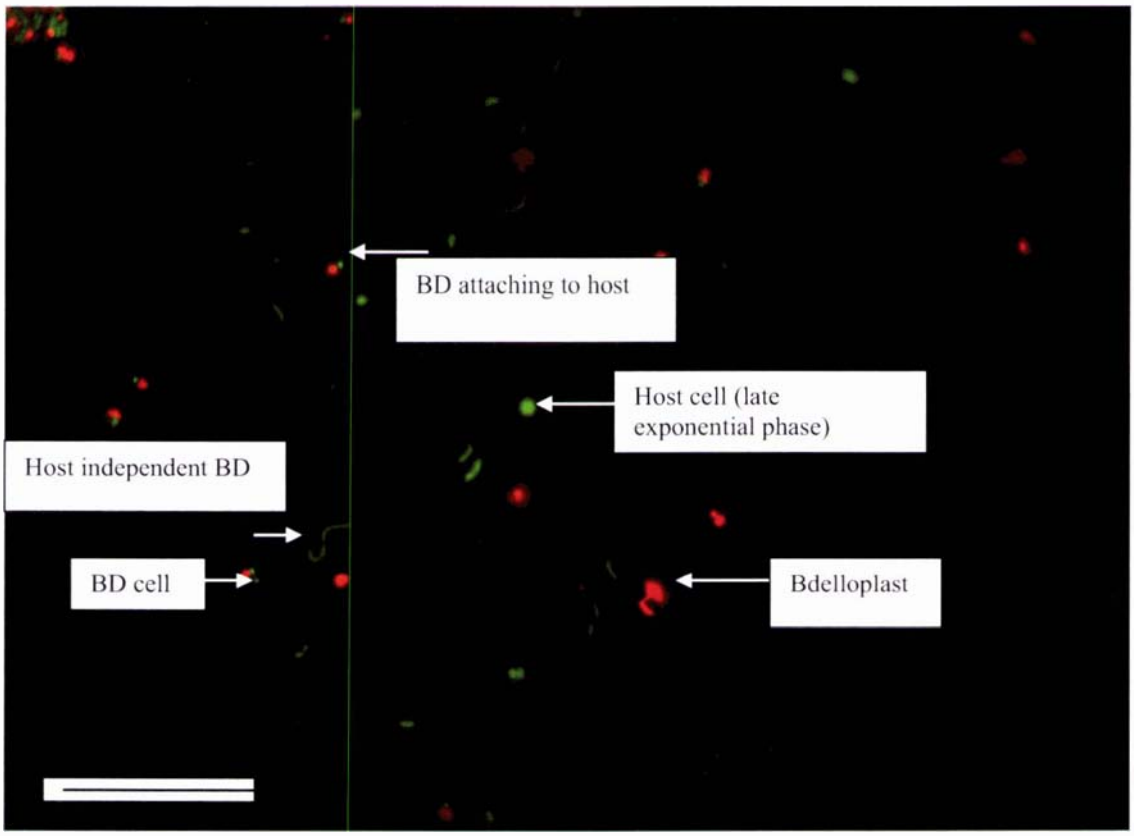


Fig. 3.4 Fluorescence microscopy image (1000x magnification) of the early lag phase cells of isolate OT2 (small green stain cells), late exponential cells of host (*V. parahaemolyticus*), attachment and penetration of OT2 into host and bdelloplast (a spherical structure of inactive host cell and BD) using Live/Dead® *Bac*Light™ dye (a mixture of 500 µL of SYTO®-9 and 500 µL of propidium iodide). Bar: 5 µm.

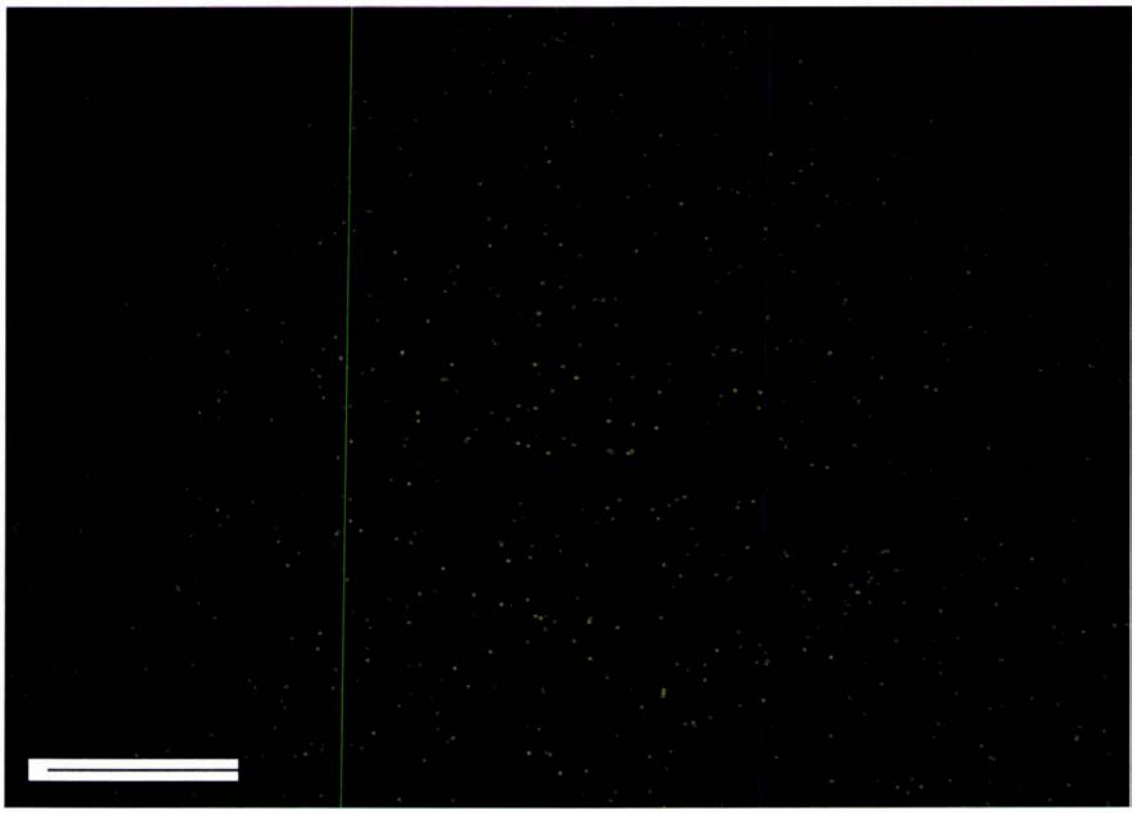


Fig. 3.5 Fluorescence microscopy image (1000x magnification) of the late exponential cells of isolate OT2 without any host cells, using Live/Dead® *Bac*Light™ dye (a mixture containing 500 µL of SYTO®-9 and 500 µL of propidium iodide). Bar: 5 µm.

3.3.3 Characterisation of BD by transmission electron microscopy

An enlarged photographic image of the late exponential cultures of a pure OT2 cell revealed characteristic comma shaped OT2 cells, each with a long flagellum (Fig. 3.6). Transmission electron microscopy observations of negatively stained specimens confirmed the presence of some characteristic comma shaped OT2 cells with long flagella and the formation of bdelloplast, the unique characteristic feature of BD (Fig. 3.7). The intraperiplasmic invasion of an early lag phase OT2 cell into *V. parahaemolyticus* and formation of bdelloplast was observed as recorded in Fig. 3.8. Note the different scales of these figures.

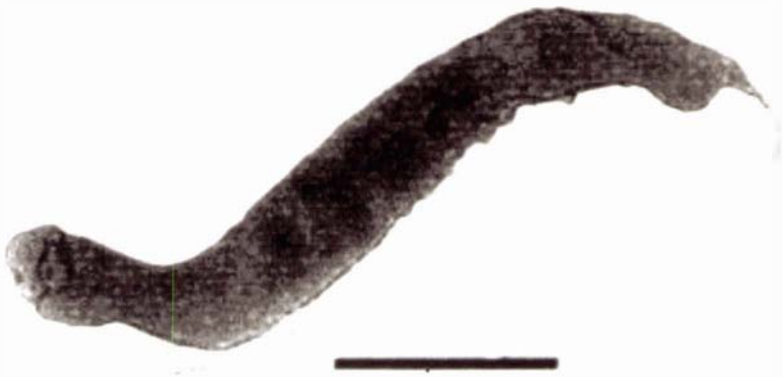


Fig. 3.6 Transmission electron microscopy of an enlarged photographic image of an attack phase cell of OT2 (100,000x magnification) using a JEOL JEM-1200 EX II transmission electron microscope at an accelerating voltage of 80 kV. Bar: 0.05 µm.

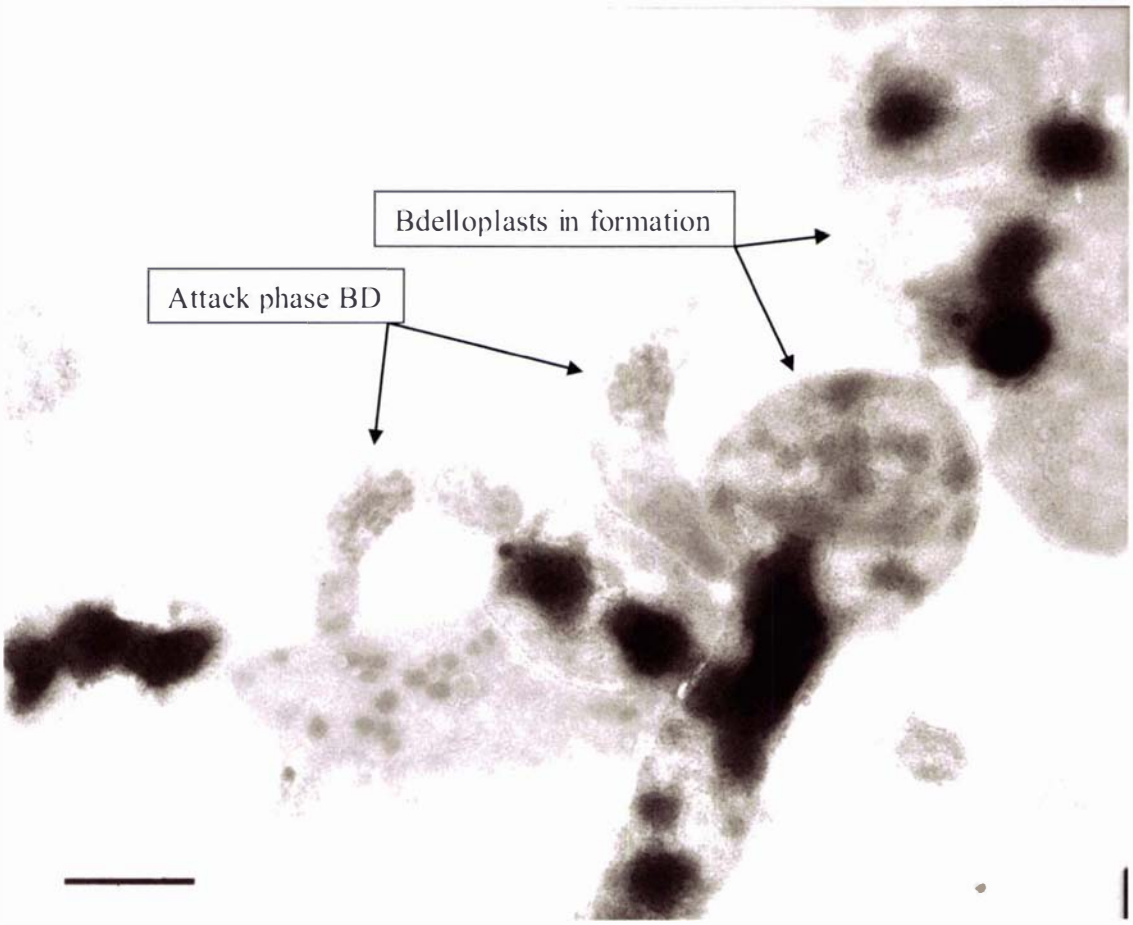


Fig. 3.7 Transmission electron microscopy image of a few attack phase cells and the formation of bdelloplast, of OT2 (100,000x magnification) using a JEOL JEM-1200 EX II transmission electron microscope at an accelerating voltage of 80 kV. Bar: 0.05 µm.

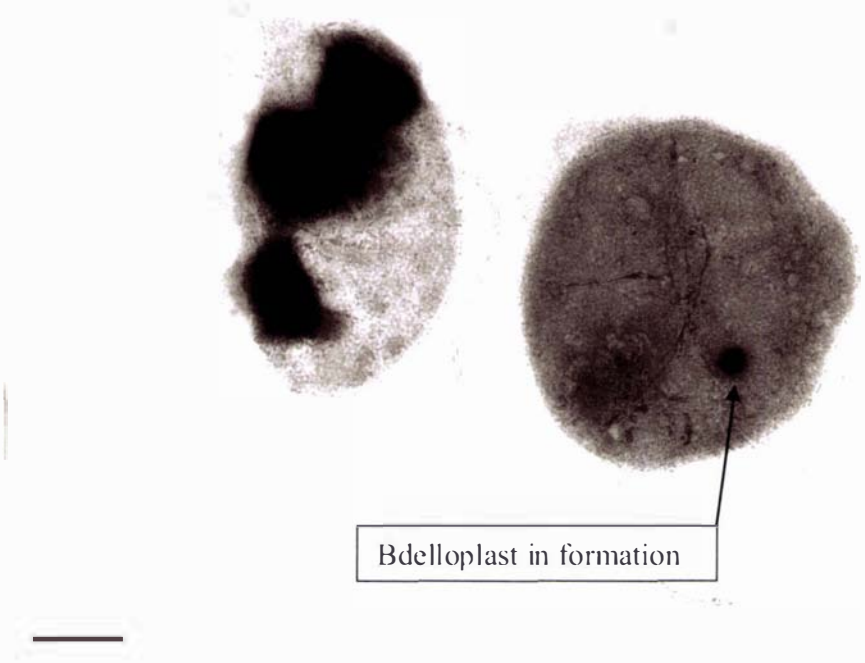


Fig. 3.8 Transmission electron microscopy image of the formation of bdelloplast within the host cell (100,000x magnification) using a JEOL JEM-1200 EX II transmission electron microscope at an accelerating voltage of 80 kV. Bar: 0.05 µm.

3.3.4 Characterisation of BD isolates using enzymic reactions

The results obtained from the enzymic reactions are reported in Table 3.4 and Appendices 3.A.1 and 3.A.2. Of the 19 enzymes tested, only two, cystine arylamidase and trypsin, yielded variable reactions. All BD isolates showed positive reactions for alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, valine arylamidase, acid phosphatase and phosphohydrolase. These results are similar to the work of Baer *et al.* (2004) except for valine arylamidase and chymotrypsin.

Table 3.4. Enzymic reactions (API ZYM system - bioMe'rieux) of thirteen NZ BD isolates. Scores of 0, 1 and 2 (red fill) corresponded to negative reactions and 3, 4 or 5 (green fill) indicate positive reactions. Values were recorded according to the table supplied by the manufacturer.

	OT1	OT2	OT3	OT4	OT5	OT-enr	TB1	TB2	TB -enr	MCB	MNA	SP	MNZ1
Control	1	1	1	1	1	1	1	1	1	1	1	1	1
Alkaline Phosphatase	5	4	5	4	5	4	5	4	5	4	5	4	5
Esterase	2	2	2	2	2	2	2	2	2	2	2	2	2
Esterase Lipase	4	3	3	3	3	3	3	3	3	3	3	3	3
Lipase	1	1	2	2	2	1	1	1	1	1	1	1	1
Leucine arylamidase	4	5	4	5	4	5	5	5	5	5	4	5	4
Valine arylamidase	3	4	3	3	3	4	4	4	3	3	4	4	4
Cystine arylamidase	3	2	3	3	2	3	2	3	2	2	2	2	2
Trypsin	3	2	2	2	2	3	4	2	2	2	2	3	2
α - chymotrypsin	0	0	1	1	1	0	1	0	1	0	1	0	0
Acid phosphatase	5	5	4	5	4	5	5	5	4	5	4	5	4
Naphthol-AS-BI-phosphohydrolase	5	5	4	5	4	5	5	5	4	5	4	5	4
α-galactosidase	1	1	1	1	1	1	1	1	1	1	1	1	1
β-galactosidase	1	1	0	1	0	1	1	1	0	1	0	1	0
β -glucuronidase	1	1	1	1	1	1	1	1	1	1	1	1	1
α -glucosidase	1	1	1	1	1	1	1	1	1	1	1	1	1
β -glucosidase	1	0	0	0	0	0	1	0	0	0	0	0	0
N-acetyl-b-glucosaminidase	1	1	1	1	1	1	1	1	1	1	1	1	1
α -mannosidase	2	0	1	0	1	0	0	0	1	0	1	0	1
α -fucosidase	2	1	0	1	0	1	0	1	0	1	0	1	0

3.3.5 Characterisation of BD isolates using antibiotic sensitivity

The results obtained from this experiment are presented in Table 3.5. The antibiogram profiles confirm that among the 29 antibiotics tested, seven (colistin, fusidic acid, sulfisoxazole, nalidixic acid, neomycin, cloxacillin and oxytetracycline) yielded resistant reactions from all the BD isolates. Only 4 antibiotics (kanamycin, tobramycin, oxacillin and polymyxin B) yielded reactions capable of differentiating the 13 tested isolates.

Table 3.5 Antibiogram profiles (antibiotic sensitivity test) of thirteen NZ BD isolates against twenty nine antibiotics. R = resistant to antibiotics (red fill), S = sensitive to antibiotics (green fill).

Antibiotics	OT1	OT2	OT3	OT4	OT5	OT-enr	TB1	TB2	TB-enr	MNA	MCB	SP	MNZ1
Ampicillin	S	S	S	S	S	S	S	S	S	S	S	S	S
Amoxicillin/Clavulminic acid	S	S	S	S	S	S	S	S	S	S	S	S	S
Amikacin	S	S	S	S	S	S	S	S	S	S	S	S	S
Bacitracin	S	S	S	S	S	S	S	S	S	S	S	S	S
Cloramphenicol	S	S	S	S	S	S	S	S	S	S	S	S	S
Carbenicillin	S	S	S	S	S	S	S	S	S	S	S	S	S
Colistin	R	R	R	R	R	R	R	R	R	R	R	R	R
Cefazolin	S	S	S	S	S	S	S	S	S	S	S	S	S
Erythromycin	S	S	S	S	S	S	S	S	S	S	S	S	S
Nitrofurantoin	S	S	S	S	S	S	S	S	S	S	S	S	S
Fusidic acid	R	R	R	R	R	R	R	R	R	R	R	R	R
Cefoxitin	S	S	S	S	S	S	S	S	S	S	S	S	S
Furazolidone	S	S	S	S	S	S	S	S	S	S	S	S	S
Sulfisoxazole	R	R	R	R	R	R	R	R	R	R	R	R	R
Gentamycin	S	S	S	S	S	S	S	S	S	S	S	S	S
Imipenem	S	S	S	S	S	S	S	S	S	S	S	S	S
Kanamycin	R	R	R	R	R	R	R	R	R	R	S	S	S

Antibiotics	OT1	OT2	OT3	OT4	OT5	OT-enr	TB1	TB2	TB-enr	MNA	MCB	SP	MNZ1
Cefamandole	S	S	S	S	S	S	S	S	S	S	S	S	S
Neomycin	R	R	R	R	R	R	R	R	R	R	R	R	R
Nalidixic acid	R	R	R	R	R	R	R	R	R	R	R	R	R
Novobiocin	S	S	S	S	S	S	S	S	S	S	S	S	S
Netilmicin	S	S	S	S	S	S	S	S	S	S	S	S	S
Tobramycin	R	R	R	R	R	R	R	R	R	R	S	S	S
Cloxacillin	R	R	R	R	R	R	R	R	R	R	R	R	R
Oxytetracycline	R	R	R	R	R	R	R	R	R	R	R	R	R
Oxacillin	R	R	R	R	R	R	R	R	R	R	S	S	S
Polymyxin B	S	S	S	S	S	S	S	S	S	S	R	S	S
Streptomycin	S	S	S	S	S	S	S	S	S	S	S	S	S
Tetracycline	S	S	S	S	S	S	S	S	S	S	S	S	S

3.3.6 Characterisation of BD isolates using 16S rDNA sequencing

Using the optimised reaction conditions, all 8 isolates of BD gave an amplified product of approximately 1500 bp using the universal primers U16a and U16b (Fig.3.9). When using the BD specific primers, 842R and 63F, amplified products of approximately 800 bp were observed (Figs 3.10 and 3.11).

Appendices 3.B.1 to 3.B.8 show the contigs obtained for each of the BD isolates using the universal primers U16a, U16b, 16F357 and 16R1087. Appendices 3.C.1 to 3.C.5 show the partial 16S rDNA sequences obtained from 5 BD isolates using primers 63F 842R, 1492R and 27F. The similarity searches using the BLAST programme (Table 3.6 for strain OT2 and Appendices 3.D.1 to 3.D.7 for other strains) confirm the identity of NZ-BD isolates to the genus level. The BD isolates were observed to be very closely related to each other and closely related (99 – 98%) to the overseas isolates of *Bacteriovorax* sp. OC71, *Bacteriovorax* sp. DA5 and *Bacteriovorax* sp. NB2. A distance tree using the Basic Local Alignment Search Tool (BLAST) programme (http://www.ncbi.nlm.nih.gov/BLAST/) confirmed the location and relatedness of OT2 with other BD isolates, also identifying it as most closely related to *Bacteriovorax* (Fig. 3.12). A neighbour-joining distance gene tree (Fig. 3.13) was estimated from the aligned sequences for each gene of 13 NZ BD isolates (OT1, OT2, OT3, OT4, OT5, OT-enr, TB1, TB2, TB-enr, SP, MCB, MNA and MNZ1); 6 overseas BD strains (NB2, DD1, DA5, OC71, NE1 and OC21) and an out-group *Vibrio parahaemolyticus* strain 070925 using PAUP v 4.0 (Swofford, 1998). The result confirmed that all the NZ BD isolates are situated in the same place, but are negligibly different to the overseas isolates of *Bacteriovorax* sp. OC71 and *Bacteriovorax* sp. DA5.

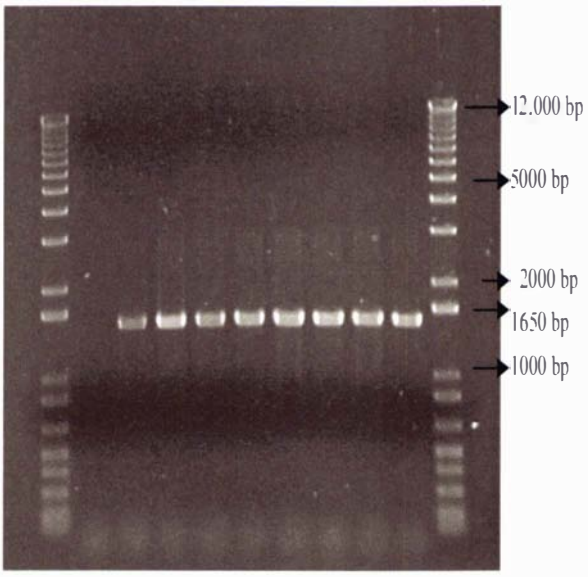


Fig. 3.9 Photograph of the PCR amplified product of 8 NZ BD isolates using the universal 16S primers U16a and U16b for generating complete 16S rDNA sequencing. Lanes 1 and 11 contain 1 kb plus DNA Ladder (Invitrogen Life Technologies, USA; Cat No. 10787-018). Lane 2 depicts the negative reaction (no BD added) sample and lanes 3 to 10 show product of isolates OT1, OT2, OT3, TB2, SP1, MCB, MNA and MNZ1 respectively.

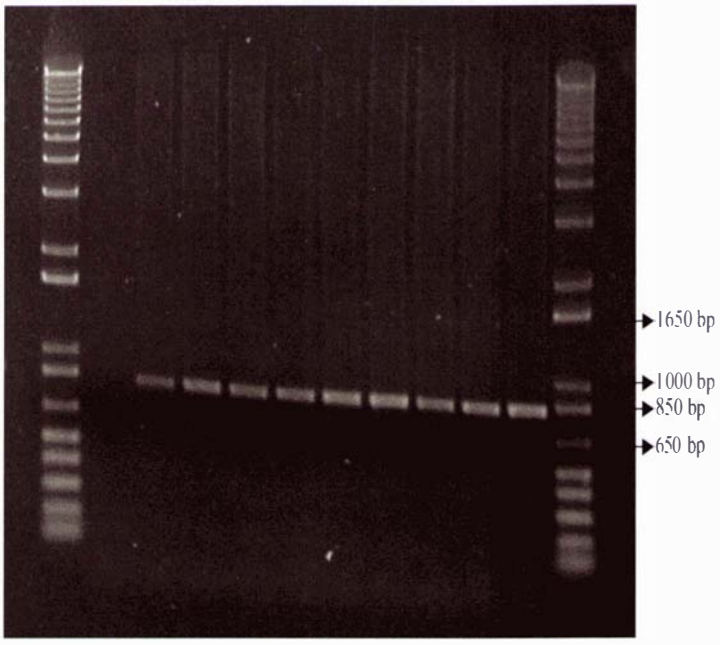


Fig. 3.10 Photograph of the PCR amplified product of 9 NZ BD isolates using the BD specific reverse primer 842R and a forward primer 63F for generating partial 16S rDNA sequencing. Lanes 1 and 11 contain 1 kb plus DNA Ladder (Invitrogen Life Technologies, USA; Cat No. 10787-018). Lane 2 depicts the negative reaction (no BD added) sample and lanes 3 to 11 show PCR amplified product of isolates OT1, OT2, OT3, OT-enr, TB2, SP1, MNA, MCB and MNZ1 respectively.

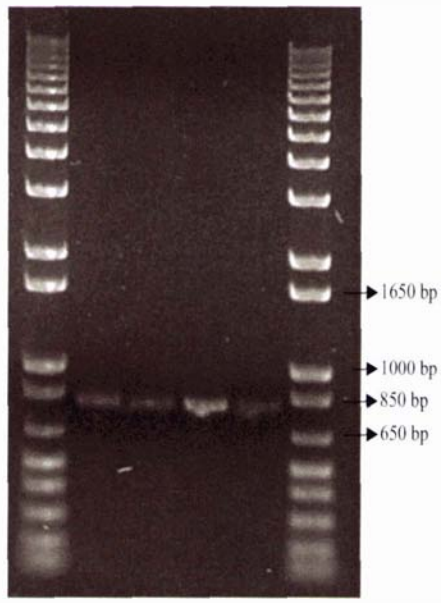


Fig. 3.11 Photograph of the PCR amplified product of 4 NZ BD isolates using the BD specific reverse primer 842R and a forward primer 63F for generating partial 16S rDNA sequencing. Lanes 1 and 6 contain 1 kb plus DNA Ladder (Invitrogen Life Technologies, USA; Cat No. 10787-018). Lanes 2 to 5 show PCR amplified product of isolates OT4, OT5, TB1 and TB-enr respectively.

Table 3.6 Similarity searches of NZ BD isolate (OT2) using the BLAST programme (http://www.ncbi.nlm.nih.gov/BLAST/) showing the closest phylogenetic relatives to the overseas BD isolates. The OT2 isolate was observed to be very closely related (99 – 98%) to the overseas isolates of *Bacteriovorax* sp. OC71, *Bacteriovorax* sp. DA5 and *Bacteriovorax* sp. NB2.

Closest phylogenetic relative	Score (bits)	Identities / Similarity (%)	Accession number
Bacteriovorax sp. DA5	2619	1418/1440 (98%)	EF092435
Bacteriovorax sp. OC71	2619	1359/1365 (99%)	DQ536436
Bacteriovorax sp. NB2	2593	1414/1439 (98%)	EF092436
Bdellovibrio sp. JS2	2097	1354/1441 (93%)	AF084856
Bdellovibrio sp. JS7	2089	1353/1441 (93%)	AF084861
Bdellovibrio sp. JS4	2089	1353/1441 (93%)	AF084858
Bdellovibrio sp. JS7	2081	1352/1441 (93%)	AF084862
Bacteriovorax sp. DD1	1865	984/997 (98%)	EF092444
Bacteriovorax sp. NE1	1861	984/997 (98%)	EF092445
Bacteriovorax sp. GSL41	1729	914/928 (98%)	DQ536440
Bdellovibrio sp. JS10	1693	964/998 (96%)	AF084863
Bdellovibrio sp. JS6	1693	964/998 (96%)	AF084860

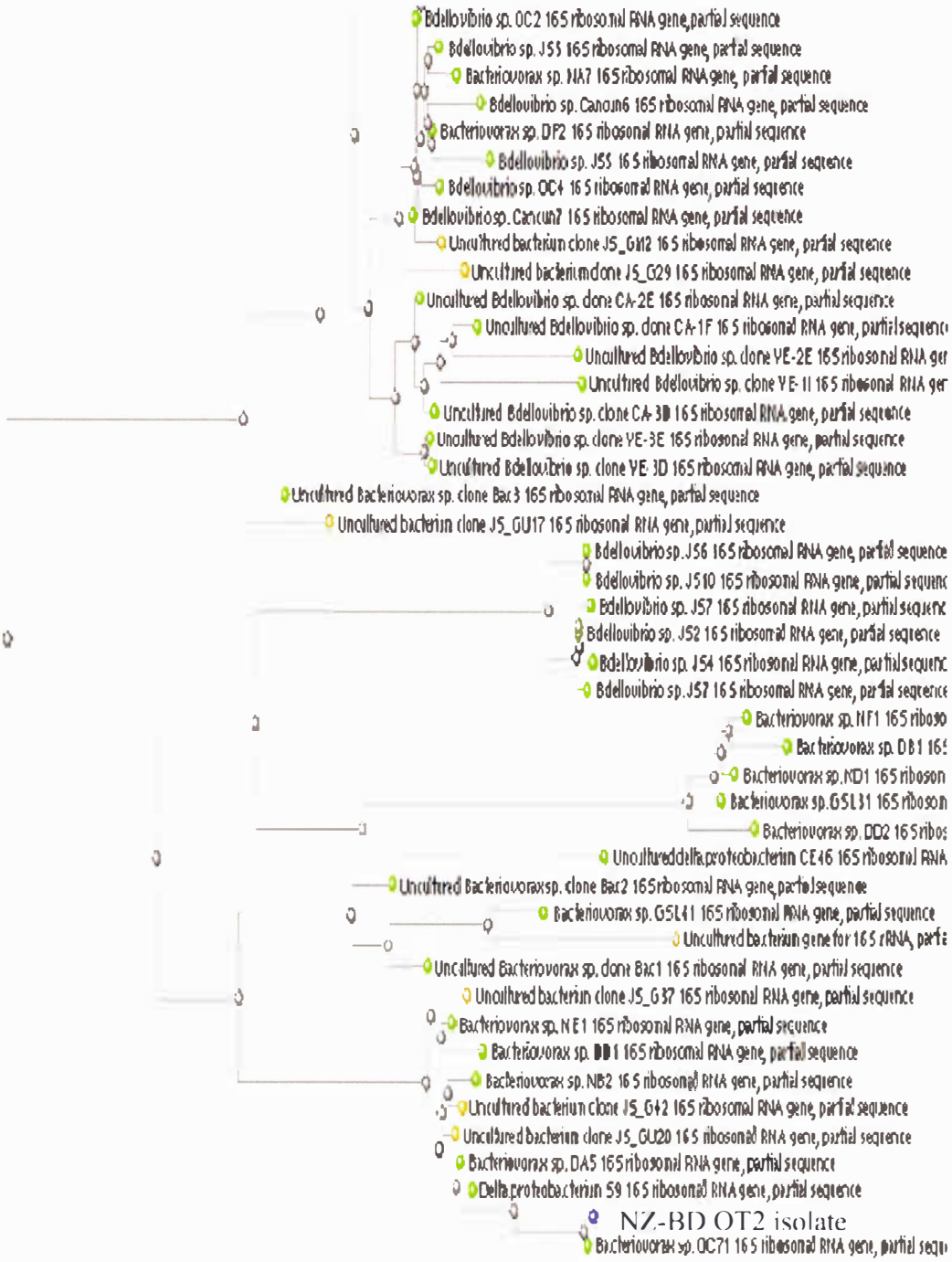


Fig. 3.12 Distance tree of a NZ BD isolate (OT2) using NCBI database using the Basic Local Alignment Search Tool (BLAST) programme available on internet (http://www.ncbi.nlm.nih.gov/BLAST/).

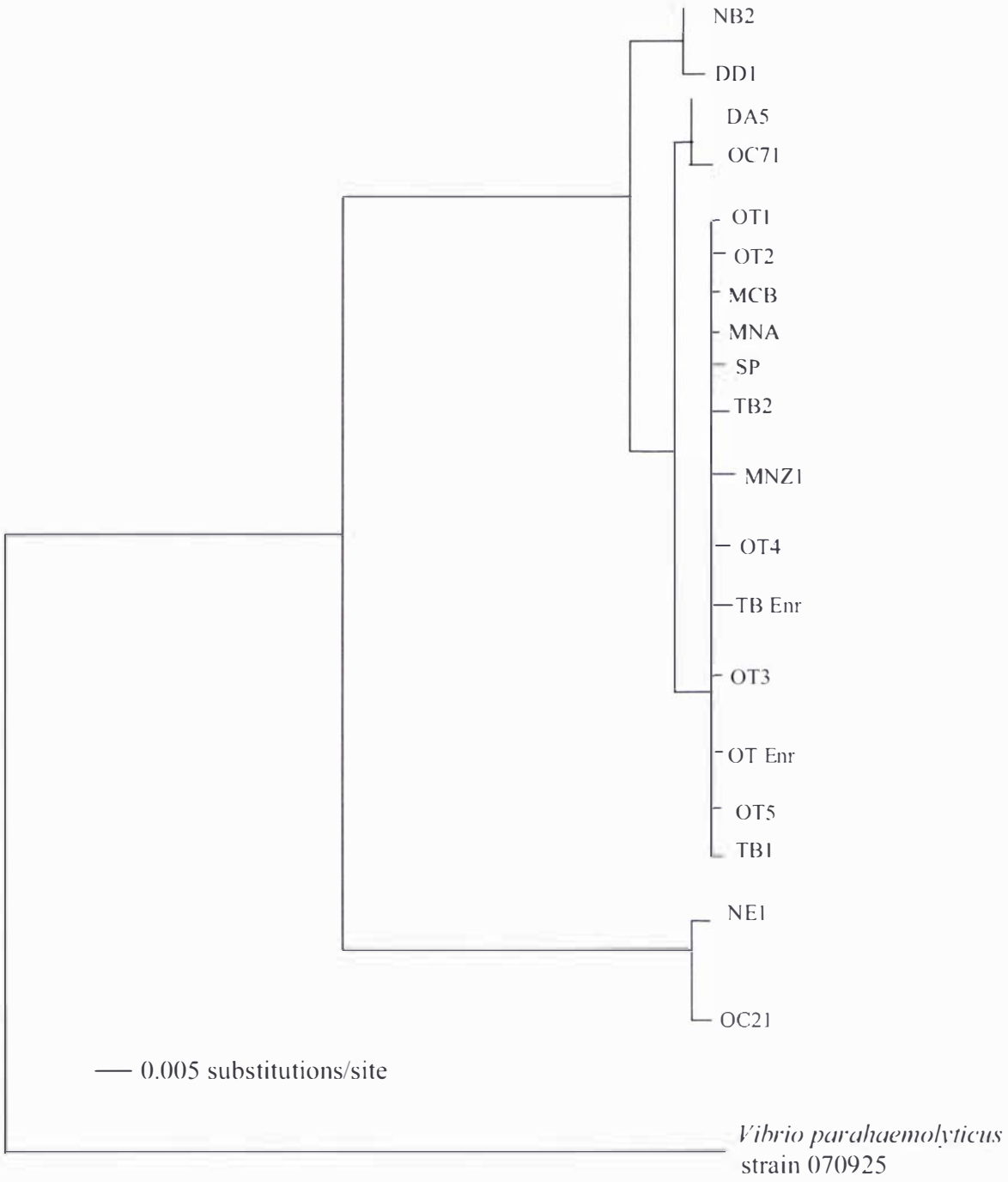


Fig. 3.13 A neighbor-joining distance gene tree generated using 16S rRNA genes of 13 NZ BD isolates (OT1, OT2, OT3, OT4, OT5, OT-enr, TB1, TB2, TB-enr, SP, MCB, MNA and MNZ1) and 6 overseas BD strains (NB2, DD1, DA5, OC71, NE1 and OC21) for comparative purposes. *Vibrio parahaemolyticus* strain 070925 was used as out-group for rooting the tree. Phylogenetic relationships were estimated from the aligned sequences for each gene using PAUP v 4.0 (Swofford, 1998).

3.3.7 Characterisation of BD isolates using SDS-PAGE

The protein banding patterns of the BD isolates are presented in Fig 3.14. At least 15 - 17 detectable protein bands were identified by SDS-PAGE, but there was no detectable variation observed among the BD strains. Molecular sizes of the bands from the BD isolates ranged between 15 and 150 kD. Major protein bands were observed at 150, 120, 100, 75, 65, 60, 46, 41, 37, 25 and 15 kD.

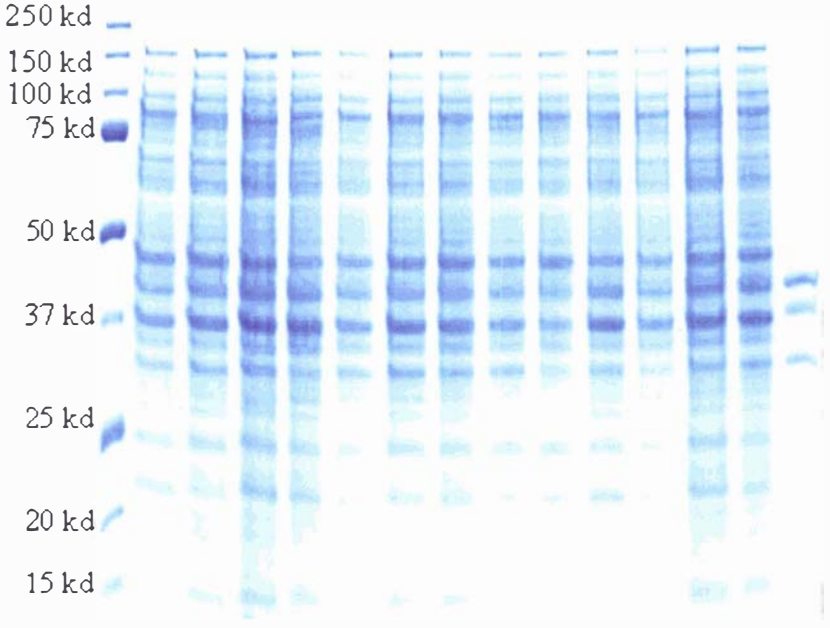


Fig. 3.14 Analysis of whole cell protein banding pattern of NZ BD isolates by SDS-PAGE. Lanes 1 and 15 contain Dual colour precision plus protein™ molecular size standards. Lanes 2 through 14 show patterns of isolates OT1, OT2, OT3, OT4, OT5, OT-enr, TB1, TB2, TB-enr, SP, MCB, MNA and MNZ1, respectively.

3.3.8 Characterisation of BD isolates using pulsed-field gel electrophoresis

The pulsotypes from 15 isolates of BD are shown in Figure 3.15. Following the standardized criteria for interpreting the PFGE patterns described by Tenover *et al.* (1995), all the isolates were deemed to be very closely related. The dendrogram of the pulsotypes of BD isolates is presented in Fig. 3.16. All the pulsotypes were branches around 0.8, which confirms that all the BD isolates tested were very closely related.

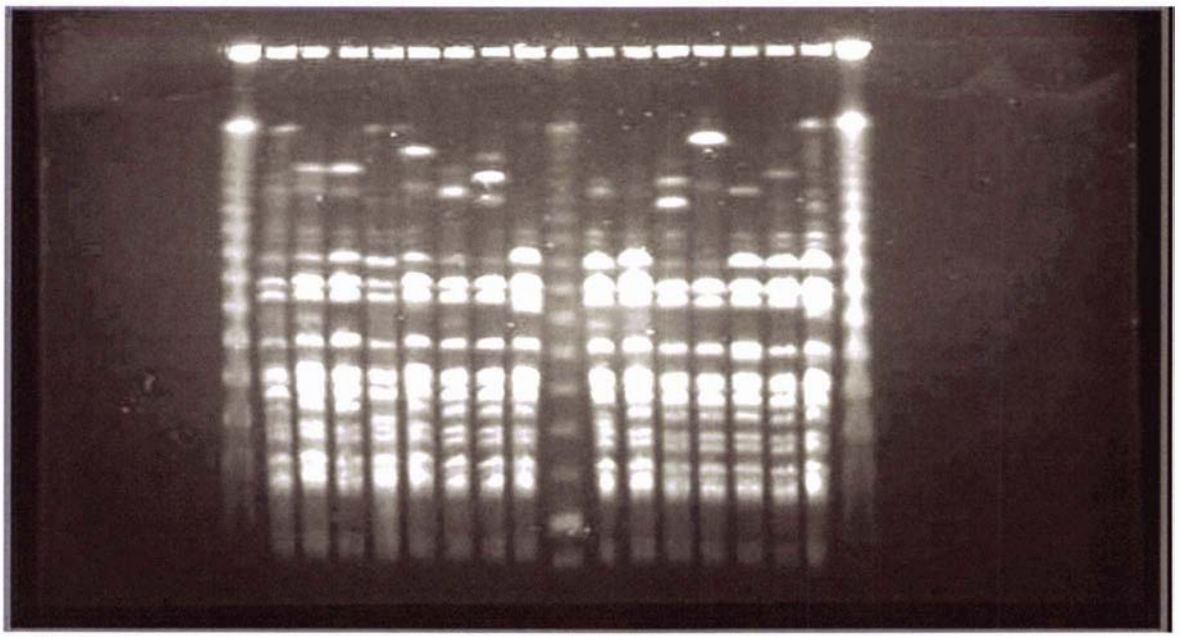


Fig. 3.15 Photograph of a PFGE gel of NZ BD isolates. Lanes 1 and 17 contain lambda ladder and lane 10 contains low range ladder. Lanes 2 through 9 show patterns of isolates OT1, OT2, OT3, OT4, OT5, OT-enr, TB1 and TB2. Lanes 11 through 17 show patterns of isolates SP, SP-enr, MCB, MCB-enr, MNA, TB-enr and MNZ1 respectively.

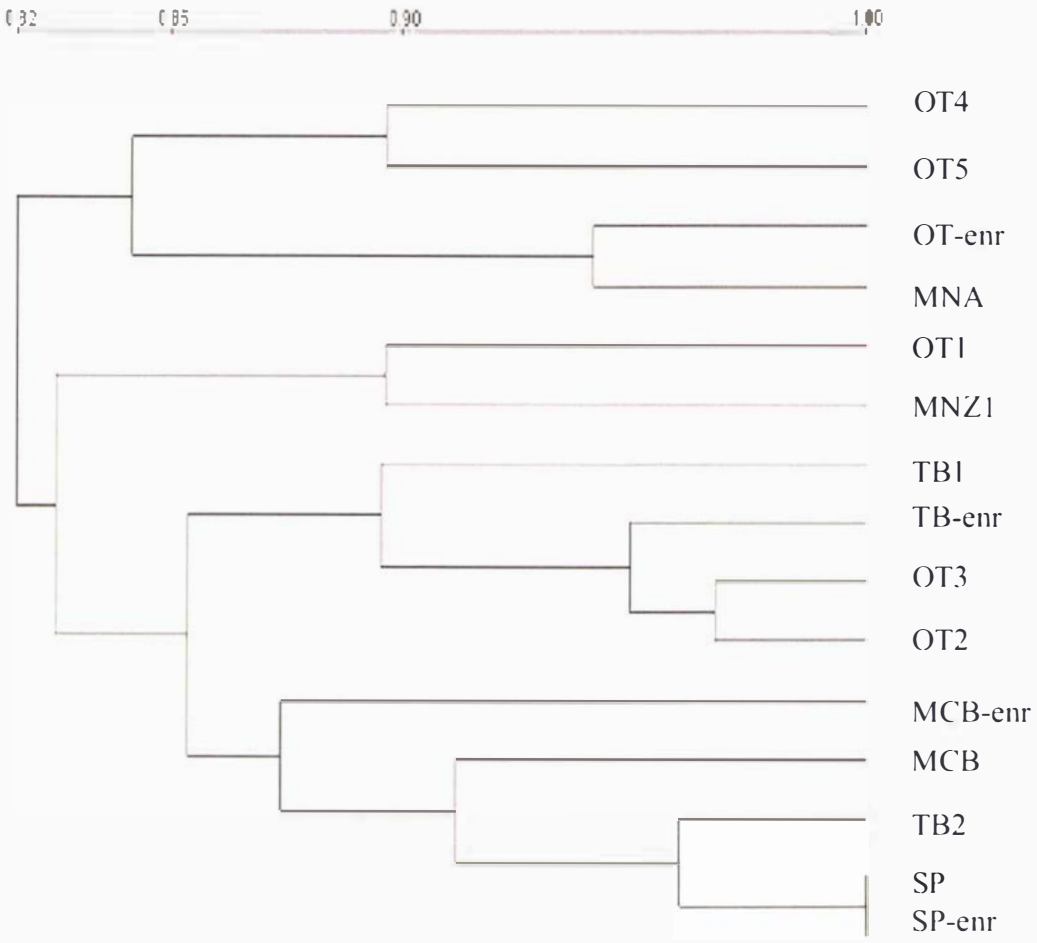


Fig. 3.16 Dendrogram of the pulsotypes of NZ BD isolates (OT1, OT2, OT3, OT4, OT5, OT-enr, TB1, TB2, SP, SP-enr, MCB, MCB-enr, MNA, TB-enr and MNZ1) using Diversity database software.

3.4 Discussion

The sample collection sites were selected based on availability, ease of access and laboratory suitability. Samples were collected during the winter and spring, 2004 (June to August and September to November). New Zealand's winter months bring colder weather and more rain to most areas of North Island and the average temperature ranges from 1°C to 14°C. The spring weather ranges from cold and frosty to warm and hot and the recorded water temperatures of 15°C – 19°C are not unusual. The recorded water salinities were also typical for New Zealand waters except for the one site where the salinity was recorded as 2.3% due to heavy rainfall before sample collection. Most of the collection points (14 out of 16) yielded BD plaques directly from the plates in almost pure form (Figs. 3.2 and 3.3), although the concentration of BD plaques varied between the water samples of different sites. Globally, variable number of BD have been recovered from sea water: these include (per litre of seawater) 40,000 - 50,000 off the coast of Israel (Shilo, 1966), 121 - 194 off the coast of Oahu, Hawaii (Taylor *et al.*, 1974), 24, 400 - 170,400 from an Aquarium in the USA (Schoeffield and Williams, 1990) and 0 - 76,260 off the coast and from aquaculture farms of Taiwan. In comparison, relatively high numbers of BD, (6,000 - 40,000) were isolated off the coast of New Zealand in this study (Table 3.3). *V. parahaemolyticus* was observed to be an efficient host bacterium in the recovery of BD suggesting an abundance of these predators in the spring months in New Zealand. However, on two occasions (OT-enr and TB-enr), enrichment techniques were needed for the isolation of BD. In the present study, collection of water samples for isolation of BD was made in only two seasons, leaving the question of their abundance in other seasons unanswered. The inability to isolate BD during the winter months suggested the presence of lesser numbers of BD in cold weather.

The purity verification of the BD plaques was inconsistent between the techniques used in this study. Gram stain reactions revealed pure Gram-negative coccobacillus cells for all the 16 isolates. However, the purity verification of pure BD cells of the 3 isolates (AH, KT and MB) using phase contrast microscopy and the SWYE plate showed impurity even after 3 purifications using 0.8, 0.45 and 0.2 μm sequential filters (Table 3.3). The reasons for the failure of purifications are not known. It is possible that some small Gram-negative bacterial cells or host-

independent BD or BD-like cells were present in the samples, which could not be separated by 0.2 μm filters.

Reduction of turbidity of the dual cultures of suspected BD and prey cells is an indicator of prey cell lysis as well as an increase of the numbers of BD cells. In this study, the reduction of turbidity was routinely examined by absorbance measurement every 6 hours (OD~0.300 dropping to OD 0.055 – 0.120 at 610 nm) (Table 3.3), and this technique was found to be an accurate and effective step in recovering BD plaques either from the primary samples or maintenance flasks.

Following isolation, the next step was to determine whether the isolated plaques were caused by actual BD cells. The isolated BD cells were confirmed and characterised by fluorescence microscopy, electron microscopy, API-Zym enzymic reactions, antibiotic sensitivity test, partial and complete 16S rDNA sequencing and similarity searches in the data bank, SDS-PAGE and pulsed field gel electrophoresis.

In fluorescence microscopy, the observed presence of small, rod-shaped, highly motile cells is an established characterization of this organism (Stolp and Starr, 1963; Abram and Davis, 1970; Burnham and Robinson, 1974) and bdelloplasts were observed, which is one of the unique features of BD (Williams *et al.*, 2003) (Figs. 3.4 and 3.5). The findings from this study confirmed that this technique is suitable for testing the purity of BD isolate and to determine the viability of BD cells, which could be an essential step for any future application study of BD isolates. This technique also validated the use of sequential syringe filters, which is one of the routine techniques for the separation of pure BD cells from their host. The negatively stained specimens in the electron microscopy study revealed characteristic comma-shaped cells with a long flagellum and the formation of bdelloplasts and the intraperiplasmic invasion of an OT2 cell into *V. parahaemolyticus* (Figs. 3.6, 3.7 and 3.8), which is the prey bacterial species suggested by Schoeffield and Williams (1990) to provide the most efficient means of detection of BD in sea water samples. Since only one isolate of BD and its host have been examined in this study, the findings are restricted to this particular isolate, but it is reasonable to assume that other isolates, would have similar structural and topological features.

Of the nineteen enzymes tested, only two, cystine arylamidase and trypsin, yielded variable reactions (Table 3.4). Notwithstanding the fact that BD has been shown to lack many of the enzymes used in metabolic processes (Reichelt and Baumann, 1974; Gadkari and Stolp, 1975), all BD isolates showed positive reactions, for alkaline phosphatase, chymotrypsin, esterase, esterase lipase, leucine arylamidase, valine arylamidase, acid phosphatase and phosphohydrolase. This is consistent with the findings of Baer *et al.* (2004), except that they had negative reactions for valine aryladmidase and chymotrypsin.

In the antibiotic sensitivity profiles, among the twenty-nine antibiotics tested, BD exhibited resistance to seven (colistin, fusidic acid, sulfisoxazole, nalidixic acid, neomycin, cloxacillin and oxytetracycline) while exhibiting differential reactions to only four antibiotics (kanamycin, tobramycin, oxacillin and polymyxin B) (Table 3.5). Baer *et al.* (2004) reported the antibiogram profiles of 3 marine isolates and they revealed that two isolates (SJ and AQ) showed similar sensitivities to ampicillin, carbenicillin, kanamycin, gentamycin and polymixin B, but were also resistant to methicillin, nalidixic acid, colistin sulphate and vancomycin. They observed variable reactions of the isolate JS5 against carbenicillin, gentamycin and polymixin B and resistant reaction against kanamycin.

Complete and partial 16S rDNA sequencing using universal and BD specific primers showed that the BD isolates were phylogenetically similar to each other and closely related to overseas isolates of *Bacteriovorax* sp. OC71, *Bacteriovorax* sp. DA5 and *Bacteriovorax* sp. NB2 as verified by the Basic Local Alignment Search Tool (BLAST) programme (http:// www.ncbi.Nlm.nih.gov/BLAST/) (Table 3.6; Appendices 3.D.1 to 3.D.7; Fig. 3.12) and by estimation of the phylogenetic relationships of NZ BD isolates and overseas BD strains using PAUP (Phylogenetic analysis using parsimony, version 4.0) (Fig. 3.13). This confirmed that the isolates belong to the *Bacteriovorax* genus. This is consistent with the findings of Donze *et al.* (1991) and Baer *et al.* (2000) that strong similarities between *Bacteriovorax* group of BD strains could be proved with partial 16S rRNA and complete 16S rDNA sequencing respectively. Subsequently, Baer *et al.* (2004) reclassified the *Bacteriovorax* genus into two species based on the characterisation of 3 marine isolates (SJ, AQ and JS5) as *Bacteriovorax marinus* (SJ and AQ)

and *Bacteriovorax litoralis* (JS5). The phylogenetic tree analysis confirmed that the New Zealand BD isolates are most closely related to *Bacteriovorax* sp. OC71. However, the species identification of *Bacteriovorax* sp. OC71, is not reported in the literature. The present results confirmed that NZ BD isolates are not genetically diverse between each other and are closely related to overseas BD isolates, which is in agreement with the global survey of saltwater BD from different regions of the world including New Zealand (8 NZ BD isolates such as MANZ2, MANZ5, MNZ1, MNZ3, NZ27, NZ9, NZ14 and NZAH13) by Piñeiro *et al.* (2007) using small subunit rRNA (ssu-rRNA) gene sequencing. They confirmed that the NZ BD isolates listed were in the same ribotype cluster with a maximum genetic distance of 0.025. They also isolated NZ7, which consisted of only a single isolate that did not align with any other clusters. They also confirmed that NZ-BD isolates have closely related ssu-rRNA sequencing patterns to USA- BD isolates, but they are not identical, suggesting a wide geographical distribution of similar ribotypic clusters of saltwater BD.

The protein banding patterns of the BD isolates were studied using SDS-PAGE. The bands were very similar among the BD strains (Fig. 3.14). The molecular sizes of the bands ranged between 15 and 150 kD.

It is now well established that pulsed field gel electrophoresis is a highly effective molecular fingerprinting method for discriminating bacterial strains. To date however, very little work has been performed on the subtyping and epidemiological study of BD isolates. Williams *et al.* (2003) described the discriminatory capability of PFGE on five prey-independent strains of *B. bacteriovorus*, two species of *Bacteriovorax*, one environmental terrestrial isolate, and two halophilic *Bdellovibrio*-like organisms using restriction enzymes *Asc*I, *Fse*I and *Not*I. They observed that the restriction patterns produced for the *B. bacteriovorus* were similar, but they observed ± 2-3 band differences between the strains.

In this study *Sma*I restriction enzyme was used for digesting the agarose embedded chromosomal DNA of BD. The result showed that *Sma*I restriction enzyme produced 12-16 bands sufficient for discrimination between isolates. The PFGE patterns of all the BD isolates were deemed to be very closely related (Fig. 3.15). The dendrogram of the pulsotypes had branches around 0.8,

which confirms that all the BD isolates tested were virtually identical (Fig. 3.16). The main disadvantages of the PFGE technique is that it requires a long and tedious procedure for isolating and restricting the genomic DNA. It also requires large quantities of expensive enzymes and reagents. Only one restriction enzyme has been used in this study, therefore the findings must be restricted and future studies using other restriction enzymes will be useful for the validation of this technique.

It is concluded that of the techniques performed to characterize and identify the isolates of BD, the fluorescence microscopy, electron microscopy, API-Zym enzymic reactions, partial and complete 16S rDNA sequencing and similarity searches in the data bank, SDS-PAGE and pulsed field gel electrophoresis established the identity of BD and the results are consistent with those of other researchers, which proved their integrity. The phylogenetic tree analysis confirmed that New Zealand BD isolates are not diverse between each other but the distribution of saltwater BD is genetically diverse in many continents. Although, they are different from overseas isolates, they are closely related to *Bacteriovorax* sp. OC71. Additionally, pulsed field gel electrophoresis showed that the BD isolates were virtually identical or very closely related. The antibiotic sensitivity profile tests differed from those of Baer *et al.* (2004) however, and further determinations may be necessary to prove the integrity of this technique.

Chapter 4

Predation pattern of BD against some pathogenic and spoilage organisms in solid and liquid media

4.1 Introduction

Bdellovibrionaceae, the obligate bacterial predators of bacteria, are attracting much attention in the food industry for their variability in predation pattern. Each predator can parasitise a few, or in some cases, many different Gram-negative host bacteria (Jurkevitch *et al.*, 2000; Sutton and Besant, 1994). The basic ability of terrestrial BD isolates to attack and lyse host bacteria indicates their potential to be biological control agents against Gram-negative phytopathogens (Epton *et al.*, 1989) or food-borne pathogenic and spoilage bacteria (Fratamico and Whiting, 1995). While the predation pattern of marine BD isolates (reviewed in Chapter 1) has been reported by a few researchers (Taylor *et al.*, 1974; Marbach *et al.*, 1976; Schoeffield and Williams, 1990; Sutton and Besant, 1994; Marbach *et al.*, 1976; Piñeiro *et al.*, 2004), there has, until now, been no detailed study in which researchers have compared the predation pattern and efficiencies of marine BD isolates against important seafood spoilage and pathogenic microbes at different temperatures using different parameters.

In this study, the New Zealand BD isolates have already been confirmed as taxonomically identical or closely related to each other (Chapter 3). Subsequently, the predation pattern of the BD isolates was examined against a variety of marine and terrestrial microbes in solid and liquid media, to establish the usefulness of BD as a biological control agent. To accomplish this, the following parameters were investigated:

- The predation pattern of BD against some common seafood and terrestrial spoilage and pathogenic microbes;
- whether the different BD isolates exhibit any differences in their predation patterns;
- the effect of temperature on predation;
- the quantified effect of a New Zealand BD isolate against twelve seafood pathogenic and spoilage organisms in liquid medium.

4.2 Experimental procedure

4.2.1 BD predation of pathogenic and spoilage bacteria in solid medium

The predation patterns of 13 New Zealand BD isolates (Appendix 2.D) were examined using lawns of host organisms in polypeptone 20 (Pp20) double agar overlay plates. The procedure used followed that described by Piñeiro *et al.* (2004) with some minor exceptions. The tested organisms used in this study were marine Gram-negative spoilage and pathogenic bacteria e.g., *Acinetobacter johnsonii*, *Aeromonas hydrophilia*, *Enterobacter cloacae*, *Morganella morganii*, *Photobacterium phosphoreum*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Pseudomonas cepacia*, *Pseudomonas fluorescens*, *Pseudomonas mendocina*, , *Pseudomonas pseudomallei*, *Shewanella putrefaciens*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*; marine Gram-positive pathogenic bacteria such as *Listeria monocytogenes* and terrestrial pathogenic bacteria such as *Escherichia coli* B, *Escherichia coli* ML 35 and *Salmonella typhimurium*. The culture ID and isolation sources of these organisms are listed in Appendix 2.B.

Pure cultures of the BD isolates were grown in a medium of 50 mL of 70% ASW and 1 mL of host *Vibrio parahaemolyticus* incubated in an orbital shaker (130 rpm) at 25°C for 2 days. The BD cultures were purified as described in Section 2.2 and were concentrated by centrifugation at 20,800 x g for 40 min. The cells were then re-suspended in an appropriate amount of the supernatant to give a final concentration of approximately 10^7 PFU/mL. The spoilage and pathogenic organisms under test were streaked onto a SWYE plate to obtain single colonies. Triplicate lawns of each tested organism were prepared by streaking a single colony onto the SWYE plate and incubating at 30°C for 24 h. The lawn of organisms was harvested in 3 mL of 70% ASW. To prepare lawns of each bacterium on Pp20 double agar overlay plates, 1 mL of each culture was added to 3 mL of molten Pp20 top agar and overlaid onto Pp20 bottom agar and allowed to solidify. The plates were incubated at 30°C for 1-2 days and only those host organisms that formed a lawn on the plates were used. Pp20 plates with lawns were divided into 8 quadrants, onto each of which 10 µL of a BD isolate were dropped. Plates were incubated at 20°C, 25°C, 30°C or 37°C, uninverted, for 24 h to allow the BD suspensions to be absorbed into the top agar. Plates were then inverted and incubated for a further 72 h. The plates were

examined daily for the appearance of a clearing zone in and around the dropping area of the BD isolates. A clearing zone was reported as positive, while the absence of clearing zone was recorded as negative. The inhibition zones were measured using an ordinary scale. Three trials were performed and the results were recorded as the average of the results obtained from these experiments.

4.2.2 BD predation of pathogenic and spoilage organisms in liquid medium

The spoilage and pathogenic strains used in this study were *Enterobacter cloacae*, *Morganella morganii*, *Pseudomonas aeruginosa*, *Pseudomonas cepacia*, *Pseudomonas fluorescens*, *Pseudomonas mendocina*, *Photobacterium phosphoreum*, *Pseudomonas pseudomallei*, *Proteus vulgaris*, *Shewanella putrefaciens*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*. The Crop & Food Research culture collection ID and isolation sources of these organisms are listed in Appendix 2.C. The BD isolate used in this trial was OT2. This isolate was selected on the basis of the experiments described in section 5.3.2 and it was assumed that the predation efficiency of OT2 would be similar to or better than those of the other BD isolates. The tested organisms were chosen in view of the future directions of this study, which would be focussed on seafood safety. The challenge inocula of the above prey cultures were prepared as described in Section 2.6. The OT2 culture was purified, propagated, and large-scale challenge inocula were prepared, as described in Sections 2.2 and 2.5.

Fifty millilitres of 70% sterile ASW was aseptically transferred to each of twenty four 250 mL Erlenmeyer flasks. One mL of each harvested culture (~ 9.7 $\log_{10}$ CFU/mL) was inoculated into one treatment and one control flask to give a final concentration of approximately 8.9 $\log_{10}$ CFU/mL. One mL of OT2 challenge inoculum (approximately 8.6 $\log_{10}$ PFU/mL) was added to 12 treatment flasks, while the other 12 were kept as controls. Flasks were incubated at 25°C in an orbital shaker (130 rpm). At time points zero, 6, 12 and 24 h, 4 mL aliquots were removed aseptically from each flask. The absorbance levels of the aliquots were measured at 610 nm using a spectrophotometer (Genova MK3 uv/visible spectrophotometer, Jenway Ltd, England) and the tested organisms were enumerated on SWYE plates (Section 2.7).

4.3 Results

4.3.1 BD predation of pathogenic and spoilage bacteria in solid medium

The findings demonstrated that all of the BD isolates tested gave the same predation patterns as each other (Appendices 4.A – 4.L); therefore, results are presented as a summary (Table 4.1). The isolates were effective against *A. johnsonii*, *A. hydrophilia*, *E. cloacae*, *M. morganii*, *P. phosphoreum*, *P. vulgaris*, *P. aeruginosa*, *P. cepacia*, *P. fluorescens*, *V. parahaemolyticus* and *V. vulnificus*, but were not effective against *L. monocytogenes*, *P. mendocina*, *P. pseudomallei* or *S. putrefaciens*. There was no consistent predatory activity against *E. coli* B, *E. coli* ML35 or *S.* Typhimurium although, some variable results were observed. The predatory capability varied at different temperatures, and the greatest effects were observed at 25°C and 30°C compared to 20°C and 37°C (Table 4.1). The predation effect was slower at 20°C and highly variable at 37°C.

Examples of the positive and negative results are shown in Fig. 4.1, 4.2 and 4.3. The inhibition zones produced by BD isolates were ~10 mm in diameter and similar clearing zones were observed in the predation by all BD isolates (Figs. 4.1 and 4.2).

Table 4.1 A summary of the predation pattern of all NZ BD isolates against some marine Gram-negative spoilage and pathogenic bacteria, a marine Gram-positive pathogenic bacteria and some terrestrial pathogenic bacteria using lawns of tested organisms in polypeptone 20 (Pp20) double agar overlay plates after 24, 48 and 72 h of incubation at 20°, 25°, 30° and 37°C.

Host bacteria	20°C			25°C			30°C			37°C		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
A. johnsonii	v	+	+	+	+	+	+	+	+	-	v	v
A. hydrophilia	v	+	+	+	+	+	+	+	+	v	v	v
E. cloacae	v	+	+	+	+	+	+	+	+	v	v	v
E. coli B	-	-	-	-	-	v	-	-	V	-	v	v
E. coli ML 35	-	-	-	-	-	v	-	-	V	-	v	v
L. monocytogenes	-	-	-	-	-	-	-	-	-	-	-	-
M. morganii	v	+	+	+	+	+	+	+	+	v	v	v
P. phosphoreum	+	+	+	+	+	+	+	+	+	v	+	+
P. vulgaris	v	+	+	+	+	+	+	+	+	v	v	v
P. aeruginosa	v	v	v	+	+	+	+	+	+	-	v	+
P. cepacia	+	+	+	+	+	+	+	+	+	-	v	v
P. fluorescens	v	+	+	+	+	+	+	+	+	-	v	v
P. mendocina	-	-	-	-	-	-	-	-	-	-	-	-
P. pseudomallei	-	-	-	-	-	-	-	-	-	-	-	-
S. Typhimurium	-	-	-	-	-	-	-	v	v	-	v	v
S. putrefaciens	-	-	-	-	-	-	-	-	-	-	-	-
V. parahaemolyticus	+	+	+	+	+	+	+	+	+	v	v	v
V. vulnificus	+	+	+	+	+	+	+	+	+	-	-	-

'+' means positive predation in all 3 replicate experiments, 'v' means the 3 replicate experiments gave variable predation, sometimes positive and sometimes negative , '-' means no predation observed in any of the 3 replicate experiments. All NZ BD isolates gave the same results.

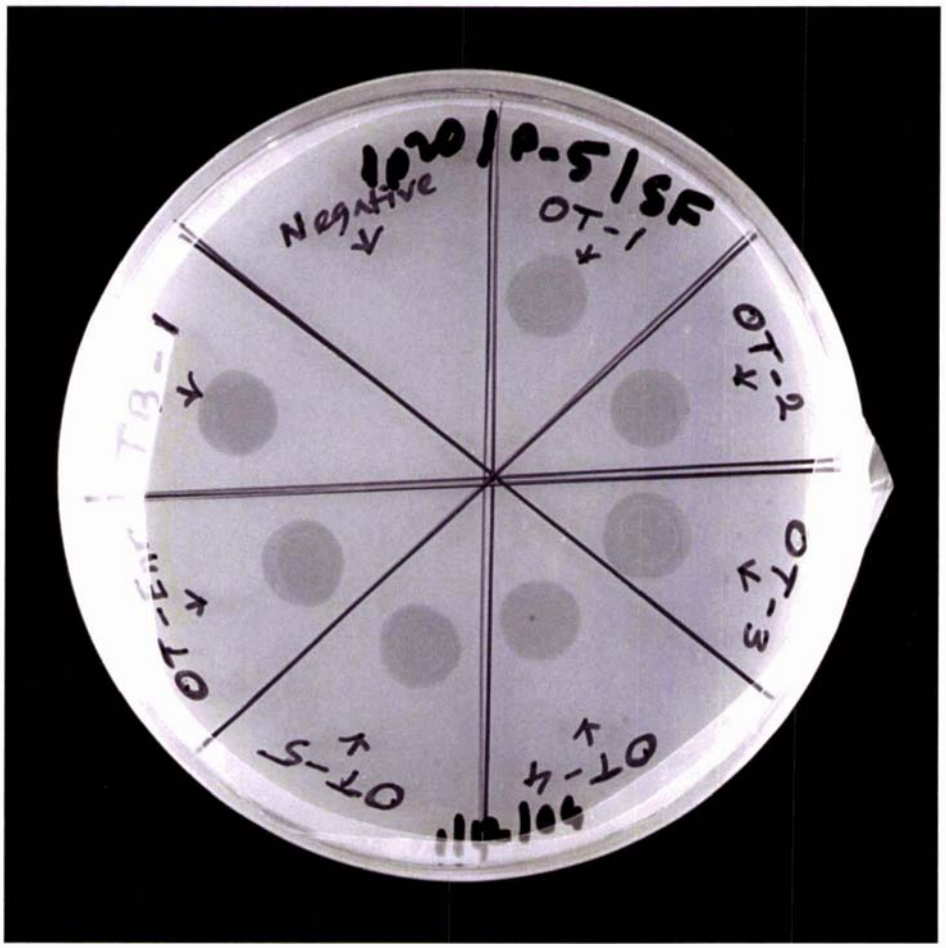


Fig. 4.1 Predation capability of BD isolates (OT1, OT2, OT3, OT4, OT5, OT-enr, TB1 and control sample) against *V. parahaemolyticus* in polypeptone 20 (Pp20) double agar overlay plates. The positive results are indicated by clearing or lysis of lawn of tested organism. Negative means no BD added and the results showed no lysis of the lawn of tested organism. Inhibition zone = ~10mm in diameter.

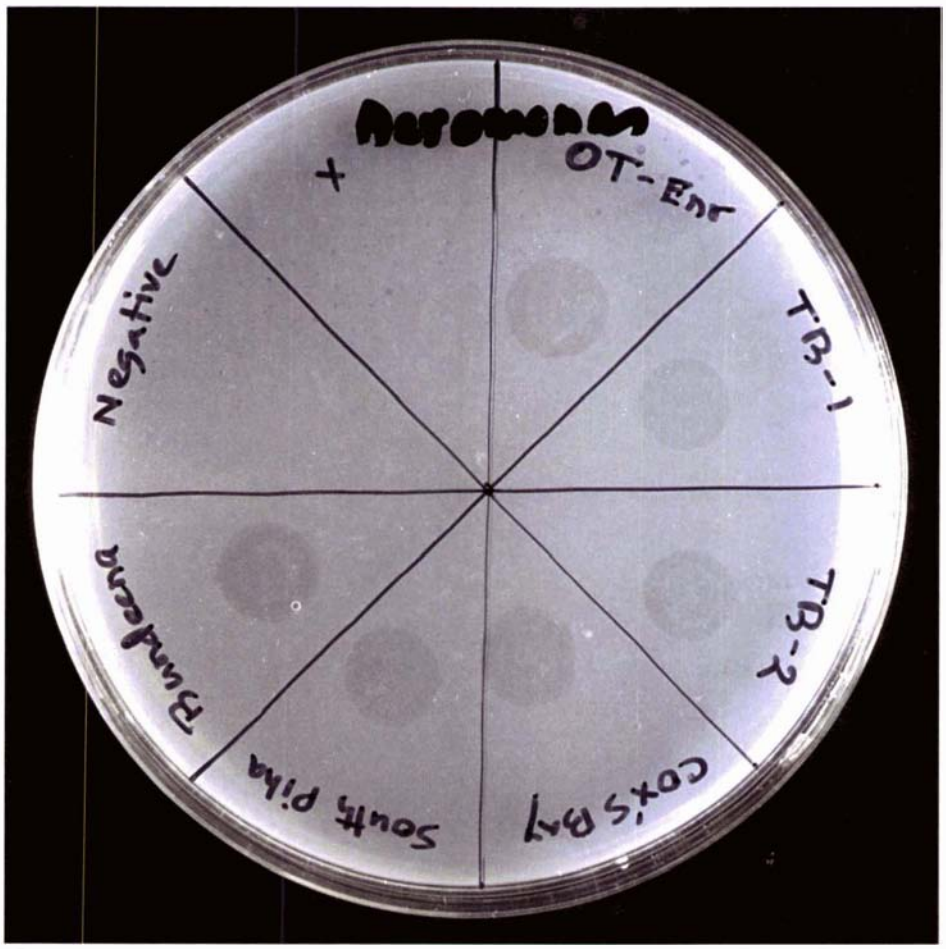


Fig. 4.2 Predation capability of BD isolates (OT-enr, TB1, TB2, MCB, SP, Bundeena and control sample) against *A. hydrophilia* in polypeptone 20 (Pp20) double agar overlay plates. The positive results are indicated by clearing or lysis of the lawn of the tested organism. Negative means no BD added and the results showed no lysis of the lawn of tested organism. Inhibition zones ≈10 mm in diameter. Bundeena was an isolate from New South Wales, Australia which was not studied further in the current research.

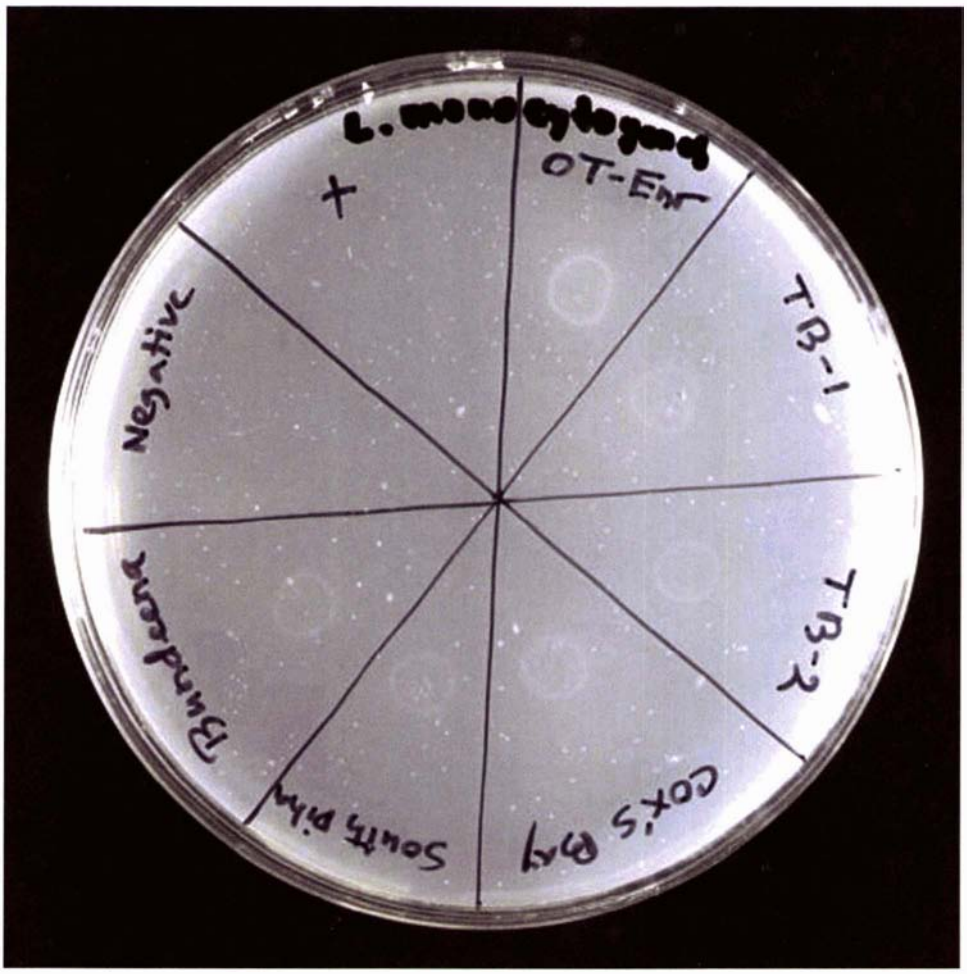


Fig. 4.3 Predation capability of BD isolates (OT-enr, TB1, TB2, MCB, SP, Bundeena and control sample) against *L. monocytogenes* in polypeptone 20 (Pp20) double agar overlay plates. Negative means no BD added and the results showed no lysis of the lawn of tested organism. Bundeena was an isolate from New South Wales, Australia which was not studied further in the current research.

4.3.2 BD predation of pathogenic and spoilage organisms in liquid medium

The ability of OT2 to reduce the absorbance of the dual cultures of twelve pathogenic and spoilage organisms after 6, 12 and 24 h incubation at 25°C is summarised in Table 4.2. After 6 h of incubation, the highest reductions in absorbance were observed in cultures of *E. cloacae*, *P. phosphoreum*, *V. parahaemolyticus*, and *V. vulnificus*. Although a moderate reduction in absorbance was observed in cultures of *P. fluorescens*, *P. cepacia*, *P. aeruginosa* and *M. morganii*, minimal or no detectable reductions were observed in the other cultures. Similar trends in reductions were observed after 12 h of incubation, as well as for *P. vulgaris*, and also after 24 h of incubation. No detectable changes in absorbance over the entire 24 h period of incubation were detected in dual cultures containing *P. mendocina*, *P. pseudomallei*, *S. putrefaciens* or in control samples.

The effect of OT2 on the population of the twelve tested organisms as assessed on SWYE plates is summarized in Figs. 4.4, 4.5, 4.6, 4.7 and Appendix 4.M. After 6 h of incubation, the highest reduction effect (~4 $\log_{10}$ unit) was observed in the viability of *P. phosphoreum*, *V. parahaemolyticus*, *V. vulnificus* and *E. cloacae*. A moderate reduction effect of ~ 2 $\log_{10}$ unit was detected in the viability of *P. fluorescens*, *P. cepacia*, *P. aeruginosa*, *M. morganii* and *P. vulgaris*. Similar effects were observed after 12 h of incubation. However, after 24 h of incubation, reductions in the mean populations of the above nine tested organisms were similar. *P. mendocina*, *P. pseudomallei*, *S. putrefaciens* and control samples exhibited little or no reductions in their total viable numbers after 6, 12 or 24 h of incubation at 25°C.

Table 4.2 The effectiveness of BD isolate OT2 (8.6 log_{10} PFU/mL) in reducing absorbance of different spoilage and pathogenic organisms after challenging for 24 hours in 70% ASW at 25°C. Absorbances were measured using a spectrophotometer at a wavelength of 610 nm.

	Initial absorbance[b]	Absorbance changes[c]		
Tested organisms[a]	0 h	6 h	12 h	24 h
E. cloacae	0.34	0.01	0.01	0.02
E. cloacae + *BD*	0.35	0.20	0.22	0.25
M. morganii	0.33	0.01	0.01	0.01
M. morganii + *BD*	0.33	0.10	0.16	0.23
P. aeruginosa	0.29	0.01	0.02	0.01
P. aeruginosa + *BD*	0.29	0.07	0.17	0.20
P. cepacia	0.33	0.00	0.01	0.02
P. cepacia + *BD*	0.33	0.11	0.17	0.24
P. fluorescens	0.34	0.00	0.01	0.01
P. fluorescens + *BD*	0.33	0.11	0.16	0.25
P. mendocina	0.31	0.00	0.01	0.01
P. mendocina + *BD*	0.30	-0.01	0.00	0.00
P. phosphoreum	0.32	0.00	0.00	0.01
P. phosphoreum + *BD*	0.32	0.20	0.22	0.23
P. pseudomallei	0.32	0.00	0.00	0.00
P. pseudomallei + *BD*	0.32	0.00	0.00	0.01
P. vulgaris	0.30	0.00	0.01	0.01
P. vulgaris + *BD*	0.30	0.03	0.18	0.21
S. putrefaciens	0.28	0.00	0.00	0.00
S. putrefaciens + *BD*	0.29	0.00	0.00	0.01
V. parahaemolyticus	0.29	0.00	0.01	0.01
V. parahaemolyticus + *BD*	0.29	0.21	0.22	0.23
V. vulnificus	0.28	0.00	0.00	0.00
V. vulnificus + *BD*	0.28	0.20	0.21	0.21

[a] *Prey organisms were either grown alone in monoculture (control) or in dual culture with BD (BD +)*

[b] *Absorbance (OD at 610 nm) of either tested organism alone (control) or dual culture of BD isolate and tested organisms at 0 h*

[c] *Mean absorbance (n=3) of either tested organism alone (control) or BD isolate and tested organisms at time 0 h minus mean absorbance of the same tested organism (control) or dual cultures of the same after 6, 12 h or 24 h at 25°C*

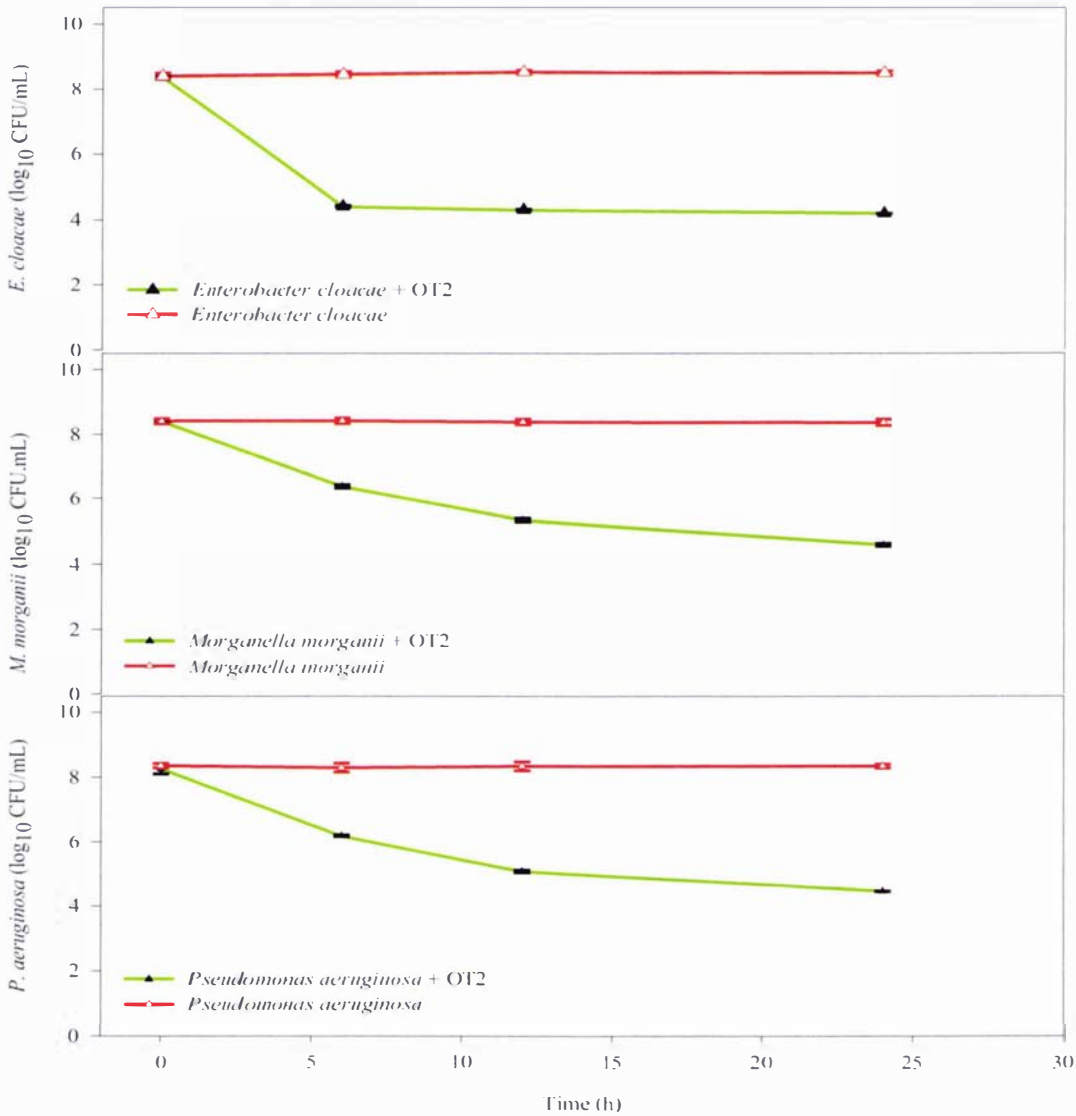


Fig. 4.4 The effectiveness of isolate OT2 (8.6 $\log_{10}$ PFU/mL) in reducing the population of *Enterobacter cloacae* (8.6 $\log_{10}$ CFU/mL), *Morganella morganii* (8.7 $\log_{10}$ CFU/mL) and *Pseudomonas aeruginosa* (8.7 $\log_{10}$ CFU/mL) in 70% ASW during a 24 h challenge at 25°C. Samples were tested after 0, 6, 12 and 24 h. Values are presented as mean ± standard error, n = 3.

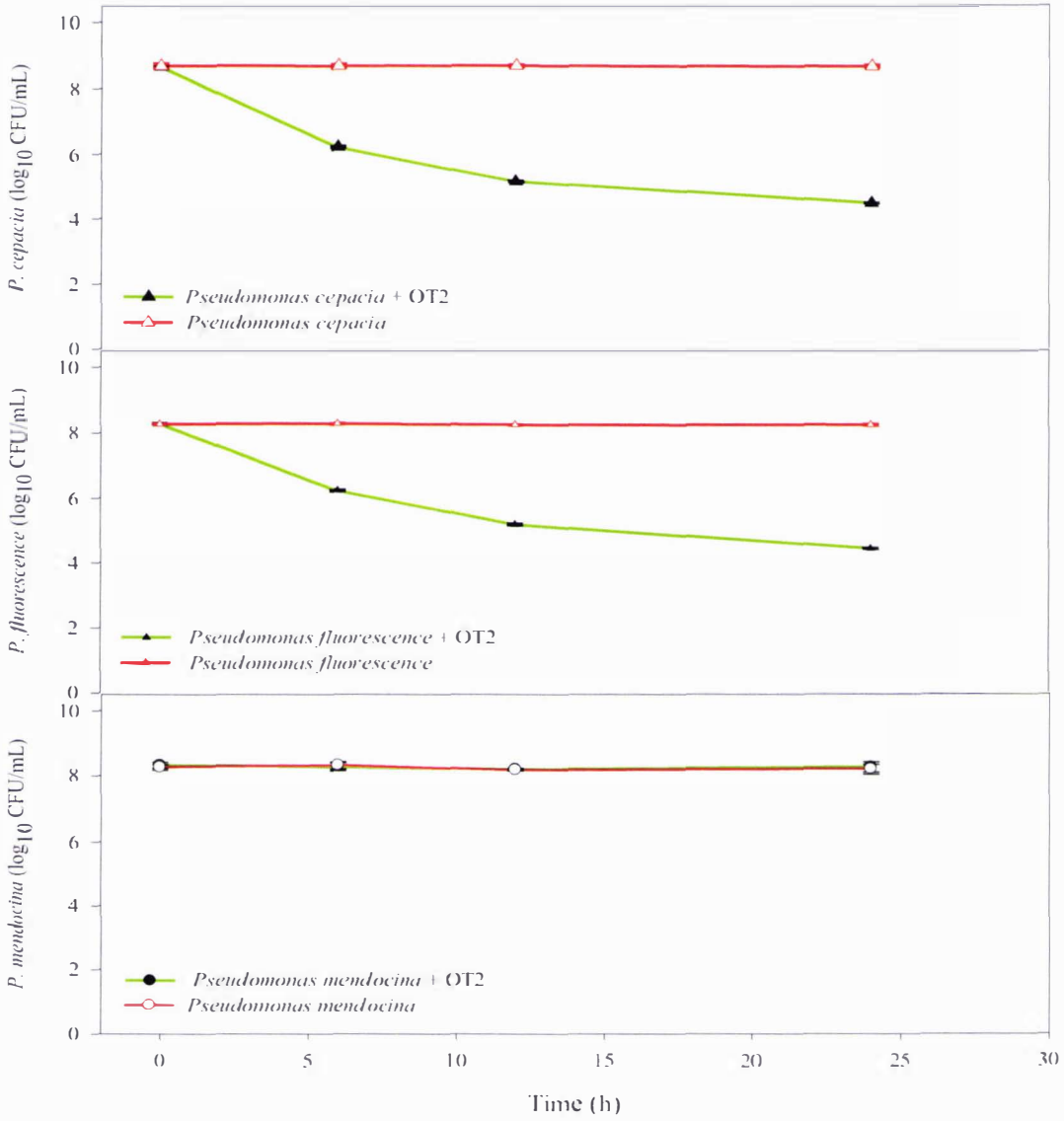


Fig. 4.5 The effectiveness of OT2 (8.6 $\log_{10}$ PFU/mL) in reducing the population of *Pseudomonas cepacia* (8.7 $\log_{10}$ CFU/mL), *Pseudomonas fluorescens* (8.6 $\log_{10}$ CFU/mL) and *Pseudomonas mendocina* (8.5 $\log_{10}$ CFU/mL) in 70% ASW during a 24 h challenge at 25°C. Samples were tested after 0, 6, 12 and 24 h. Values are presented as mean ± standard error, n = 3.

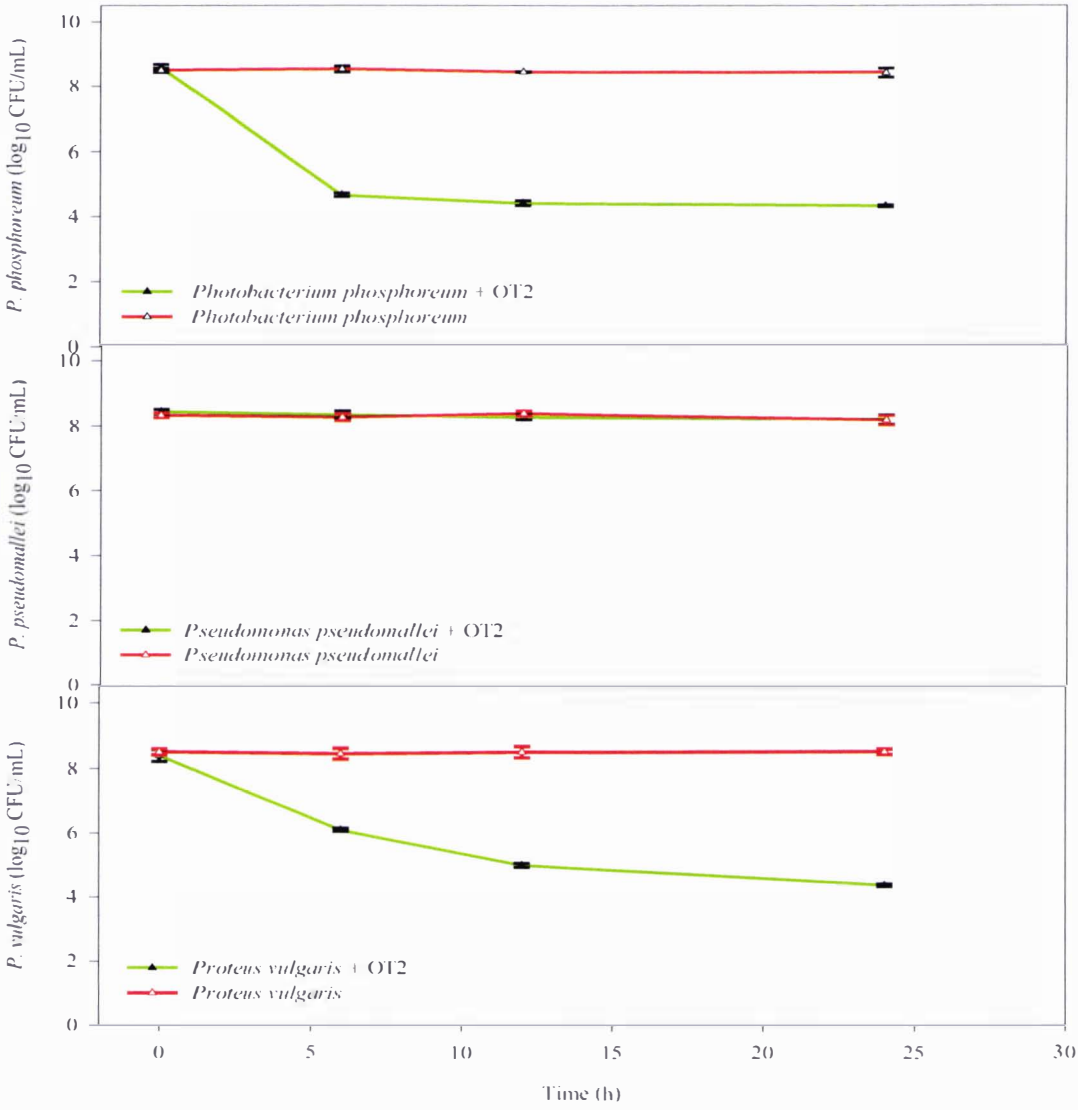


Fig. 4.6 The effectiveness of isolate OT2 (8.6 $\log_{10}$ PFU/mL) in reducing the population of *Photobacterium phosphoreum* (8.8 $\log_{10}$ CFU/mL), *Pseudomonas pseudomallei* (8.6 $\log_{10}$ CFU/mL) and *Proteus vulgaris* (8.8 $\log_{10}$ CFU/mL) in 70% ASW during a 24 h challenge at 25°C. Samples were tested after 0, 6, 12 and 24 h. Values are presented as mean ± standard error, n = 3.

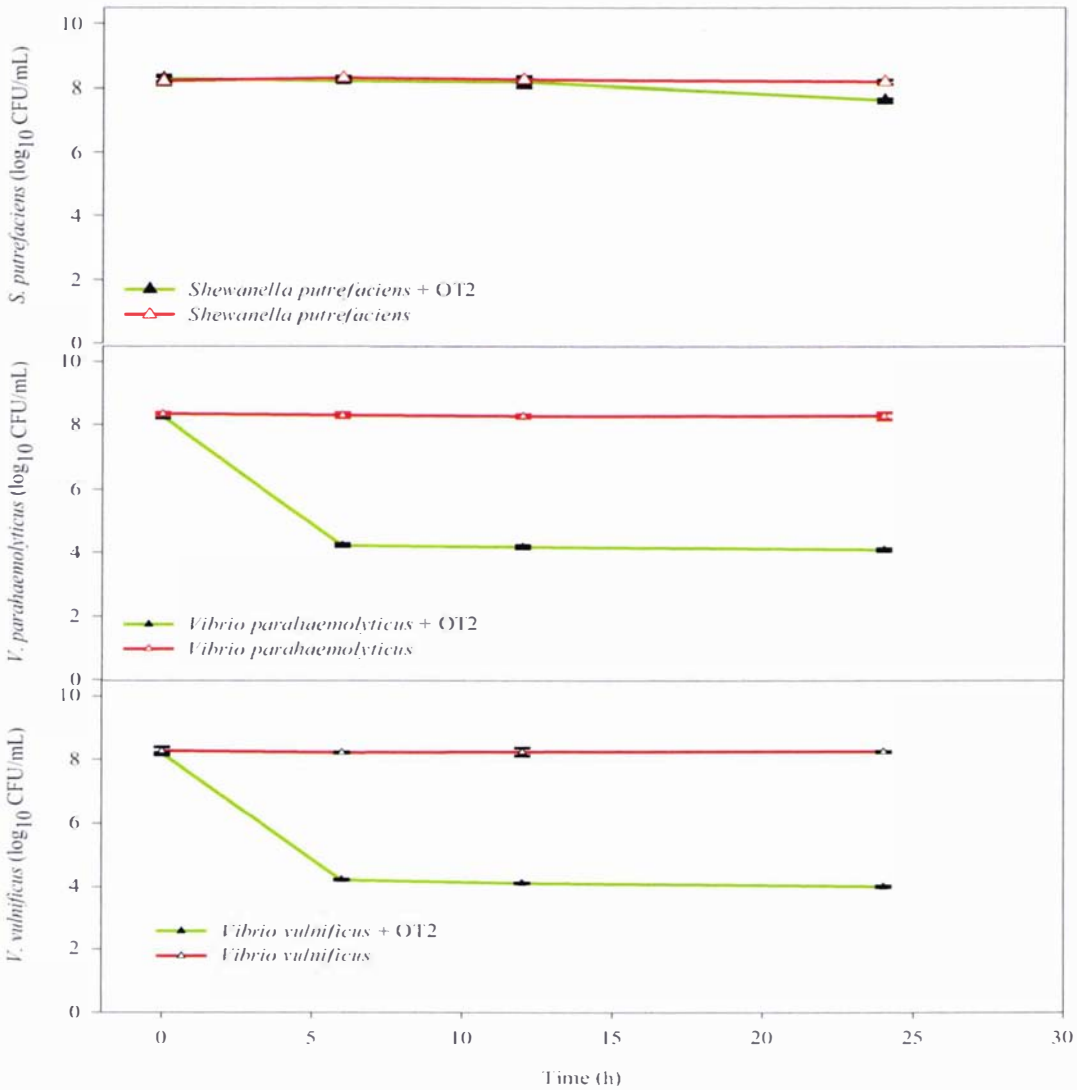


Fig. 4.7 The effectiveness of isolate OT2 (8.6 $\log_{10}$ PFU/mL) in reducing the population of *Shewanella putrefaciens* (8.5 $\log_{10}$ CFU/mL), *Vibrio parahaemolyticus* (8.6 $\log_{10}$ CFU/mL) and *V. vulnificus* (8.8 $\log_{10}$ CFU/mL) in 70% ASW during a 24 h challenge at 25°C. Samples were tested after 0, 6, 12 and 24 h. Values are presented as mean ± standard error, n = 3.

4.4 Discussion

4.4.1 BD predation of pathogenic and spoilage bacteria in solid medium

This study was designed to determine the predation pattern of several BD isolates in solid medium. The predation patterns obtained support the view that the isolates are identical or closely related to each other as shown in the taxonomic characterisation (Chapter 3). The observations showed that the BD isolates can attack many but not all Gram-negative bacteria. They can attack *A. johnsonii*, *A. hydrophilia*, *E. cloacae*, *M. morganii*, *P. phosphoreum*, *P. vulgaris*, *P. aeruginosa*, *P. cepacia*, *P. fluorescens*, *V. parahaemolyticus* and *V. vulnificus*, but cannot attack *P. mendocina*, *P. pseudomallei*, *S. putrefaciens* or the Gram positive *L. monocytogenes* (Table 4.1; Appendices 4.A – 4.L). The results are in contrast to those of Sutton and Besant (1994) and Taylor *et al.* (1974), whose BD isolates were not effective against *P. aeruginosa*, but were similar to their findings on the predatory effects against *Photobacterium* and *Vibrio*, and the lack of predatory effect against *E. coli*.

The present results confirmed that marine BD cannot effectively attack terrestrial isolates such as *E. coli* B, *E. coli* ML35 or *S.* Typhimurium (Table 4.1). The reasons for the ineffectiveness against terrestrial isolates are not known but clearly need further investigation. It is possible that the medium used for this study suited only the attack of marine isolates.

The most effective predation temperatures were observed at 25°C and 30°C, which is in keeping with the mesophilic character of BD (Table 4.1; Appendices 4.A – 4.L).

4.4.2 BD predation of pathogenic and spoilage organisms in liquid medium

The predation patterns and efficiencies of BD isolates against seafood pathogenic and spoilage organisms in solid and liquid media were similar, confirming that BD isolates are selective in their choice of prey. Piñeiro *et al.* (2004) observed different predation patterns on agar plates by different isolates of BD against different Gram-negative bacteria isolated from the high saline environment of the Great Salt Lake, Utah, USA. These authors suggested the probability of results being influenced by growth media and incubation temperatures.

The present findings (Table 4.2; Figs. 4.4, 4.5, 4.6 and 4.7) confirm that a New Zealand BD isolate (OT2) is effective against *P. phosphoreum*, *V. parahaemolyticus*, *V. vulnificus*, *E. cloacae*, *P. fluorescens*, *P. cepacia*, *P. aeruginosa*, *M. morganii* and *P. vulgaris*, but is not effective against *P. mendocina*, *P. pseudomallei* or *S. putrefaciens*. The predation efficiencies of BD were also found to vary among the different prey organisms. BD quickly attacked *P. phosphoreum*, *V. parahaemolyticus*, *V. vulnificus* and *E. cloacae*, but only slowly attacked *P. fluorescens*, *P. cepacia*, *P. aeruginosa*, *M. morganii* and *P. vulgaris*. Marbach *et al.* (1976) also observed differences in the predation of BD isolates against different Gram-negative bacteria. Taylor *et al.* (1974) examined the predation capability of BD isolates against 42 species of facultatively anaerobic marine bacteria and they were able to parasitize 17-32 of the species. Jurkevitch *et al.* (2000) challenged 22 phytopathogens with BD isolates and their selection of hosts varied from 6 – 10 of the pathogens tested. Rogosky *et. al.* (2006) observed that BD efficiently killed *Pantoea agglomerans, E. coli* and *Serratia marcescens* but was less effective against *Enterobacter aerogenes*, *Erwinia carotovora* ssp. *Carotovora* or *Salmonella enterica* LT2. Thus, the results of the present study are in agreement with the observations of overseas studies that BD can prey on various Gram-negative bacteria but not all. The reasons for the variability in selected Gram-negative bacteria are not known. Rogosky *et al.* (2006) observed variable results in the predation pattern of BD isolates and concluded that the degree of domestication of the prey does not easily explain the predation difference. It is possible that BD interacts with specific receptors, providing specificity, as well as interacting with more general components of the cell well.

As stated above, the reason why some organisms are predated and others are not is unknown at this time. The reason for the different rates of predation is also unknown. Several groups of investigators (Schoeffield and Williams, 1990; Varon and Shilo, 1969b; Luderitz *et al.*, 1966), however, have implicated the R antigen of the lipopolysaccharide layer of Gram-negative bacterial cell walls as the component necessary for specific interaction with BD. It is possible that the R antigen in some species of Gram-negative bacteria is made inaccessible to BD by variable polysaccharide chains on the surface of the cell wall (O antigen) (Varon and Shilo, 1969b; Luderitz *et al.*, 1966). If so, the R antigen of the predated bacteria may simply be more accessible to the predator, allowing for more efficient interaction between the two microorganisms. This being the case, the susceptibility of prey bacteria to predation by BD may not only be genus-specific but also species-specific and even a strain-specific phenomenon, dependent on the characteristics of the O antigen of the prey strain. However, evidence to support this is inconclusive. Miyamoto and Kuroda (1975) demonstrated that a single strain of BD parasitized 17 strains of *V. parahaemoticus* to approximately the same degree. In the present study, the predation pattern of BD was studied against different groups of Gram-negative spoilage and pathogenic organisms. It has been observed that the effectiveness of BD is not group-specific or genus-specific. BD predated *P. fluorescens*, *P. cepacia*, *P. aeruginosa*, and *P. vulgaris*, but not *P. mendocina* or *P. pseudomallei*. Schoeffield and Williams (1990) observed that *V. parahaemoticus* (P-5) yielded up to 10 times more BD plaques from estuarine water samples than did *V. parahaemoticus* (P-15). Further investigation into the factors which influence the efficiency of predation is warranted.

The present study confirms that the incubation temperature had an effect on predation probably because of the growth range of marine BD isolates. Baer *et al.* (2004) examined the temperature growth range of marine BD at 10, 15, 20, 25, 30, 35 and 40°C for 2 weeks and revealed the growth range of marine BD was 15-30°C. The optimum growth temperatures of prey bacteria used in this study range from 20°-37°C. In the present work, predation was observed at all temperatures tested (20°, 25°, 30° and 37°C), but the rate was highest at 25°-30°C, and only a little was observed at 37°C. While a longer incubation time may have resulted in increased predation, it would not affect the predation rate. It is probable that the predation rate is influenced by the growth rate of the predator at that particular temperature and possibly, also by

the growth rate of the prey. Hence, the predation rate at any particular temperature may vary with the prey. This implies that more effective predation may sometimes occur at the optimum growth temperature of the prey organism. However, given that the BD isolate used in the present work grows optimally at 25°-30°C, it is probable that high predation rates will not be observed at 37°C, even when the optimum growth temperature of the prey approaches this temperature.

The reason for the ~4 log unit predation limit of BD and <4.0 $\log_{10}$ CFU/mL predation threshold of host bacteria is not clearly understood, and needs further investigation. It is possible that BD encounter susceptible prey bacteria by random collision (Rittenberg, 1982) or by chemotaxis (Section 1.1.4). Low numbers of prey may simply reduce the chances for random collisions or it may be that the soluble nutrients required for chemoattraction are not produced in required amounts by the bacteria in such low concentrations. It has been shown that an absorbance reduction of ~0.2 unit at the wavelength of 610nm always equates with approximately 4 log unit reductions of host cells in enumeration (plate counts), which confirms the application of absorbance measurement as a useful indicator for measuring the predation efficiency of BD isolates against tested organisms. In terms of ecological significance, the concept that the predator does not completely eliminate the prey ensures its own survival, since without prey, it would itself be unable to grow. Hence, the results observed in this work appear logical for a predator / prey relationship and are unlikely to be an experimental artefact. However, with respect to a food environment, this failure to reduce the prey numbers below a particular level may mitigate against if being used as a biopreservative, particularly where pathogens are concerned.

Chapter 5

In vitro study of BD against *Photobacterium phosphoreum*

5.1 Introduction

The issue of food safety is of importance to the food industry, government regulatory agencies and to all consumers, because 25% of all food produced is lost post-harvest due to microbial spoilage (Fratamico and Whiting, 1995). The shelf life of fresh fish products depends on storage conditions such as time, temperature, atmosphere and the initial fish quality. In fact, the hygienic quality of fish and fishery products rapidly declines because of cross-contamination from various sources (Gram and Huss, 1996). Bacterial spoilage of saltwater fish is caused by several Gram-negative bacteria; in particular, *Shewanella* spp., *Photobacterium phosphoreum*, *Pseudomonas* spp., *Acinetobacter* spp., *Flavobacterium* spp. and *Aeromonas* spp. (Dalgaard, 1995). Modified atmosphere packaging (MAP) has been increasingly popular in many countries in the storage and distribution of fresh marine fish. Of particular concern is *P. phosphoreum*, which is one of the major spoilage organism that limits the shelf life of MAP stored fish (Mejlholm and Dalgaard, 2002). To further extend shelf life it is therefore important to develop new packed products using preservation techniques that inhibit *P. phosphoreum* (Mejlholm and Dalgaard, 2002).

Some potential preservation techniques to control the level of microbial spoilage in foods include the use of low dose irradiation, bacteriocins, organic acids and other chemical agents. The use of microorganisms which are antagonistic to other bacteria, particularly to Gram-negative bacteria, has been explored to only a limited degree. In the last few years some papers have been published dealing with biopreservation, including studies on the application of *Leuconostoc* species (Jacobsen *et al*., 2003) and *Lactococcus lactis* ssp. *lactis* (Wessels and Huss, 1996) but these can only suppress the growth of the aerobic Gram-positive bacteria associated with spoilage of refrigerated food.

Similar to their proposed use as biological control agents to selectively eliminate bacterial blooms, Bdellovibrionaceae are now being investigated as useful preventive agents against the

Gram-negative microbes that cause food spoilage and food poisoning. The capability of BD to reduce numbers of spoilage and pathogenic organisms is well established and has been reviewed in Chapter 1 (General Review of Literature). Although there have been some studies focusing on the efficacy and probable use of terrestrial BD against pathogenic and spoilage organisms in industry as a buffered spray or dip (Jackson and Whiting, 1992; Fratamico and Whiting, 1995; Fratamico and Cooke, 1996; Kadouri and O'Toole, 2005) there is no published work on the possible application of marine BD against spoilage and pathogenic organisms in the food industry.

The objectives of this study were to determine the *in vitro* effects of New Zealand seawater BD isolates against *P. phosphoreum* and to determine suitable salinity, pH, nutrient, prey and predator concentrations and their ratios in effectively reducing the growth of *P. phosphoreum*. The following investigations were undertaken to achieve these goals.

- To evaluate the survival of pure BD cultures at different temperatures so that BD isolates can be stored for longer periods for long term trials or industrial use with minimal variation of the characteristics or efficacy.
- Screening of seawater BD against *P. phosphoreum* to select the best isolate for *in vitro* application.
- To study the effect of salinity on the predation of *P. phosphoreum* by BD and to determine the optimum salinity conditions.
- To evaluate the effect of pH on the predation of *P. phosphoreum* by BD and to determine the optimum pH value.
- To study the time course of BD against *P. phosphoreum* and the growth of BD in the presence of *P. phosphoreum*.
- To study the effect of nutrient, prey and predator concentrations on the reduction of *P. phosphoreum* numbers by BD.
- To study the effect of prey : predator ratios on the reduction of *P. phosphoreum* numbers.

5.2. Experimental procedure

5.2.1 Survival of two BD isolates at different temperatures

The BD isolates used in this study were MNA and MCB (Chapter 3). The pure cultures of MNA and MCB were grown in a medium of 50 mL of 70% ASW and 1 mL of host *V. parahaemolyticus* incubated in an orbital shaker (130 rpm) at 25°C for 2 days. Large-scale cultures were prepared as described in Section 2.5. The pellets were resuspended in an appropriate amount of 70% ASW, giving final concentrations of approximately 10^{10} PFU/mL. The homogenised suspensions of MNA cultures were divided into 78 tubes, each of which contained 3 mL x 10.3 $\log_{10}$ units of BD/mL. Each set of tubes (n=13) containing MNA cultures was kept at 4°C, 10°C, 20°C, 25°C, 30°C or 37°C. Aliquots of MCB cultures were divided into 128 tubes, each of which contained 3 mL x 10.3 $\log_{10}$ unit of BD/mL. Each set of tubes (n=16) containing MCB cultures were kept at –18°C, 0°C, 4°C, 10°C, 20°C, 25°C, 30°C or 37°C. One tube of each culture from each temperature was removed daily and diluted appropriately to prepare 10-fold serial dilutions in triplicate. 1 mL from each dilution was plated onto polypeptone 20 (Pp20) medium using the double agar technique for the enumeration of BD described in Section 2.3. The plates were incubated aerobically at 25°C until the appearance of a characteristic BD plaque.

5.2.2 Screening of different seawater BD against *P. phosphoreum*

Inocula of *P. phosphoreum* were prepared as described in Section 2.6, while large-scale cultures of thirteen BD isolates were prepared as described in Section 2.5. The experiments were performed in fourteen 250-mL sterile Erlenmeyer flasks; thirteen experimental flasks and one control flask, all of which contained 50 mL of 70% ASW. One mL of harvested *P. phosphoreum* (9.7 $\log_{10}$ CFU/mL) was inoculated into all experimental and control flasks to give a final concentration of 8.0 $\log_{10}$ CFU/mL. One-mL of each BD isolate (approximately 9.0 $\log_{10}$ PFU/mL cells) was added to each experimental flask to give the final concentrations shown in Table 5.1. Flasks were incubated at 25°C in an orbital shaker (Lab line SHK A2000 orbital shaker, Barnstead International, USA) running at 130 rpm. Aliquots (10 mL) were aseptically

taken from each flask at times 0, 10 and 24 h. Changes in bacterial numbers were evaluated by absorbance and by using SWYE plates as described in Section 2.7.

5.2.3 Ability of BD (OT2) to reduce numbers of *P. phosphoreum* at different salinities

A culture of *P. phosphoreum* was prepared as described in Section 2.6. The BD isolate used in this study was OT2. The cultures were purified, propagated, and large-scale challenge inocula were prepared as described in Sections 2.2 and 2.5. The effect of BD on the population of *P. phosphoreum* at different salinities was studied in liquid media. Batches (500 mL each) of 0%, 12.5%, 25%, 50%, 70%, 100% and 150% ASW (artificial sea water; Instant Ocean; Aquarium system; USA) were prepared (Appendices 1.E – 1.K). The salt concentrations of the solutions were measured (Table 5.2) using a hand-held refractometer (Atago Hand Refractometer, USA).

Fifty mL of each salt solution were aseptically transferred to each of two 250 mL Erlenmeyer flasks. One mL of harvested *P. phosphoreum* culture (10.1 $\log_{10}$ CFU/mL) was inoculated into each flask to give a final concentration of 8.4 $\log_{10}$ CFU/mL and 1 mL of OT2 challenge inoculum (final concentration 7.2 $\log_{10}$ PFU/mL) was added to the 7 treatment flasks, while the other 7 were kept as controls. Flasks were incubated at 25°C in an orbital shaker (130 rpm). At times 0, 10, 24 and 48 h, 4 mL aliquots were removed aseptically from the flasks. The absorbance levels of the aliquots were measured at 610 nm using a spectrophotometer and *P. phosphoreum* was enumerated using SWYE plates (Section 2.7).

5.2.4 Ability of BD (OT2) to reduce numbers of *P. phosphoreum* at different pH values

The bacterial challenge strain used in this study was *P. phosphoreum*, while the BD isolate was OT2. The cultures were purified, propagated and large-scale challenge inocula were prepared as described in Sections 2.2 and 2.5. The pH buffer used in the experiment was 0.1 M Citric acid – 0.2 M Na_2HPO_4 (pH 4.0 to 6.6) and was prepared according to the protocol described by Dawson *et al.* (1986). The buffer solutions were autoclaved at 121°C for 15 min and their pH stability was verified for at least 2 days. This study was performed using fourteen 250 mL Erlenmeyer flasks divided into 7 groups. Each group comprised two flasks – one of which was the treatment and the other was the control. Six groups of flasks contained 49 mL of 70% ASW and 1 mL of the relevant buffer. One mL of harvested *P. phosphoreum* culture (10.2 $\log_{10}$ CFU/mL) was inoculated into all treatment and control flasks, which gave a final concentration of 8.5 $\log_{10}$ CFU/mL. One mL of OT2 challenge inoculum (final concentration 7.3 $\log_{10}$ PFU/mL) was added to the seven treatment flasks. Flasks were incubated at 25°C in an orbital shaker (130 rpm). At times 0, 24, and 48 h, 5 mL aliquots were removed aseptically from the flasks. The absorbance levels of the aliquots were measured at 610 nm using a spectrophotometer and *P. phosphoreum* was enumerated using SWYE plates (Section 2.7).

5.2.5 Co-culture of BD (OT2) with *P. phosphoreum* in 70% ASW

An inoculum of OT2 was prepared as described in Section 2.5. High, medium and low dose BD dilutions were plated onto polypeptone 20 (Pp20) medium using a double layer overlay technique and the results were recorded as $\log_{10}$ PFU/mL. A lawn of *P. phosphoreum* was prepared as previously described in Section 2.6. The experiment was performed in four 250 mL sterile Erlenmeyer flasks, three of which were treatment flasks and one was a control flask. All flasks contained 50 mL of 70% ASW and 1 mL of harvested *P. phosphoreum* (final concentration 8.6 $\log_{10}$ CFU/mL). The three treatment flasks were inoculated with different doses of OT2 (7.8; 4.7; 3.5 $\log_{10}$ PFU/mL), while the other was kept as a control (no BD added). The flasks were incubated at 25°C in an orbital shaker (130 rpm). Aliquots (4 mL) were taken from each flask after 0, 2, 3, 4, 5, 6, 8, 9, 10 and 24 h of incubation and SWYE plates were used

to enumerate *P. phosphoreum*. A portion of the aliquots was filtered through 0.8 µm, 0.45 µm and 0.2 µm filters and plated onto Polypeptone 20 plates (Section 2.3) to enumerate BD plaques.

5.2.6 Effect of different nutrient concentrations on the reduction of *P. phosphoreum* by BD (OT2)

An initial experiment was designed to study the effect of different nutrient levels on the reduction of *P. phosphoreum* by BD. The predator and prey bacteria were prepared as previously described (Sections 2.2, 2.3 and 2.6). From the maintenance flask, a 2 day old dual culture (OT2 + prey cells) was filtered through 0.8 µm to 0.45 to 0.2 µm syringe filters to remove the prey cells. A series of different dilutions (1%, 2%, 4%, 8% and 16%) of standard SWYE broth was prepared using 70% ASW as diluents.

This experiment consisted of five experimental and one control flask. Each flask contained 50 mL of a concentration of SWYE broth respectively diluted to 1, 2, 4, 8, and 16% of full strength,. The control flask contained 50 mL of 16% diluted SWYE broth. One mL of harvested *P. phosphoreum* (final concentration 8.3 $\log_{10}$ CFU/mL) was inoculated into each of the six flasks, followed by 1 mL of a dense suspension of isolate OT2 to a final concentration of 6.4 $\log_{10}$ PFU/mL in the five experimental flasks. Flasks were incubated at 25°C in an orbital shaker at 130 rpm. At times 0, 24 and 48 h, 5 mL aliquots were removed aseptically from the flasks. The viability of *P. phosphoreum* was enumerated using SWYE plates (Section 2.7).

5.2.7 Effect of prey concentrations on the predation of *P. phosphoreum* by BD (OT2)

To examine the effect of different doses of OT2 against different concentrations of *P. phosphoreum* in the presence or absence of nutrients (diluted SWYE (16%) broth or 70% ASW respectively), an inoculum of OT2 was prepared as described in Section 2.5. The final concentration was adjusted to McFarland standard 5. Ten-fold serial dilutions were prepared using 70% ASW to obtain different doses of OT2. A 24 h grown lawn of *P. phosphoreum* was prepared as described in Section 2.6 and harvested with an appropriate amount of 70% ASW to give McFarland standard 5. Dilutions were then prepared to obtain different host cell concentrations (8.1, 3.7 and 2.8 $\log_{10}$ CFU/mL).

This study was performed with 2 sets of 12 flasks divided into six groups. One set of 12 flasks contained 50 mL of diluted SWYE (16%) broth in each flask, and another set of 12 flasks contained 50 mL of 70% ASW in each flask. Each set of flasks was divided into three groups: the first group contained 1 mL of a high concentration of *P. phosphoreum* (final concentration 8.1 $\log_{10}$ CFU/mL); the second group contained 1 mL of a medium concentration of *P. phosphoreum* (final concentration 3.7 $\log_{10}$ CFU/mL); and the third group contained 1 mL of a low concentration of *P. phosphoreum* (final concentration 2.8 $\log_{10}$ CFU/mL). Three flasks of each group were inoculated with different doses of OT2 (final concentration 7.2; 3.9; or 2.1 $\log_{10}$ PFU/mL). OT2 was not added into one flask of each group, which together became the control flasks.

5.2.8 Effect of predator levels on the reduction of *P. phosphoreum* numbers

The previous experiment had studied the effect of different doses of BD against different concentrations of *P. phosphoreum* in nutrient or 70% ASW medium. The present experiment was designed to further evaluate the effect of different doses of BD against a high levels of *P. phosphoreum* with more regular sampling.

The bacterial challenge strain used in this study was *P. phosphoreum*. An inoculum of OT2 was prepared as described in Section 2.5. 10-fold dilutions were prepared using 70% ASW to obtain a range of different serial concentrations (Table 5.6).

This experiment was performed in seven 250-mL sterile Erlenmeyer flasks, each containing 50 mL of 70% ASW and 1 mL of harvested *P. phosphoreum* (final concentration 8.4 $\log_{10}$ CFU/mL). OT2 challenge inocula (8.3 $\log_{10}$ PFU/mL) were added to give the cell ratios shown in Table 5.6. Aliquots (4 mL) were taken at times 0, 2, 4, 6, 8, 10 and 24 h of incubation from each flask aseptically. The absorbance levels of the aliquots were measured using a spectrophotometer at 610 nm and *P. phosphoreum* was enumerated using SWYE plates (Section 2.7).

5.3. Results

5.3.1 Survival of two BD isolates at different temperatures

The survival of dense suspensions of MNA culture without host bacteria at different temperatures for 13 days is presented in Fig. 5.1 and Appendix 5.A. After 13 days of storage, only minor decreases in viability were observed at 4°C, 10°C and 20°C. However, at the higher temperatures, there was a significant reduction in numbers, this reduction being most pronounced at 37°C, where no viable cell was observed after 10 days of storage. After 13 days of storage, $\log_{10}$ reduction values of 1.3, 0.9, 1.5, 2.9, 5.9 and >7.8 were estimated at 4°C, 10°C, 20°C, 25°C, 30°C and 37°C respectively (Fig. 5.2).

The survival of MCB culture without a host organism was recorded for 16 days at different temperatures and the results are summarized in Fig. 5.3 and Appendix 5.B. In addition to the storage temperatures used in previous experiment, this isolate was also kept at –18°C and 0°C for 16 days. The mean plaque-forming unit of this isolate was found to exhibit a similar pattern to that in the previous experiment.

At temperatures of 4°C, 10°C and 20°C only slight decreases in viability were observed after 16 days of storage. At higher temperatures, however, there was a significant reduction in viability. Interestingly, viability was also significantly reduced at temperatures of 0°C and –18°C. After 16 days of storage, $\log_{10}$ reduction values of >7.8, 2.2, 0.8, 0.6, 0.9, 3.1 and >7.8 were estimated at –18°C, 0°C, 4°C, 10°C, 20°C, 25°C, 30°C and 37°C respectively (Fig. 5.4).

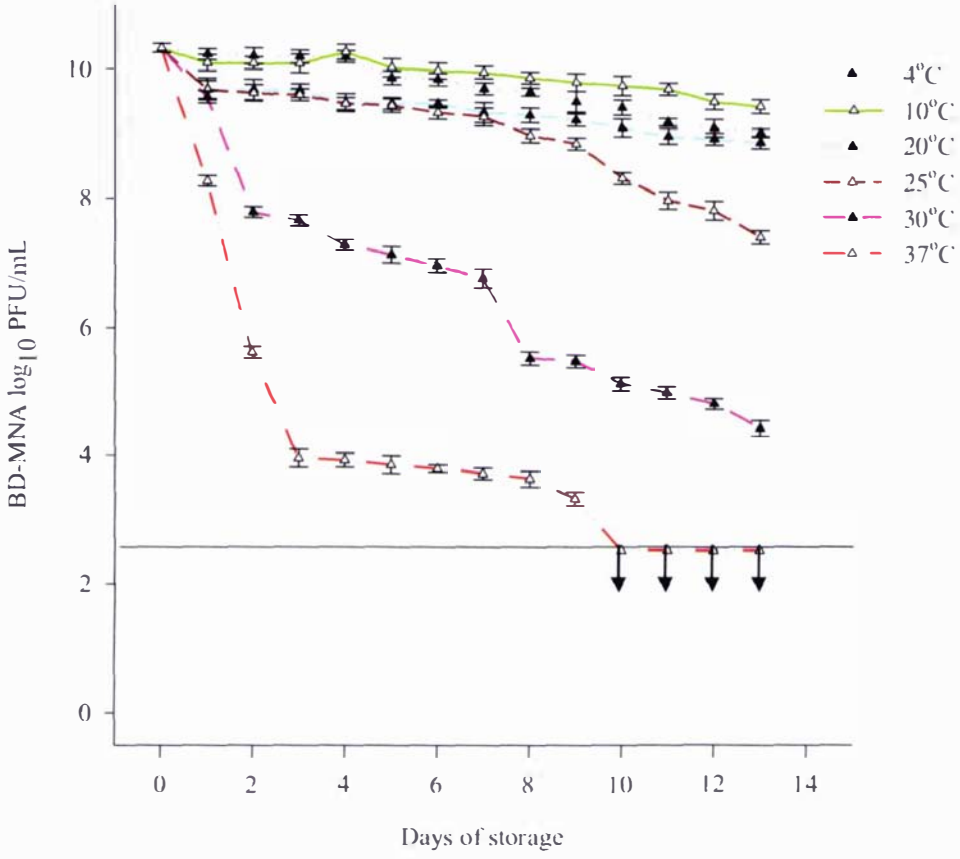


Fig. 5.1 Survival of a dense suspension of a BD isolate (MNA) in 70% ASW without host bacteria stored at 4°C, 10°C, 20°C, 25°C, 30°C and 37°C for 13 days. Error bars represent the standard deviation of 3 replicates. Down arrow (↓) means less than.

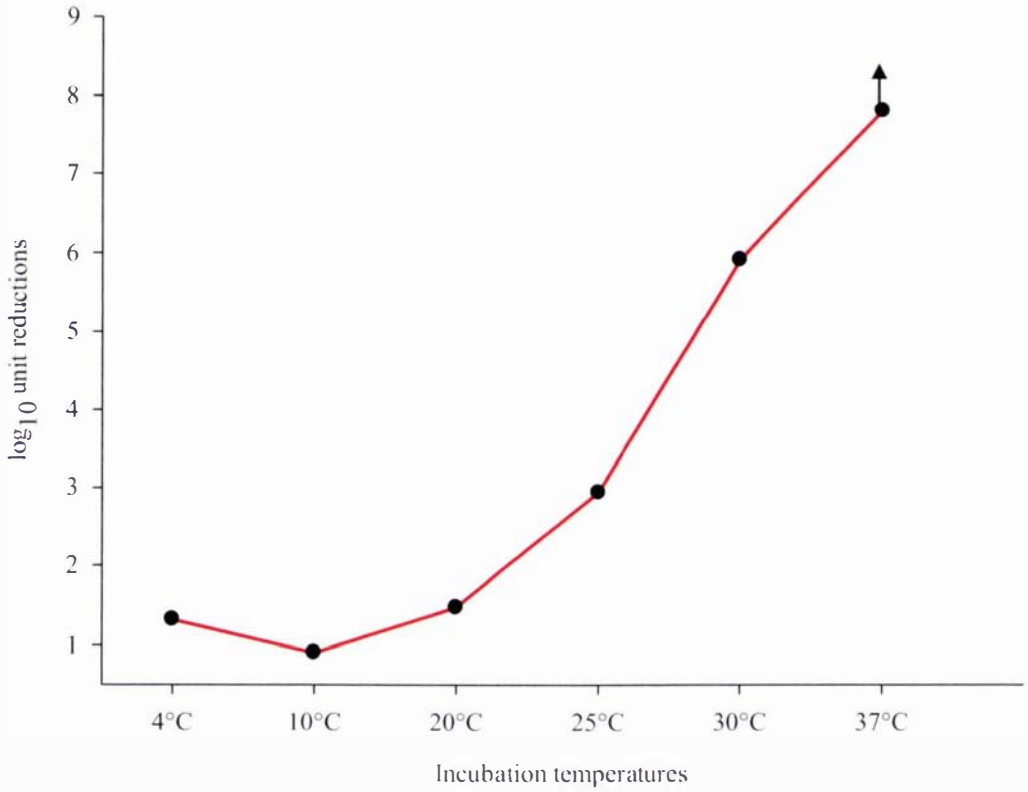


Fig. 5.2 Log_{10} reduction of a dense suspension of a BD isolate (MNA) after 13 days of storage in 70% ASW at 4°C, 10°C, 20°C, 25°C, 30°C and 37°C. Up arrow (↑) means greater than.

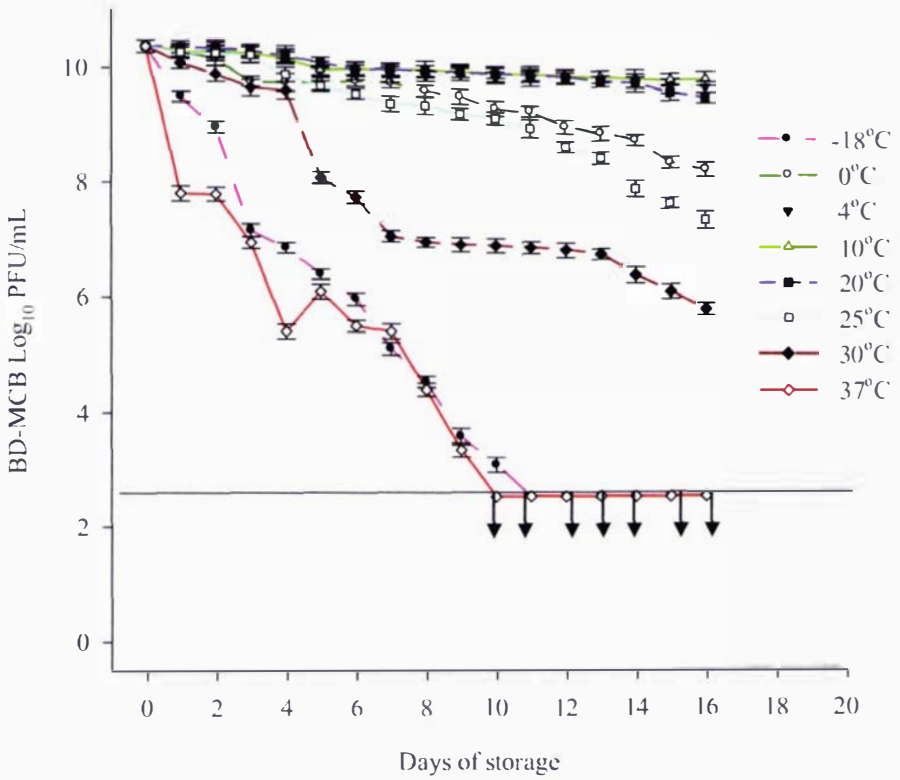


Fig. 5.3 Survival of a dense suspension of a BD isolate (MCB) in 70% ASW without host bacteria stored at -18°C, 0°C, 4°C, 10°C, 20°C, 25°C, 30°C and 37°C for 16 days. Error bars represent the standard deviation of 3 replicates. Down arrow (↓) means less than.

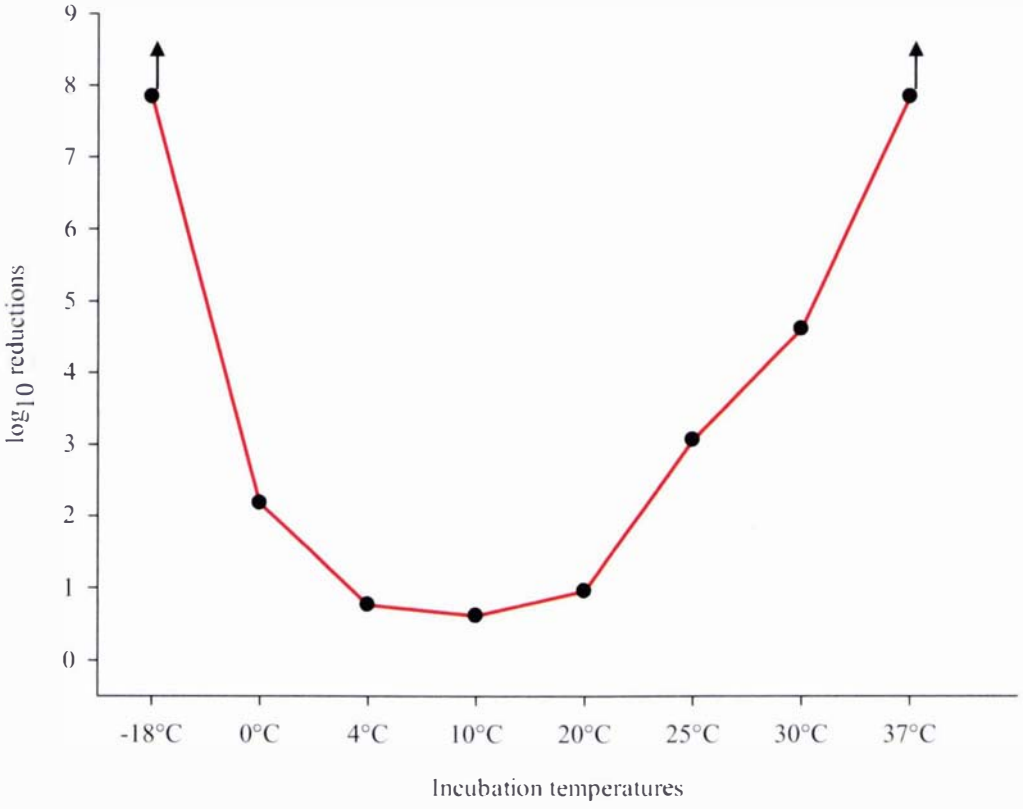


Fig. 5.4 Log_{10} reduction of a dense suspension of a BD isolate (MNA) after 16 days of storage in 70% ASW at -18°C, 0°C, 4°C, 10°C, 20°C, 25°C, 30°C and 37°C for 16 days. Up arrow (↑) means greater than.

5.3.2 Screening of different seawater BD against *P. phosphoreum*

The results are summarized in Table 5.1. After 10 h of incubation, the highest reductions in absorbance were observed in cultures challenged with OT2 and TB1. These strains also displayed the highest reductions in absorbance after 24 h. Moderate to high reductions in absorbance were detected in cultures challenged with OT1, OT3, OT4, OT5, MCB, MNA and SP after 10 and 24 h. Lower reductions of absorbance were observed in the cultures challenged with other isolates (OT-enr, TB2, TB-enr and MNZ1) after 10 and 24 h. The control samples showed minimal changes in the absorbance after 10 and 24 h of incubation at 25°C.

The impact of the different BD isolates on the population of *P. phosphoreum* in 70% ASW, as assessed on SWYE plates, is summarized in Figs 5.5, 5.6 and Appendix 5.C. After 10 h of incubation, highest reductions in viable *P. phosphoreum* were observed in cultures challenged with OT2 and OT1. Although a medium to high reduction in the mean viability of *P. phosphoreum* numbers was detected in samples challenged with OT5, OT4, MNA, MCB, TB1, TB2, OT3, OT-enr and SP, a lower reduction was observed with TB-enr and MNZ1. However, after 24 h of incubation, the reduction in the mean population of *P. phosphoreum* was found to be similar in all cultures. The control sample (no BD added) did not show any changes in the total viable numbers of *P. phosphoreum* after 10 or 24 h of incubation at 25°C.

On the basis of these results, OT2 was selected for more detailed research as a strain exhibiting high attacking efficacy against *P. phosphoreum*.

Table 5.1 The effectiveness of different isolates of BD in reducing numbers of *P. phosphoreum* (8.0 log_{10} CFU/mL) in 70% ASW after 10 and 24 h at 25°C. Absorbances were measured using a spectrophotometer at the wavelength of 610 nm.

		Initial Absorbance[a]	Absorbance change [b]	
BD isolate (initial number – log_{10} PFU/mL)	Prey to predator ratio	0 h	10 h	24 h
OT1 (7.2)	6	0.32	0.18	0.20
OT2 (7.3)	5	0.33	0.25	0.27
OT3 (7.2)	6	0.33	0.21	0.22
OT4 (7.2)	6	0.33	0.22	0.23
OT5 (7.3)	5	0.33	0.19	0.20
OT-enr (7.3)	5	0.32	0.13	0.15
TB1 (7.2)	6	0.35	0.25	0.27
TB2 (7.1)	8	0.31	0.14	0.16
TB-enr (7.3)	5	0.31	0.13	0.14
MNA (7.3)	5	0.31	0.20	0.24
MCB (7.2)	6	0.34	0.20	0.21
SP (7.2)	6	0.37	0.22	0.24
MNZ1 (7.1)	8	0.36	0.17	0.20
Control (no BD)	n/a	0.34	0.01	0.02

[a] *Absorbance (OD at 610 nm) of dual culture of BD isolates and* P. phosphoreum *in 70% ASW at 0 h*

[b] *Mean absorbance (n=3) of BD isolate and* P. phosphoreum *at time 0 h minus mean absorbance of the same dual culture after 10 h or 24 h at 25°C.*

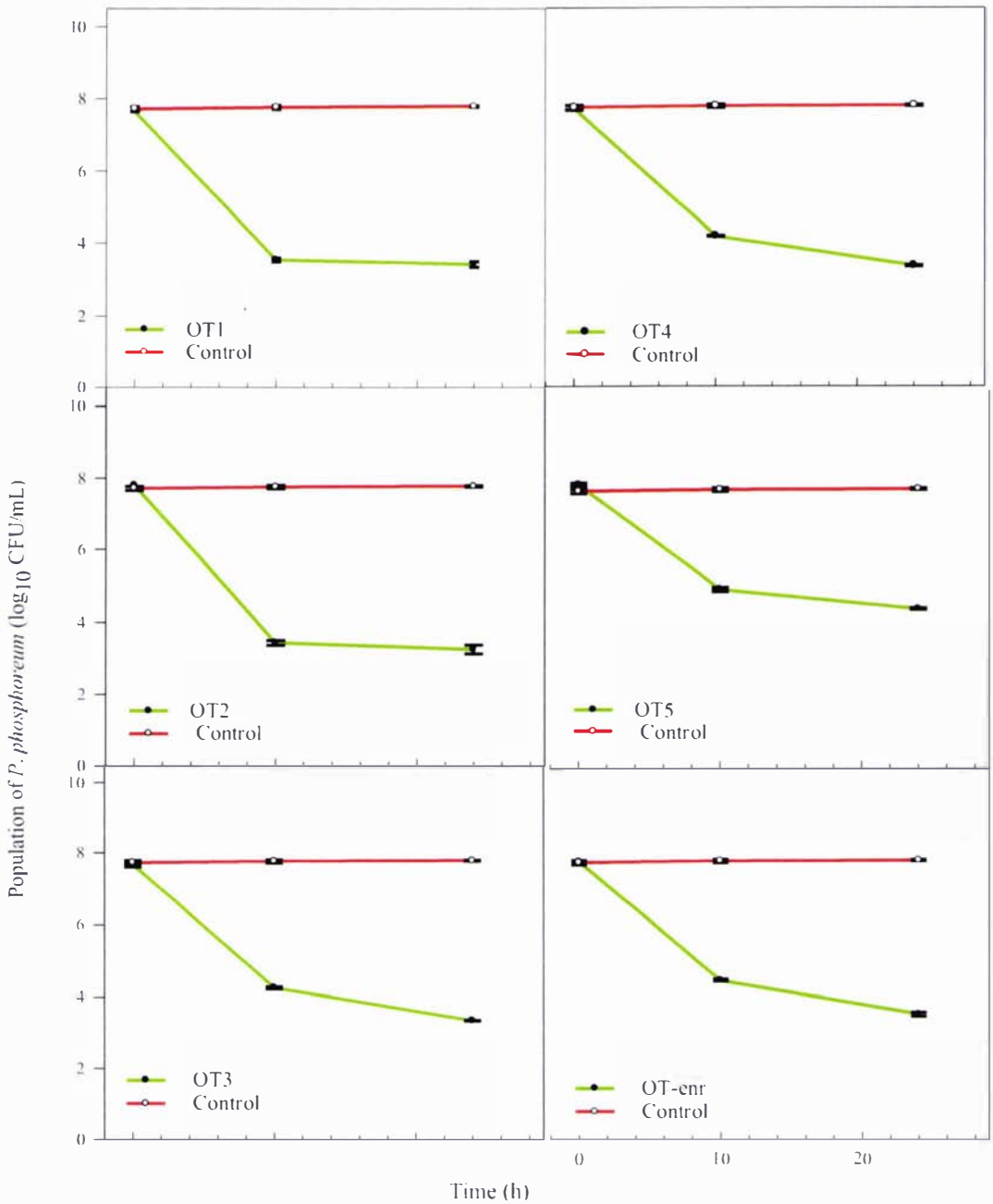


Fig. 5.5 The effectiveness of BD isolates (OT1, OT2, OT3, OT4, OT5 and OT-enr) in reducing the population of *P. phosphoreum* (8.0 $\log_{10}$ CFU/mL) after a 24 h challenge in 70% ASW at 25°C. Error bars represent the standard deviation of 3 replicates.

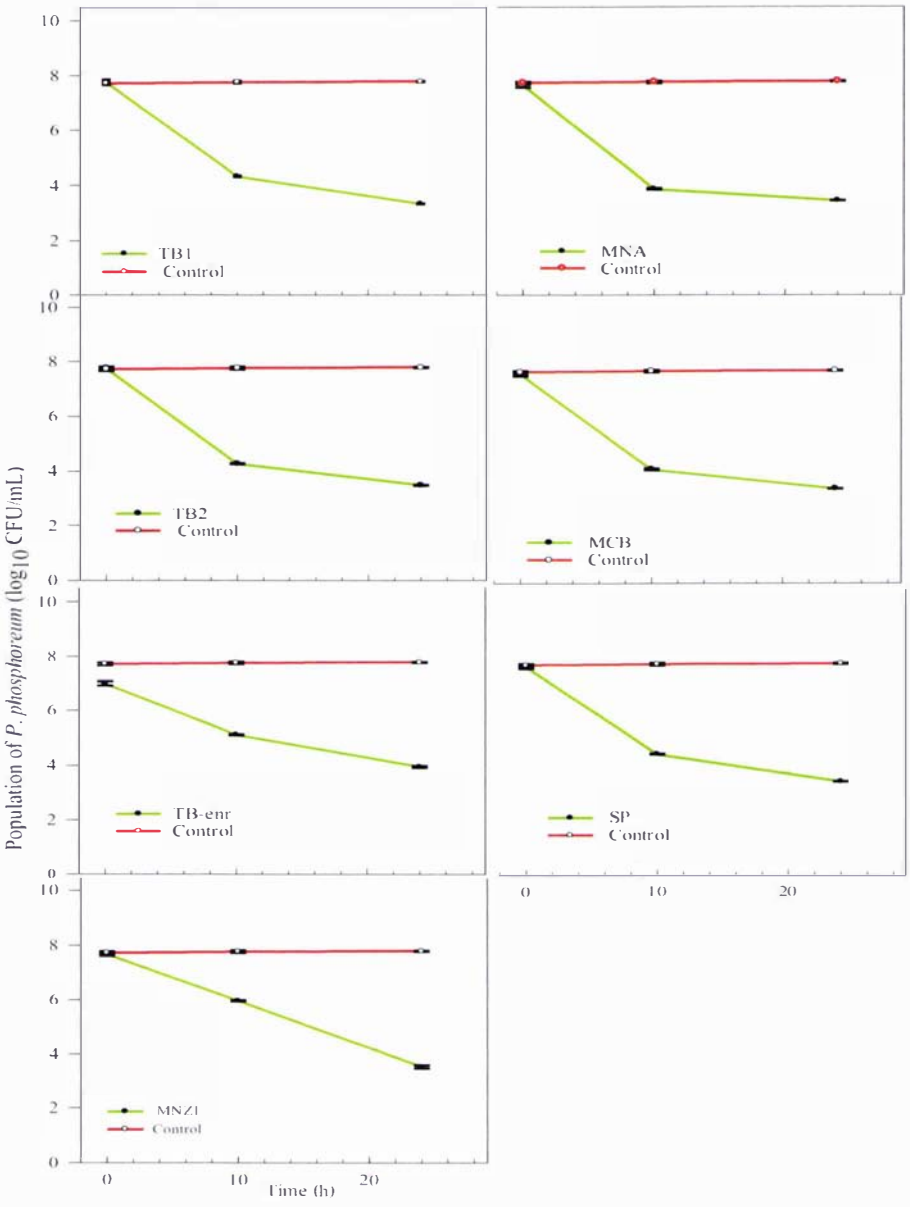


Fig. 5.6 The effectiveness of BD isolates (TB1, TB2, TB-enr, MNZ1, MNA, MCB and SP) in reducing the population of *P. phosphoreum* (8.0 $\log_{10}$ CFU/mL) after a 24 h challenge in 70% ASW at 25^{0}C. Error bars represent the standard deviation of 3 replicates.

5.3.3 Ability of BD (OT2) to reduce numbers of *P. phosphoreum* at different salinities

Table 5.2 shows the measurement of salt concentrations of the solutions prepared by diluting artificial seawater. The effects of different salt concentrations on the reduction of absorbance of the dual cultures of OT2 and *P. phosphoreum* after 10, 24 and 48 h of incubation at 25°C are summarized in Table 5.3. After 10 h of incubation, the highest reductions in absorbance were observed at salinities 1.5 and 2.1%. Although a moderate absorbance reduction was measured after 10 h at 3.0% salinity, minimal reductions were detected in the other salt solutions. After 24 h of incubation, solutions of 1.5, 2.1 and 3.0% salt showed the highest reductions in absorbance, while medium reductions were observed at salinities of 0.9 and 4.5%. After 48 h of incubation, there was evidence of the absorbance starting to increase at salinities of 0.9, 2.1, 3.0 and 4.5%. There were no detectable changes in the absorbance over the entire 48 h of incubation at 0 or 0.5% salinity or in the control samples.

Fig. 5.7 and Appendix 5.D show the effects of the different salt solutions on the total viable counts of *P. phosphoreum* (CFU/mL) after 10, 24 and 48 h of challenge with OT2. The population of *P. phosphoreum* remained unchanged at 0 and 0.5% salinity. After 10 h of incubation, the largest reduction in the population of *P. phosphoreum* was observed at 1.5% salinity, while lower reductions were observed at 2.1% and 3.0% salinity. The mean population of *P. phosphoreum* was virtually unchanged after 10 h at salinities of ≤0.9 or 4.5%. After 24 h of challenge the reduction in the mean population of *P. phosphoreum* was found to be similar at salinity values of 1.5, 2.1 and 3.0%, while slight reductions were observed at salinities of 0.9 and 4.5%. After 48 h of challenge, the changes in the population of *P. phosphoreum* were similar at salinity values of 0.9, 1.5, 2.1 and 3.0%. There were no detectable changes in the population of *P. phosphoreum* after 48 h of incubation at salinities of ≤0.5 or 4.5%. The control samples of each salt solution showed only marginal changes in the mean population of *P. phosphoreum* after 10, 24 and 48 h of incubation at 25°C.

Table 5.2 Salinity measurement using hand refractometer.

Artificial sea water (ASW) %[a]	salt concentration %(w/v) [b]
0	0
12.5	0.5
25	0.9
50	1.5
70	2.1
100	3.0
150	4.5

[a]*Different percentages of artificial seawater medium in water*
[b]*Salt concentration, measured after autoclaving, using Atago hand refractometer.*

Table 5.3 Effect of salinity on the reduction of absorbance of dual cultures of BD isolate OT2 and *P. phosphoreum* at a prey to predator ratio of 16 after 10, 24 and 48 h of incubation at 25°C. Absorbances were measured using a spectrophotometer at the wavelength of 610 nm.

	Initial absorbance[b]	Absorbance change [c]		
Salinity %[a]	0 h	10 h	24 h	48 h
0.0 (treatment)	0.33	0.00	0.00	-0.01
0.0 (control)	0.33	0.00	0.00	-0.01
0.5 (treatment)	0.34	0.01	0.01	0.04
0.5 (control)	0.34	0.00	0.00	-0.01
0.9 (treatment)	0.35	0.03	0.19	0.25
0.9 (control)	0.35	0.01	0.01	0.00
1.5 (treatment)	0.35	0.22	0.27	0.26
1.5 (control)	0.36	0.00	0.01	0.01
2.1 (treatment)	0.36	0.20	0.27	0.20
2.1 (control)	0.36	0.00	0.00	-0.01
3.0 (treatment)	0.37	0.11	0.23	0.19
3.0 (control)	0.38	0.01	0.02	0.00
4.5 (treatment)	0.39	0.01	0.18	0.20
4.5 (control)	0.40	0.01	0.01	0.00

[a] *Challenge inocula of 1-mL* P. phosphoreum *(8.4 log_{10} CFU/mL) and 1-mL of OT2 (7.2 log_{10} PFU/mL). Controls contained no BD.*

[b] *Mean absorbance of dual culture of BD isolate and* P. phosphoreum *at time 0 h*

[c] *Mean absorbance of dual culture of BD and* P. phosphoreum *at time 0 h of incubation minus mean absorbance of the same culture at 10, 24 or 48 h of incubation.*

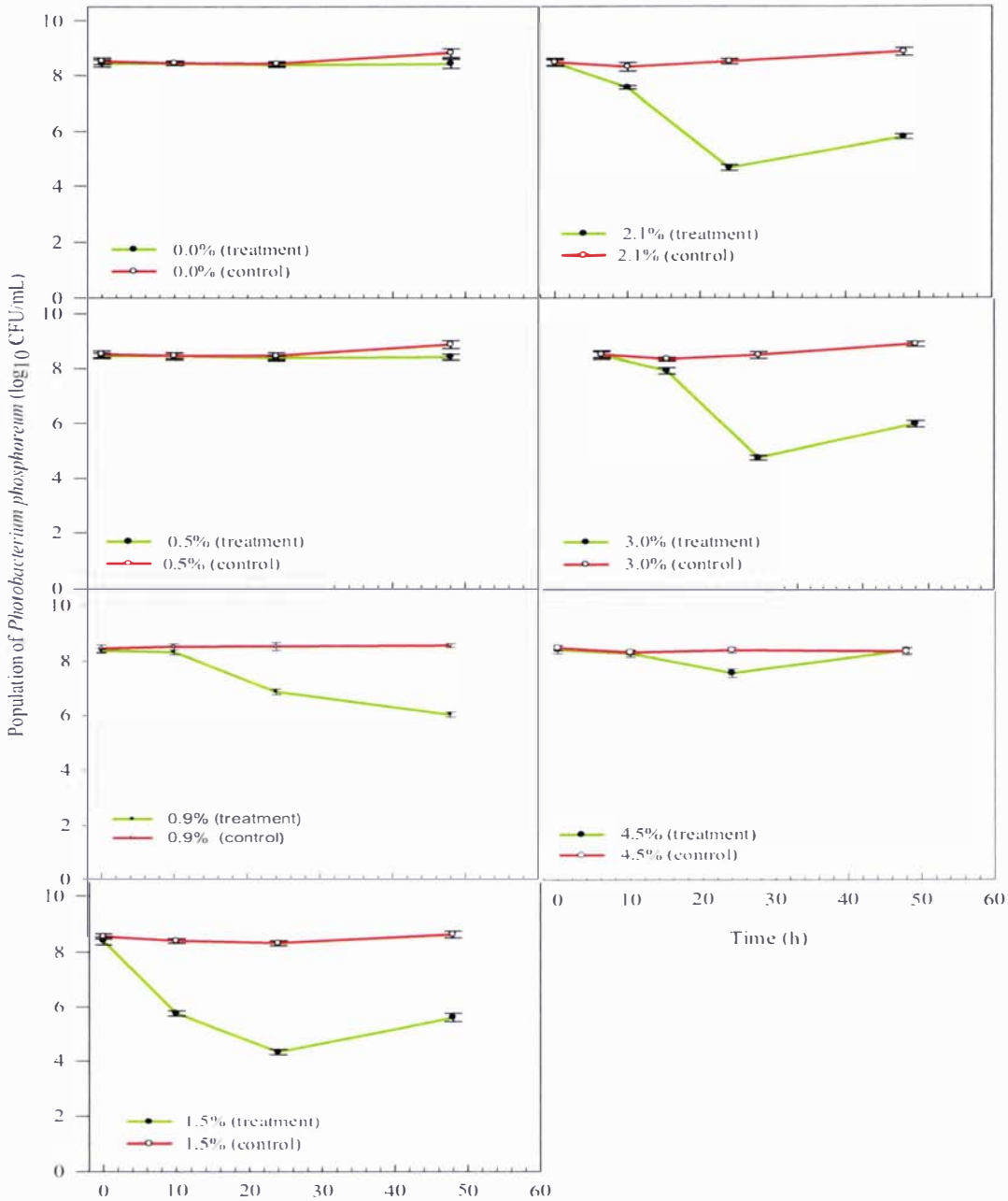


Fig. 5.7 Effect of salinity on the population of *P. phosphoreum* after challenging BD isolate OT2 (7.2 $\log_{10}$ PFU/mL) against *P. phosphoreum* (8.4 $\log_{10}$ CFU/mL) in 70% ASW at a prey to predator ratio of 16 after 10, 24 and 48 h of incubation at 25°C. Error bars represent the standard deviation of 3 replicates.

5.3.4 Ability of BD (OT2) to reduce numbers of *P. phosphoreum* at different pH values

Table 5.4 shows the effect of pH on the reduction of absorbance of the culture of OT2 and *P. phosphoreum* after 24 and 48 h of incubation at 25°C. The absorbance of the dual culture (OD at 610 nm) remained unchanged at pH 4.0, 4.6, and 5.0 after 24 and 48 h of incubation at 25°C. Although a slight reduction in the absorbance of the culture of OT2 and *P. phosphoreum* was observed at pH 5.6 after 24 and 48 h of incubation, the highest reductions in absorbance were detected at pH 6.0 to 8.2 after 24 and 48 h of incubation. The absorbance of the control samples (no OT2 added) did not show any notable changes at any tested pH value.

Fig. 5.8 and Appendix 5.E show the effect of pH on the population of *P. phosphoreum* after challenge with OT2 after 0, 24 and 48 h of incubation at 25°C. At pH 4.0 and 4.6, no detectable colonies of *P. phosphoreum* were observed in either the treatment or control at the lowest dilution (CFU/mL at 10^2) after 24 or 48 h of incubation. This indicates that *P. phosphoreum* did not survive at these values. Therefore the effect of BD could not be studied. At pH 5.0 the population of *P. phosphoreum* was almost unchanged (reduction of < 0.4 $\log_{10}$ unit) after 24 and 48 h of incubation, while at pH 5.6 there was a (1 log) reduction in numbers. The largest effects were observed between pH 6.0 and 8.2 after 24 h of incubation where the populations of *P. phosphoreum* were reduced by 3 to 4.3 $\log_{10}$ units. *P. phosphoreum* populations appeared to recover slightly between 24 and 48 h incubation. The mean CFU/mL of the control samples (no OT2 added) did not show any notable changes in the population of *P. phosphoreum* at pH 5.0 or higher.

Table 5.4 Effect of pH on the reduction of absorbance of the dual cultures of BD isolate OT2 and *P. phosphoreum* in 70% ASW at a prey to predator ratio of 16 after 24 and 48 h of incubation at 25°C. Absorbances were measured using a spectrophotometer at the wavelength of 610 nm.

	Initial absorbance[b]	Absorbance change [c]	
pH [a]	0 h	24 h	48 h
4.0 (treatment)	0.77	-0.06	-0.07
4.0 (control)	0.76	-0.03	-0.03
4.6 (treatment)	0.77	-0.02	-0.05
4.6 (control)	0.77	0.00	-0.02
5.0 (treatment)	0.77	0.00	0.02
5.0 (control)	0.73	-0.02	-0.03
5.6 (treatment)	0.77	0.05	0.07
5.6 (control)	0.74	0.03	0.05
6.0 (treatment)	0.78	0.50	0.59
6.0 (control)	0.78	0.04	-0.09
6.6 (treatment)	1.31	0.47	0.49
6.6 (control)	1.30	0.00	-0.06
8.2 (treatment)	0.75	0.58	0.59
8.2 (control)	0.74	-0.01	0.01

[a] *Challenge inocula of 1-mL* P. phosphoreum *(8.4 log_{10} CFU/mL) and 1-mL of OT2 (7.3 log_{10} PFU/mL). Controls contained no BD. pH 8.2 solutions contained no buffer, only 70% ASW*

[b] *Mean absorbance of dual culture of BD isolate and* P. phosphoreum *(treatment) or* P. phosphoreum *alone (control) at time 0 h*

[c] *Mean absorbance of dual culture of BD and* P. phosphoreum *or* P. phosphoreum *alone (control) at time 0 h of incubation minus mean absorbance of the same culture at 24 or 48 h of incubation.*

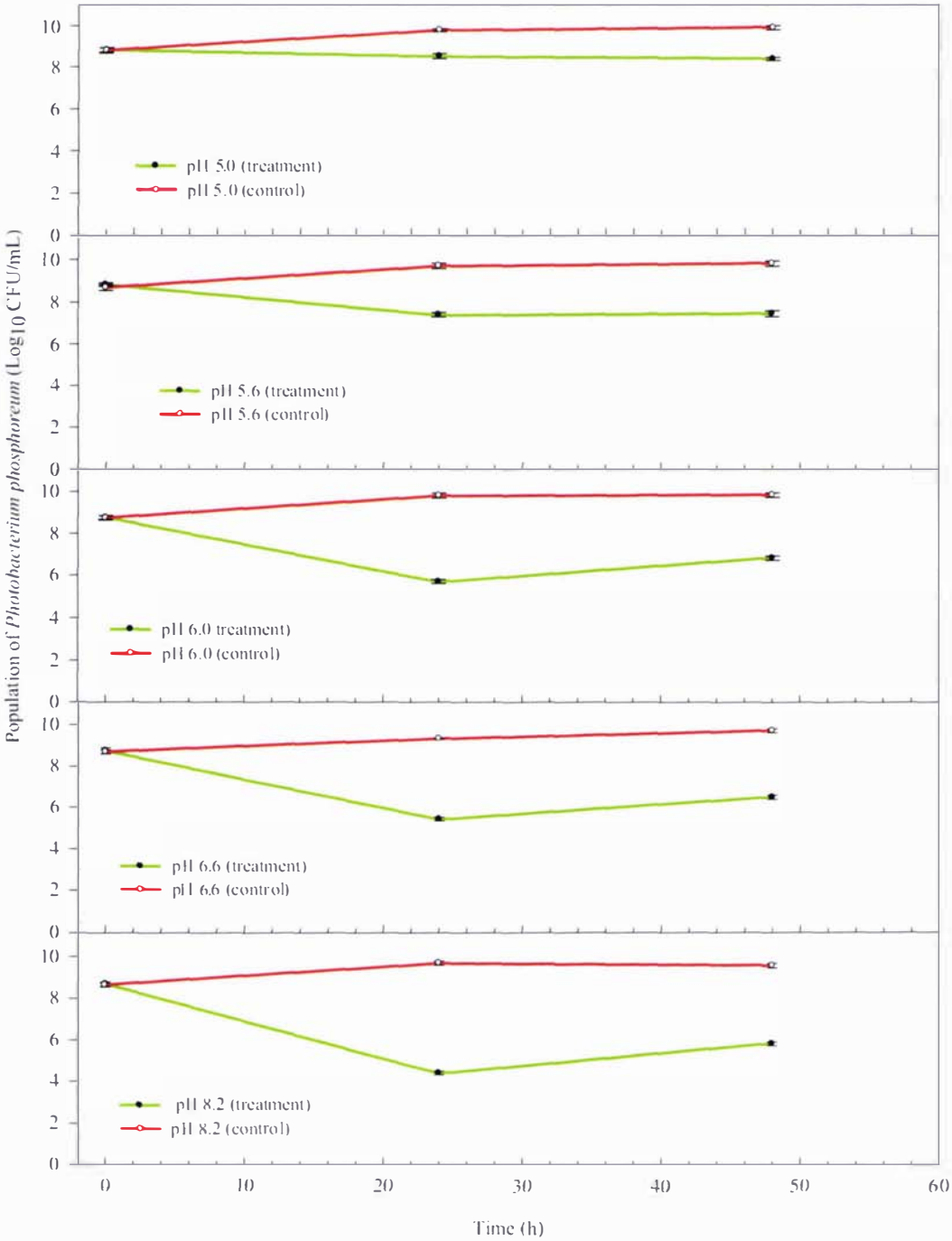


Fig. 5.8 Effect of pH on the population of *P. phosphoreum* after challenging BD isolate OT2 (7.3 $\log_{10}$ PFU/mL) against *P. phosphoreum* (8.4 $\log_{10}$ CFU/mL) in 70% ASW at a prey to predator ratio of 16 after 24 and 48 h of incubation at 25°C. Error bars represent the standard deviation of 3 replicates.

5.3.5 Co-culture of BD (OT2) with *P. phosphoreum* in 70% ASW

Counts of *P. phosphoreum* and BD during 24 h of co-culture at 25°C are shown in Figures 5.9 and 5.10 respectively and in Appendices 5.F and 5.G respectively. Although the effect of the highest concentration of OT2, at a prey to predator ratio of 6.2, was first noted after 5-6 h of incubation, when the population of *P. phosphoreum* was reduced by 0.3 - 0.4 $\log_{10}$ unit, reductions peaked after 8-10 h of incubation when a 4.4 $\log_{10}$ unit reduction was recorded. After this time, there was no further decrease in numbers of *P. phosphoreum*.

When using the medium concentration of OT2 against *P. phosphoreum*, at a prey to predator ratio of 7.5 x 10^3, the reduction in absorbance was first noted after 10 h of incubation (0.95 $\log_{10}$ unit reduction) and the maximum reduction was observed after 24 h of incubation (4.53 $\log_{10}$ unit). Similarly, the effect of the lowest concentration of OT2 was only 0.6-log unit after 10 h of incubation, while the reduction effect was greatest after 24 h of incubation (4.63 $\log_{10}$ unit reduction). The control flask (no BD added) showed almost non-detectable changes in the population of *P. phosphoreum* after 24 h of incubation at 25°C.

When using the highest OT2 concentration, BD growth was observed after 6 h of incubation and the maximum increase of ~3.0 $\log_{10}$ unit was observed within 9 – 10 h. No further increase in BD numbers was observed after this time.

Although the effect of the medium concentration of OT2 was first observed within 7 h of incubation, the maximum increase of ~5.0 $\log_{10}$ unit of BD numbers was observed after 24 h of incubation. At the lower concentration of OT2, the increase in BD numbers was slower. However, the greatest increase in BD numbers (~6 $\log_{10}$ unit) was observed in samples challenged with this low concentration of OT2.

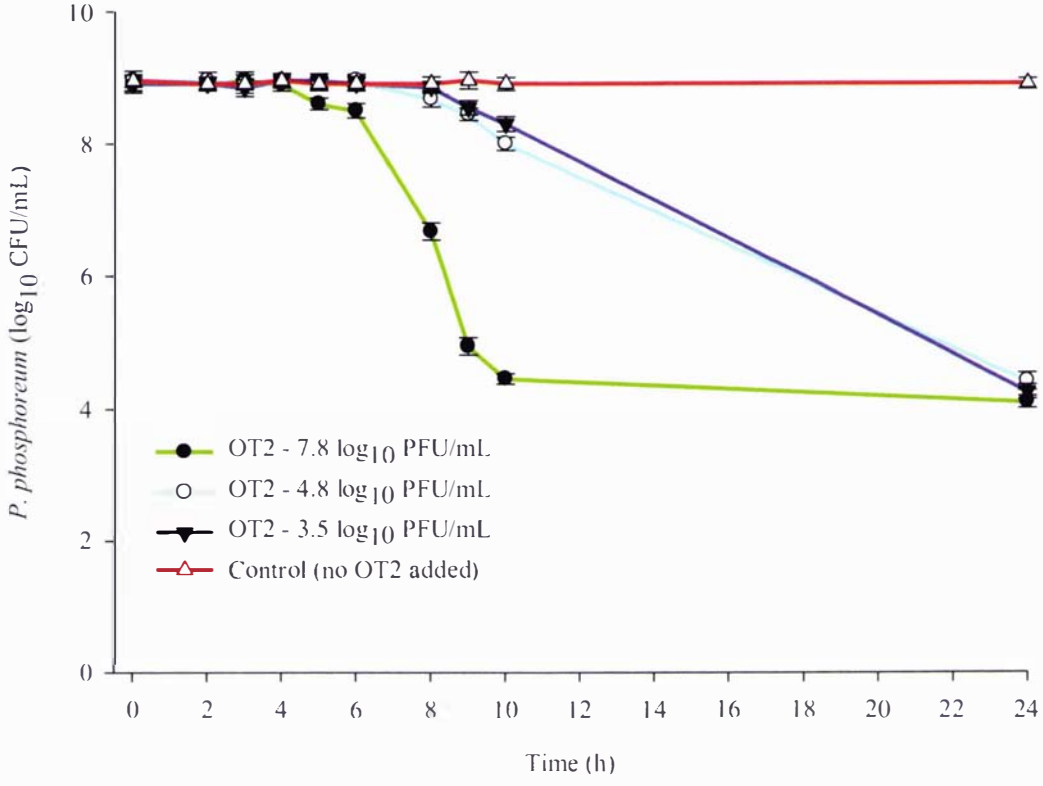


Fig. 5.9 Time course of different doses of BD isolate OT2 (7.8, 4.8 and 3.5 log_{10} PFU/mL) challenged against against *P. phosphoreum* at prey to predator ratios of 6 x 10^0, 7x 10^3 and 1x 10^5 in 70% ASW for 24 h of incubation at 25°C. Samples were tested after 0, 2, 3, 4, 5, 6, 8, 9, 10, 12 and 24 h. Values are presented as mean ± standard error, n = 3.

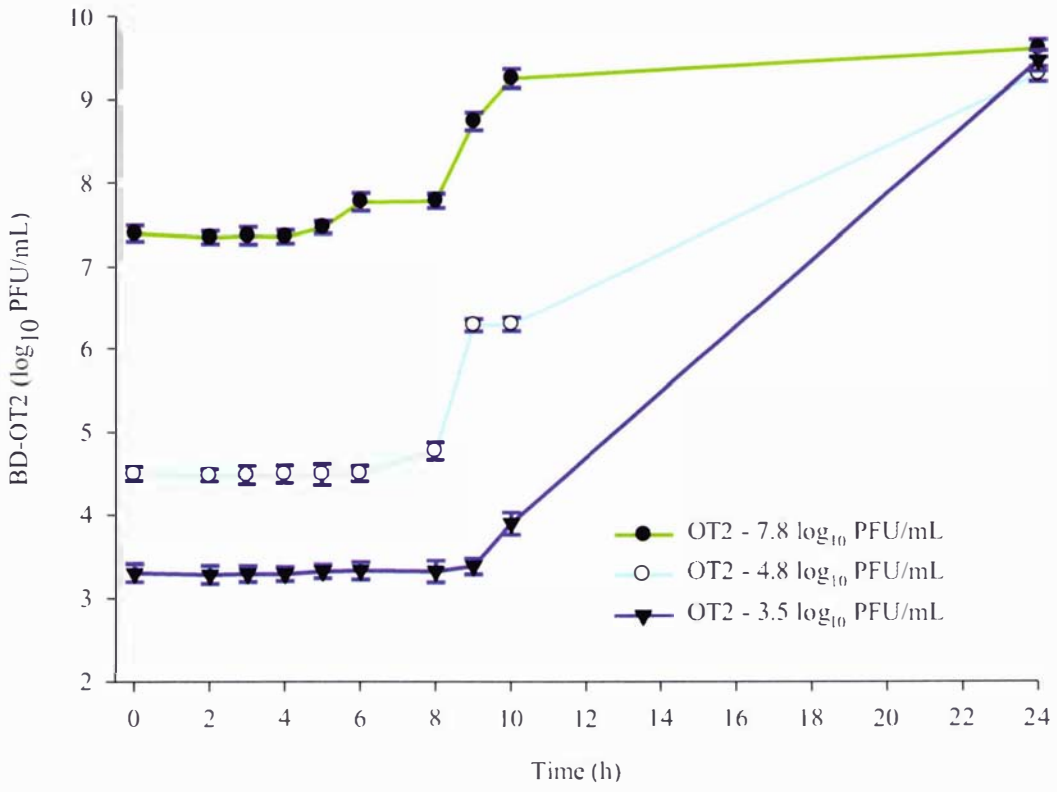


Fig. 5.10 Effect of different doses of BD isolate OT2 (7.8, 4.8 and 3.5 log_{10} PFU/mL) on its growth in the presence of *P. phosphoreum* (8.6 log_{10} CFU/mL) at prey to predator ratios of 6 x 10^0, 7 x 10^3 and 1 x 10^5 in 70% ASW during 24 h of incubation at 25°C. Samples were tested after 0, 2, 3, 4, 5, 6, 8, 9, 10, 12 and 24 h. Values are presented as mean ± standard error, n = 3.

5.3.6 Effect of different nutrient concentrations on the reduction of *P. phosphoreum* numbers by BD

The reason for choosing 1% to 16% standard SWYE broth to study the effect of different nutrient levels on the reduction of *P. phosphoreum* is that there are much lower concentrations of nutrients in a natural sea water system than those in the SWYE broth from which the BD were isolated. A particular concentration of SWYE was considered, which was suitable for prey growth and predation of BD. Overseas workers (Shilo and Bruff, 1965; Seidler and Starr, 1969; Baer *et al.*, 2004) also used dilute nutrient broth (NB/10) for maintaining HI or terrestrial BD. The highest reductions in numbers of *P. phosphoreum* were observed after 24 h of incubation at dilutions of 1 and 2% of standard SWYE, although significant reductions were seen at all nutrient concentrations tested (Table 5.5). However, re-growth of *P. phosphoreum*, observed after 48 h of incubation, was more rapid in the higher nutrient levels of standard SWYE broth.

Table 5.5 Reduction of *P. phosphoreum* populations by BD isolate OT2 at a prey to predator ratio of 44 in diluted SWYE broth.

SWYE broth concentration[a]	Mean initial count (log_{10} CFU / mL)[b]	Mean reductions (log_{10} CFU / mL)[c]	
	0 h	24 h	48 h
1%	8.22	4.47	4.52
2 %	8.18	3.50	3.33
4 %	8.23	2.97	2.87
8%	8.16	1.39	0.37
16%	8.18	0.35	-0.04
Control (16%)	8.22	-0.53	-0.83

[a] *Different dilutions of SWYE broth were prepared according to the specified proportions and inoculated with 1 mL of OT2 (6.40 log_{10} PFU/mL) + 1 mL of* P. phosphoreum *(8.51 log_{10} CFU/mL). Control samples contained 50 mL of 16% standard SWYE broth inoculated with just 1 mL of* P. phosphoreum *(8.51 log_{10} CFU/mL).*

[b] *Counts of mean* P. phosphoreum *population (log_{10} CFU/mL) at 0 h of incubation*

[c] *Mean population of* P. phosphoreum *(log_{10} CFU/mL) at time 0 h of incubation minus mean population at time 24 h or 48 h of incubation.*

5.3.7 Effect of prey concentrations on the predation of *P. phosphoreum* by BD

The changes in high concentrations (8.1 $\log_{10}$ CFU/mL) of *P. phosphoreum* upon challenging with the three different doses of OT2 in diluted SWYE (16%) or 70% ASW after 8, 16 or 24 h of incubation at 25°C are shown in Fig. 5.11 and Appendices 5.H and 5.I. There was no real reduction of the numbers of *P. phosphoreum* in diluted SWYE with any dose size of BD, while in 70% ASW, the mean *P. phosphoreum* counts were reduced significantly within 16 h of incubation only in samples challenged with the high dose of OT2. The medium dose of OT2 reduced *P. phosphoreum* counts only after 24 h of incubation, and there was minimal reduction of *P. phosphoreum* population in samples with the low dose of OT2. Control samples did not show any change in the mean *P. phosphoreum* population over 24h.

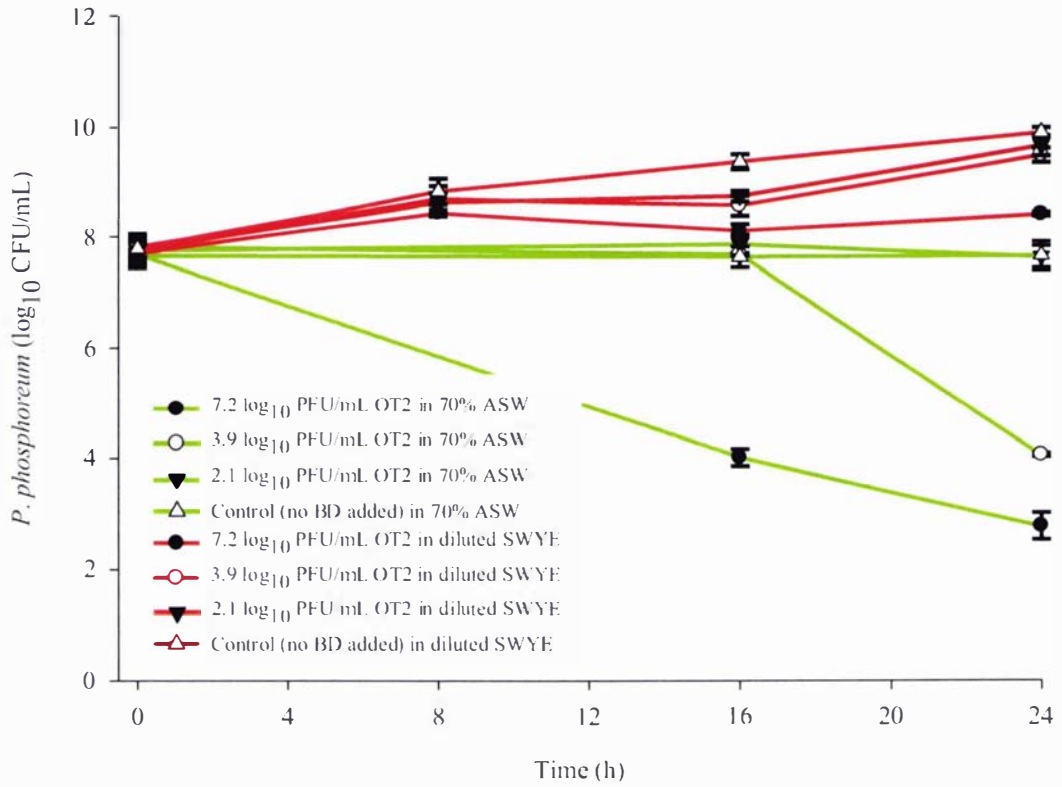


Fig. 5.11 Effect of different doses of B/D isolate OT2 (7.2, 3.9 and 2.1 $\log_{10}$ PFU/mL) against a high concentration of *P. phosphoreum* (8.1 $\log_{10}$ CFU/mL) in diluted SWYE (16% SWYE broth) or 70% ASW at 25°C. Values are presented as mean ± standard deviation, n = 3.

When using a medium concentration of *P. phosphoreum* (3.7 $\log_{10}$ CFU/mL) in diluted SWYE broth (Fig. 5.12 and Appendices 5.J and 5.K), there were no significant effects of the low or medium doses of BD on the numbers of *P. phosphoreum*, but the high dose resulted in a reduced growth rate and final concentration of host cells. In 70% ASW, the high dose of BD actually decreased the mean counts of *P. phosphoreum*, the medium dose BD reduced the growth rate somewhat, but the low dose of BD did not show any effect.

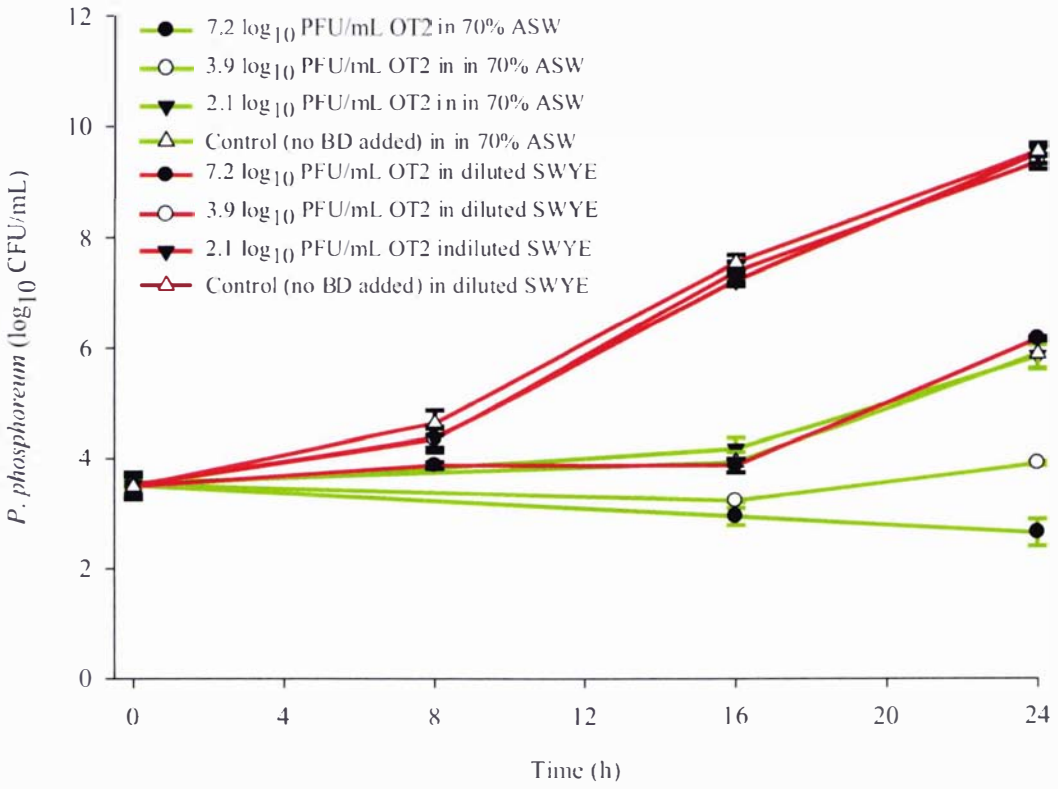


Fig. 5.12 Effect of different doses of BD isolate OT2 (7.2, 3.9 and 2.1 $\log_{10}$ PFU/mL) against a medium concentration of *P. phosphoreum* (3.7 $\log_{10}$ CFU/mL) in diluted SWYE (16% SWYE broth) or 70% ASW at 25°C. Values are presented as mean ± standard deviation, n = 3.

The impacts of the different doses of OT2 against a low concentration of *P. phosphoreum* (2.8 $\log_{10}$ CFU/mL) in diluted SWYE broth or 70% ASW are summarized in Fig. 5.13 and Appendices 5.L and 5.M. There were minimal effects of the low or medium doses of BD on the numbers of *P. phosphoreum* in diluted SWYE broth, while the high dose substantially depressed the growth of host cells. In 70% ASW *P. phosphoreum* did not grow in either the presence or absence of BD.

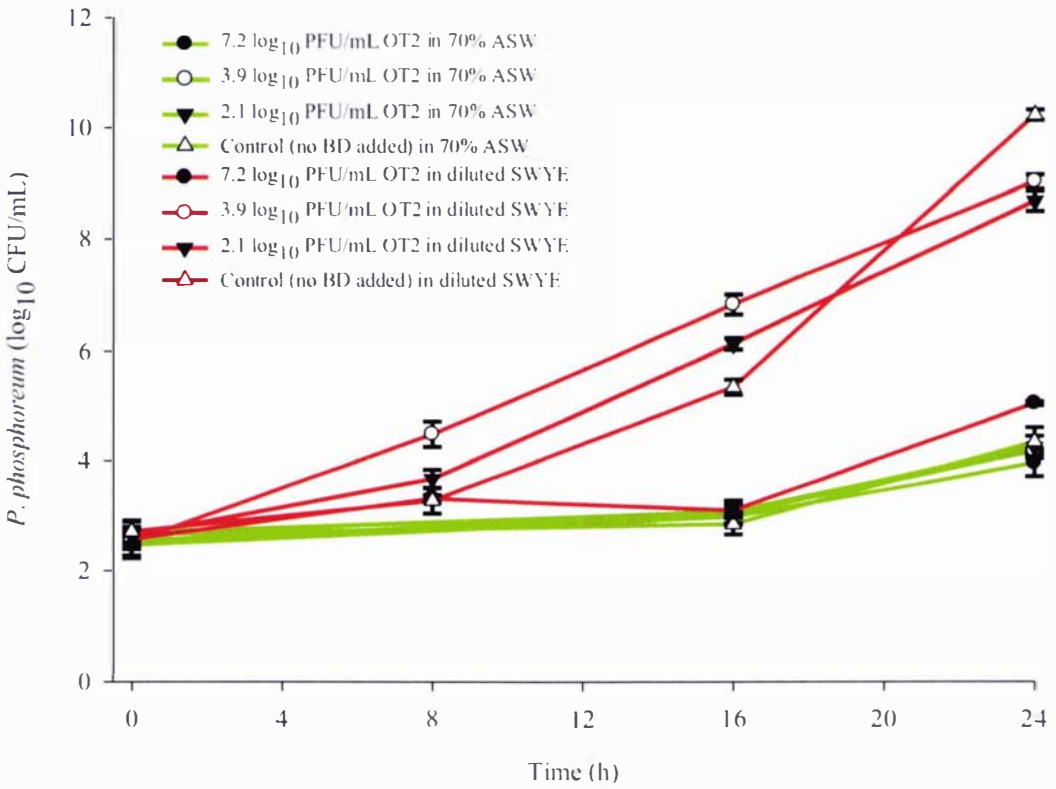


Fig. 5.13 Effect of different doses of BD isolate OT2 (7.2, 3.9 and 2.1 $\log_{10}$ PFU/mL) against a low concentration of *P. phosphoreum* (2.8 $\log_{10}$ CFU/mL) in diluted SWYE (16% SWYE broth) or 70% ASW at 25°C. Values are presented as mean ± standard deviation, n = 3.

5.3.8 Effect of predator levels on the reduction of *P. phosphoreum* numbers

Predator levels of 7.4 to 8.3 $\log_{10}$ PFU/ml had a significant effect on reducing of absorbance of dual cultures of *P. phosphoreum* and OT2 in 70% ASW throughout the incubation period (Table 5.6). However, at a BD level of 3.4 $\log_{10}$ PFU/ml reduced absorbance was only observed some time after 12 h and at 2.4 PFU/ml there were no changes in the culture absorbance over 24 h of incubation.

The changes in the population of *P. phosphoreum* after challenging with the different prey levels are shown in Fig. 5.14 and Appendix 5.N. At high levels of *P. phosphoreum* (8.4 $\log_{10}$ CFU/mL), predator levels of 8.1 and 8.3 PFU/ml resulted in a rapid effect which then started to reverse. After 9h of incubation, re-growth of *P. phosphoreum* was observed. In contrast, a slow but more prolonged effect was observed at prey levels of 7.7, 7.4 and 3.4 PFU/mL with >4.0 $\log_{10}$ unit reduction of *P. phosphoreum* numbers after 24 h of incubation. Prey levels of 2.4 PFU/mL had minimal effect on reducing *P. phosphoreum* numbers over the 24 h period.

The results from OT2 levels of 2.4, 3.4 and 7.1 $\log_{10}$ PFU/mL against high levels (8.4 CFU/mL) of *P. phosphoreum* in 70% ASW in this experiment are similar to those recorded in Section 5.3.7 for OT2 levels of 2.1, 3.9 and 7.2 $\log_{10}$ PFU/mL also against high levels (8.1 CFU/mL) of *P. phosphoreum* in 70% ASW.

Table 5.6 Effect of predator levels of 8.3, 8.1, 7.7, 7.4, 3.4 and 2.4 log_{10} PFU/mL on the absorbance changes of the dual cultures of *P. phosphoreum* (8.3 log_{10} CFU/mL) and BD isolate OT2 in 70% ASW at 25°C for 24 h. Samples were tested after 0, 2, 4, 6, 8, 10, 12 and 24 h and the absorbances were measured using a spectrophotometer at the wavelength of 610 nm.

BD inocula[a] (log_{10} PFU/mL)	*P. phosphoreum* inocula (log_{10} CFU/mL)	Prey: predator ratio	Initial absorbance[b]	Absorbance change [c]						
			0 h	2 h	4 h	6 h	8 h	10 h	12 h	24 h
8.3	8.4	1.3	0.5	0.1	0.1	0.2	0.2	0.2	0.2	0.2
8.1	8.4	2.3	0.4	0.1	0.1	0.2	0.2	0.2	0.2	0.2
7.7	8.4	5.4	0.3	0.1	0.1	0.2	0.2	0.2	0.2	0.2
7.4	8.4	10	0.3	0.0	0.1	0.2	0.2	0.2	0.2	0.2
3.4	8.4	10^5	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.2
2.4	8.4	10^6	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Nil	8.4	Control	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[a] *Prey : predator ratio prepared as dilutions of OT2 (8.32 log_{10} PFU/mL) and* P. phosphoreum *(8.43 log_{10} CFU/mL) in 50-mL of 70% ASW*

[b] *Absorbance (OD at 610 nm) of dual culture of BD isolates and* P. phosphoreum *at 0 h*

[c] *Mean absorbance (n=3) of BD isolate and* P. phosphoreum *at time 0 h minus mean absorbance of the same dual culture after 2, 4, 6, 8, 10, 12 or 24 h of incubation.*

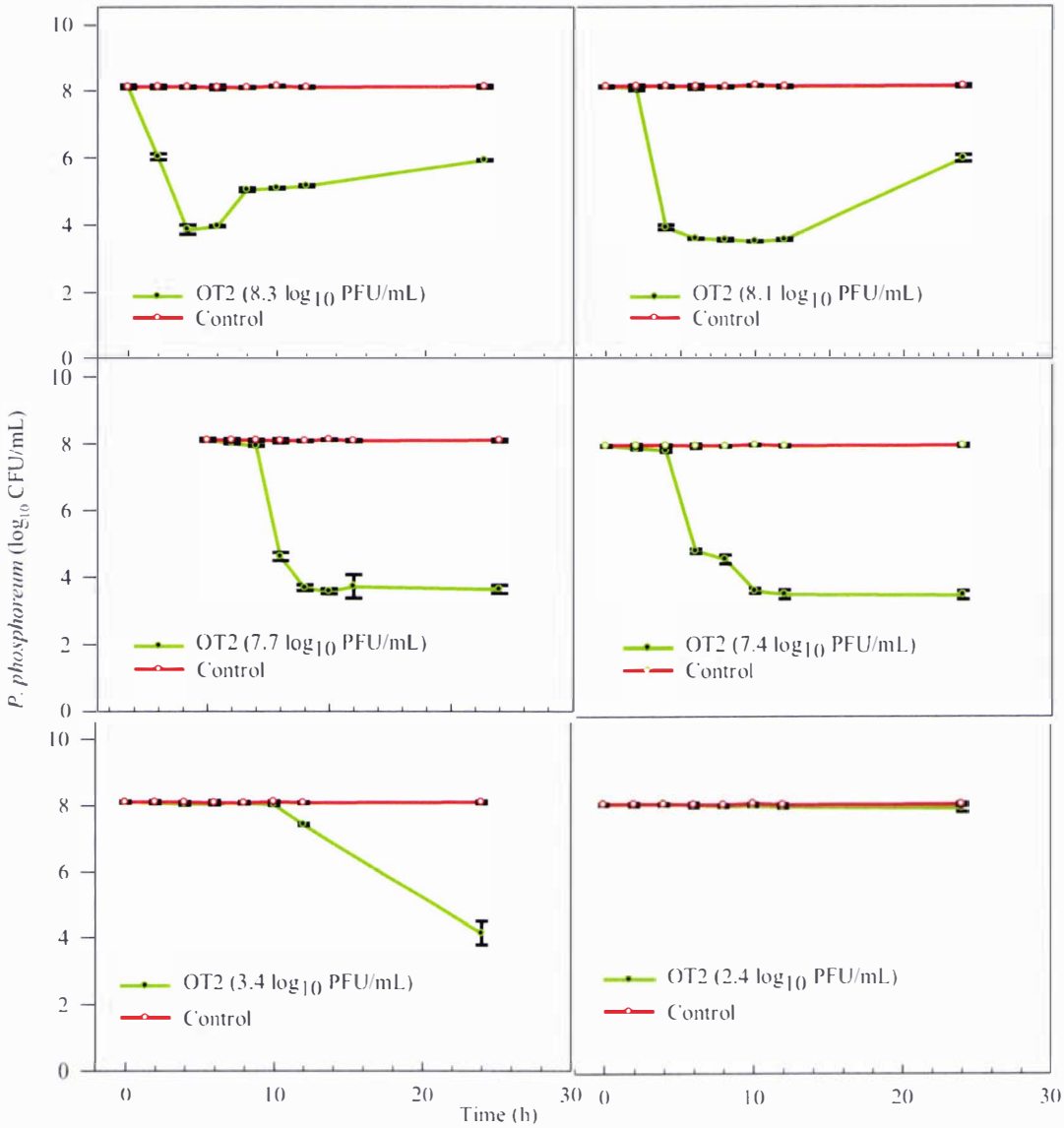


Fig. 5.14 Effect of predator (BD isolate OT2) levels of 8.3, 8.1, 7.7, 7.4, 3.4 and 2.4 log_{10} PFU/mL on reducing high numbers of *P. phosphoreum* (8.3 log_{10} CFU/mL) in 70% ASW. Samples were incubated at 25°C for 24 h and tested on 0, 2, 4, 6, 8, 10, 12 and 24 h. Values are shown as means with the standard error bars.

5.3.9 Effect of prey : predator ratios on the predation of *P. phosphoreum* by BD

The coexistence of prey and predator populations plays an important role in the natural ecosystem and the predator – prey system was suggested as a convenient model for studying the phenomenon of predation in simulating the natural environment (Markelova and Gariev, 2005). The *in vitro* results showed that the effects of BD against *P. phosphoreum* are related to the concentrations of both prey and predator. The purpose this work is to evaluate the prey-predator interaction under laboratory conditions at different prey and predator densities. This will provide more information on the biological potential of predator bacteria to eliminate or reduce undesirable bacteria in the environment. Compiled results from the above experiments on the reduction of *P. phosphoreum* numbers, at different prey : predator ratios (*P. phosphoreum* : OT2), in 70% ASW after 24 h of incubation at 25°C, are summarized in Table 5.7 and Fig 5.15. The prey-predator ratios were hugely spread, capturing some of the boundaries to the model. However no data were recorded with *P. phosphoreum* concentrations of 8.0 and 3.7 $\log_{10}$ CFU/mL and predator concentrations of 7.2 and 3.9 $\log_{10}$ PFU/mL. A regression model fitted to the reduction data showed the *P. phosphoreum* concentration as the best single predictor (70% variance; $p<0.001$). Adding either the BD concentration or the $\log_{10}$ of the prey : predator ratio gave a very similar model ($p=0.004$ for either variable; variance accounted for by the two variable model, rises to 81%).

The two-variable models are

Reduction = -7.00 + 1.355 * *P. phosphoreum* concentration ($\log_{10}$ CFU/mL) - 0.448 * $\log_{10}$ Prey : predator ratio

or

Reduction = -7.32 + 0.964 * *P. phosphoreum* concentration ($\log_{10}$ CFU/mL) + 0.432 * BD_inoculum ($\log_{10}$ PFU/mL)

The 3 D graph (Fig 5.15) and Table 5.7 show that the prey population will remain constant when the prey inoculum is high and the predator is absent; the prey population will grow exponentially when the prey numbers are low and the predator is absent; the predator population will starve when the prey population is low. The model informs that at high concentrations of prey (~8 $\log_{10}$

CFU/mL) and medium to high concentrations of predator (3.4 – 7.3 $\log_{10}$ PFU/mL), and when the $\log_{10}$ prey : predator ratio was >5.0, a greater reduction of *P. phosphoreum* numbers was observed. At high concentration of prey (~8.4 $\log_{10}$ CFU/mL) and predator (~7.5 $\log_{10}$ PFU/mL), and when the $\log_{10}$ prey : predator ratio was <5.0, the reduction in *P. phosphoreum* numbers was less. When the concentration of prey was ~3.7 $\log_{10}$ CFU/mL, irrespective of either the predator concentration or prey : predator ratio, the reduction of *P. phosphoreum* numbers was minimal over the 24 h period. At low prey : predator ratios of >6, where *P. phosphoreum* was ~8.4 $\log_{10}$ CFU/mL, the reduction of *P. phosphoreum* numbers was also minimal.

The regression model has gaps in it, and it is unclear if the bimodal spread of data distorts the model. A broader data set would be advantageous in that it would allow detection of any curvature within the model, but would probably not affect the conclusions that can be drawn at the boundaries.

Table 5.7 Summary of the reduction of *P. phosphoreum* at different prey to predator ratios in 70% ASW after 24 h of incubation at 25°C (prey - *P. phosphoreum*; predator – BD isolate OT2).

Section / Appendix	BD inocula (log_{10} PFU/mL)	*P. phosphoreum* concentrations (log_{10} CFU/mL)	Prey : predator ratio[a]	Reduction of *P. phosphoreum* numbers[b]
5.3.2 / 5C	7.3	8.0	5	4.6 ± 0.13
5.3.3 / 5D	7.2	8.4	16	3.8 ± 0.03
5.3.4 / 5E	7.3	8.5	15	4.3 ± 0.08
5.3.5 / 5F	7.8	8.6	6	4.8 ±0.09
5.3.5 / 5F	4.8	8.6	7×10^3	4.5 ±0.13
5.3.5 / 5F	3.5	8.6	1×10^5	4.6 ±0.11
5.3.7 / 5I	7.2	8.1	8	4.9 ± 0.12
5.3.7 / 5I	3.9	8.1	1.7×10^4	3.8 ± 0.22
5.3.7 / 5I	2.1	8.1	1.0×10^6	0.1 ± 0.21
5.3.7 / 5I	7.2	3.7	3.1×10^{-4}	0.9 ± 0.12
5.3.7 / 5I	3.9	3.7	0.6	-(0.4 ± 0.15)
5.3.7 / 5I	2.1	3.7	38	-(2.4 ± 0.11)
5.3.7 / 5I	7.2	2.8	3.8×10^{-5}	-(1.5 ± 0.18)
5.3.7 / 5I	3.9	2.8	7.6×10^{-2}	-(1.6 ± 0.12)
5.3.7 / 5I	2.1	2.8	4.6	-(1.6 ± 0.11)
5.3.8 / 5I	8.3	8.4	1.3	2.2 ± 0.02
5.3.8 /5M	8.1	8.4	2.3	2.2 ± 0.10
5.3.8 /5M	7.7	8.4	5	4.9 ± 0.12
5.3.8 /5M	7.4	8.4	10	4.5 ± 0.12
5.3.8 /5M	3.4	8.4	10^5	4.0 ± 0.36
5.3.8 /5M	2.4	8.4	10^6	0.1 ± 0.11

[a] *Prey: predator ratio prepared as dilutions of OT2 and* P. phosphoreum *in 70% ASW*
[b] *Reduction in* P. phosphoreum *(log_{10} CFU/mL) after 24 h at 25°C in 70% ASW. Negative values in parentheses represent increases in* P. phosphoreum *numbers rather than reductions of less than 1 CFU/mL.*

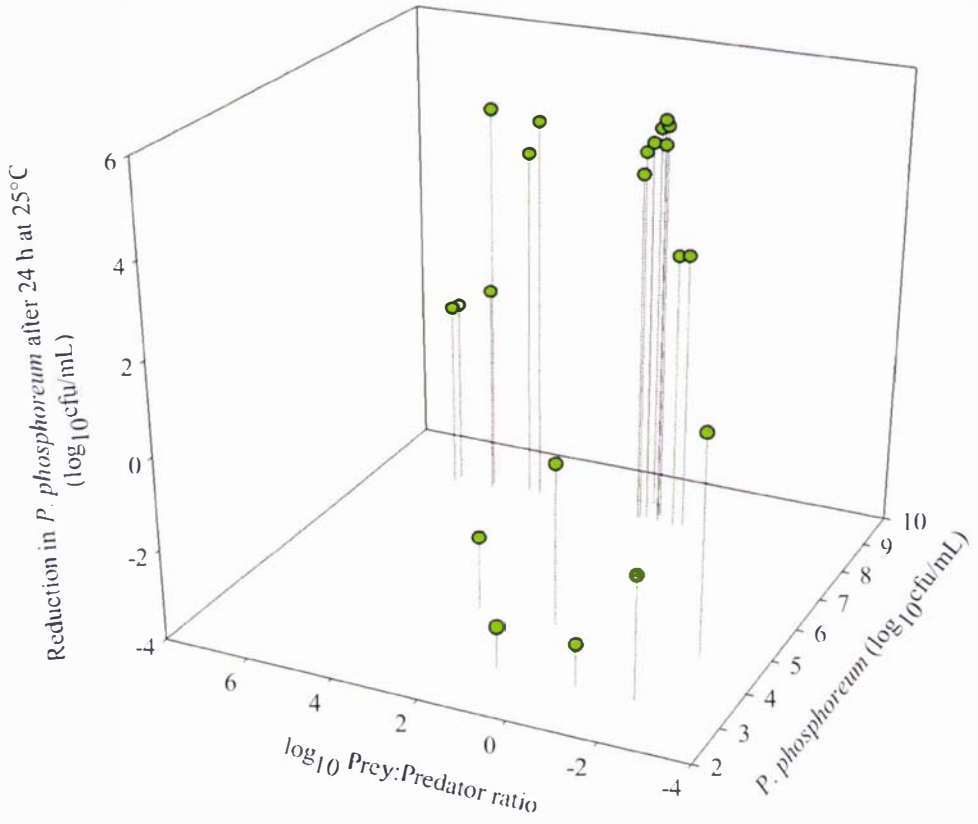


Fig. 5.15 Summary of the effect of prey: predator ratios in 70% ASW after 24 h of incubation at 25°C. Negative values in reductions in *P. phosphoreum* represent increases in *P. phosphoreum* numbers rather than reductions of less than 1 CFU/mL.

5.4 Discussion

5.4.1 Survival of BD isolates at different temperatures

The absence of an exogenous energy source, i.e. host bacteria, can lead to a relatively rapid loss of culture viability of BD at room temperature (Hespell *et al.*, 1973; 1974), which poses a problem in practical application. However, no studies have been reported to date on the survival of pure cultures of BD at different temperatures. Hence this study was performed to determine the survival rate of BD in dense suspensions without host bacteria at different temperatures. Several studies have been done on the storage of BD with their hosts in vegetative, bdelloplast or bdellocyst forms. *Bdellovibrio* cell viability can be enhanced by maintaining the BD with host cells as bdelloplast or bdellocysts (Tudor and Conti, 1977) and many laboratories maintain BD cultures by transferring at weekly intervals in the presence of host cells. However, this practice may lead to the fixation of unknown variations in the activities or characteristics of the strain over time (Talley *et al.*, 1987).

Long-term storage of dual cultures of BD and host cells at –20°C and –80°C in a cryoprotectant such as glycerol, or lyophilization of skim-milk suspensions of both parasitic and saprophytic BD has been successfully practiced (Stolp, 1981; Ruby, 1992; Williams *et al.*, 2003). However, the revival of BD, particularly from the freeze-dried form, is time consuming and inconsistent (Starr and Huang, 1972).

In this study (Section 5.3.1), the results show that in dense form, pure BD cultures can be stored for 13 to 16 days with minimal reduction in numbers at temperatures ranging from 4°C to 20°C (Figs. 5.1 and 5.3). However, significant reductions in numbers were observed at –18°C, 30°C and 37°C after 10-13 days. Thus the present study confirms the suitability of storing BD at 4°C to 20°C for a relatively long-term period which will allow the preparation of a large stock culture of pure BD in multiple vials and the opening of a new vial for each subsequent study. Since only two isolates of BD (MNA and MCB) were examined in this study, the evidence of these findings must be restricted to these particular isolates, but it is reasonable to assume that other taxonomically similar strains, e.g. OT2, would behave in a similar manner. The reason for the

rapid loss of viability of BD when stored at –18°C is unclear, but it may be due to freezing and / or thawing rates rather than the storage temperature *per se*.

5.4.2 Screening of seawater BD against *P. phosphoreum*

The present results obtained from Section 5.3.2 demonstrated that all the New Zealand BD isolates tested have considerable reduction effect against *P. phosphoreum*, but the predation efficiency of different isolates against this host was variable. Previous researchers have demonstrated differential predation and non-random attachment among prey by a variety of BD strains (Varon, 1981; Rogosky *et al.*, 2006). However, the current work was carried out on only 13 taxonomically similar strains isolated from seawater at different locations of the North Island of New Zealand, and should be repeated on a wider scale.

Although they are in fact taxonomically the same, or very closely related, strains isolated from different locations, absorbance measurements showed OT2 and TB1 to be the most effective isolates against *P. phosphoreum* followed by OT1, OT3, OT4, MCB, MNA and SP. Strains OT-enr, TB2, TB-enr and MNZ1 were the least effective against *P. phosphoreum* (Table 5.1).

The enumeration of total viable cells showed a slightly different scenario in terms of their effects. The initial predation pattern (10 h) suggested that OT2 and OT1 were the most effective isolates compared to OT5, OT4, MNA, MCB, TB1, TB2, OT3, OT-enr and SP, whereas TB-enr and MNZ1 were the least effective (Figs 5.5 and 5.6). However, after 24 h of incubation the efficiency of all BD isolates was found to be similar. It is not clearly understood why there is a difference in the initial predation effect of the different isolates of BD, but the present work showed that some isolates are more effective than others, although they are taxonomically similar or very closely related to each other (Chapter 3). The enumeration of total viable count is the more accurate technique compared to absorbance measurement. The isolate OT2 was used in the subsequent studies described in this thesis.

5.4.3 Ability of BD (OT2) to reduce numbers of *P. phosphoreum* at different salinities

The results confirmed that salinity exerts an effect on BD isolates in the reduction of a *P. phosphoreum* population (Section 5.3.3). Both the absorbance measurements and the viable counts revealed the reduction effect between salinities 0.9 – 4.5%, with reductions most marked between 1.5 – 3.0% (Table 5.3 and Fig. 5.7). The effectiveness of the BD isolate at salinity greater than 4.5% (w/v) was not studied.

In China, Song *et al.* (2005) studied the lysis abilities and growth conditions of four BD isolates and their results showed that all four isolates grew at salinity values in the range of 1% and 3.5%, with 3% salinity being the most suitable. Kelley *et al.* (1997) recovered a greater number of halophilic BD from the water surface of the Patuxent River, a tributary of the Chesapeake Bay, Baltimore, U.S., at 1.1% or greater salinity compared with those in lower salinity water. They also recovered BD from salinities below 0.5%. Marbach *et al.* (1976) screened and characterized marine BD isolated from the Israeli littoral of the Mediterranean Sea and observed that salinity tolerances ranged from 1.19 to 5.96%. Taylor *et al.* (1974) found that marine isolates from the coast of Oahu, Hawaii required a minimum of 0.44% sodium chloride for plaque formation and the optimum was 0.73 – 0.88%. In contrast, the present study confirmed a requirement of 0.9 – 4.5% sodium chloride for reduction of numbers of New Zealand marine strains of *P. phosphoreum* and the optimum was 1.5 – 3.0% (Fig. 5.7). The worldwide differences in salt tolerances of BD isolates appear to largely reflect the salinities of the waterways from which they were isolated.

5.4.4 Ability of BD (OT2) to reduce numbers of *P. phosphoreum* at different pH values

The results obtained from Section 5.3.4 are in agreement with the findings of earlier work with terrestrial BD in the U.S., which claimed that BD isolates worked best at pH values near neutrality in the reduction of an *E. coli* population (Jackson and Whiting, 1992; Fratamico and Whiting, 1995). In the present study both the absorbance measurement and the total viable counts showed a considerable reduction of *P. phosphoreum* population at pH values between 6.0 and 8.2 after 24 h of incubation (Table 5.4 and Fig. 5.8).

As *P. phosphoreum* was unable to form colonies at pH values less than 5.0, it was not possible to evaluate the efficacy of BD isolates at these pH values. Varon and Shilo (1968) reported that BD lost their motility at pH 5.0 and were therefore unable to attach to host cells. Filip *et al.* (1991) failed to grow plaques at pH values less than 6.0; however the present study has shown that BD can slightly reduce the *P. phosphoreum* population at pH 5.6.

The pH buffer (0.1 M citric acid – 0.2 M Na_2 HPO_4) using 70% ASW as diluents was found to maintain a stable pH over 48 h of incubation at 25°C.

5.4.5 Co-culture of BD (OT2) with *P. phosphoreum* in 70% ASW

The results showed that different inoculum sizes of BD initiated the reduction of *P. phosphoreum* at different times. It can be assumed that (as shown in Fig. 5.9 and 5.10 and Appendices 5F - 5G) the competing power of different amounts is proportional to the capacity of BD to attack and reduce the *P. phosphoreum* population. Although the maximum final reduction effects of different BD inoculum levels were similar (>4.50 $\log_{10}$ unit reduction) after 24 h of attack, the initial attacking effect varied between the different BD inocula. After the initial reduction the *P. phosphoreum* population remained constant over longer incubation times (Fig. 5.9; Appendix 5.F). This suggests that the difference between initial inoculum levels leads to differences in the time of commencement of the attacking effect but not on the final magnitude of the effect.

The population of BD increased in conjunction with the decreases in *P. phosphoreum*. The maximum increase of the BD population was observed after 24 h of attack using all three inoculum sizes, while the initial increase varied according to the inoculum size (Fig. 5.10; Appendix 5.H). In a U.S. study, Kadouri and O'Toole (2005) reported that a reduction of an *E. coli* biofilm started within 3 h of exposure to *B. bacteriovorus* strain 109J and the maximum 4-log reduction was recorded after 24 h of attack. The results of the present study are similar to the work of this previous investigation.

5.4.6 Effect of different nutrient concentrations on the reduction of *P. phosphoreum* numbers by BD (OT2)

The present study showed reduction effects at all nutrient concentrations tested with the highest found at dilutions of 1 and 2% of standard SWYE. The re-growth of *P. phosphoreum* was also more rapid in higher nutrient concentrations (Table 5.5). Although, from these results, it appeared that 16% of standard SWYE, the highest nutrient concentration used in this work, was the least suitable for cultivation of prey organisms and predation of BD, this concentration was chosen for further study as the ultimate aim was the biopreservation study in fish, which is a nutrient dense food.

5.4.7 Effect of prey concentrations on the predation of *P. phosphoreum* by BD

This study demonstrated that the prey concentration is the most important factor in the predation of *P. phosphoreum* by BD. Statistical analysis using a regression model showed that seventy percent of the variation of observed predation was due to prey concentration (Section 5.3.9). Efficient predation was achieved only when prey concentrations exceeded 4 $\log_{10}$ CFU/mL. This is consistent with the mathematical prediction of Varon and Zeigler (1978) using the Lotka-Volterra model. They suggested that a high prey density of 3 x 10^6 CFU/mL was necessary for a 50% chance for the survival of BD and 7 x 10^5 CFU/mL of prey concentrations was required for establishing an equilibrium in actively growing populations of prey and predator. This contrasted with studies of Varon *et al.* (1984) where it was found that maintenance of BD was possible with concentrations of prey organisms of 2 to 5 x 10^4 CFU/mL. In the present study, high prey concentrations (~8 $\log_{10}$ CFU/mL), coupled with a predator concentration of 3.4 – 7.3 $\log_{10}$

PFU/mL and a prey : predator ratio of >5.0 were required to give optimum predation (Table 5.7 and Fig. 5.15). The present model concluded that when the prey population is low, the predators can consume a proportion of the prey, but cannot eliminate the prey and the predator population will starve when the prey population is low. There is no environmental complexity, which means both prey and predators co-exist together or move randomly in a homogenous environment. The dynamics of the situation may be that, as predator numbers rise, prey numbers fall and *vice versa*. Hence, the numbers of both will continuously fluctuate in a cyclic manner. The necessity for this level of prey suggests that these organisms might be limited for biopreservation of BD. Dalgaard *et al.* (1997) detected that sensory signs of microbiological spoilage only occur when the level of *P. phosphoreum* exceeded 10^7 CFU/mL and at least 10^6 CFU/mL *V. parahaemolyticus* are required to cause disease (U.S. Food and Drug Administration, 2006). This conclusion was arrived at using 21 different concentrations of BD and prey : predator ratios (Table 5.7; Fig. 5.15). However, only five prey concentrations (8.6, 8.4, 8.0, 3.7 and 3.2 $\log_{10}$ CFU/mL) were used in the study, therefore the conclusions must be restricted. The reason for the lack of reduction of *P. phosphoreum* numbers at concentrations of <3.7 $\log_{10}$ CFU/mL is not clearly understood, and needs further investigation. BD encounter susceptible prey bacteria by random collision (Rittenberg, 1982) or by chemotaxis (Section 1.1.4). Low numbers of prey may simply reduce the chances for random collisions or it may be that the soluble nutrients required for chemoattraction are not produced in required amounts by the bacteria in such low concentrations.

5.4.8 Effect of predator levels on the reduction of *P. phosphoreum* numbers

This investigation (Section 5.3.7; Figs. 5.11, 5.12 and 5.13) has demonstrated that the effects of BD against *P. phosphoreum* are related to the concentrations of both organisms (host and predator) and the nutrient environment. High doses of BD exhibited significant effects against high and medium concentrations of prey organisms, but no effect against low concentrations of prey in 70% ASW. Interestingly, high doses of BD showed an effect against medium and low concentrations of *P. phosphoreum* in the nutrient medium at an early stage of incubation, but did not show any effect against higher concentrations of prey organisms. These results are consistent with the findings of Nunez *et al.* (2005), who demonstrated that under dilute nutrient conditions,

B. bacteriovorus 109J prevented the formation of simple bacterial biofilms (5 x 10^7 CFU/mL) and destroyed established biofilms. In contrast, medium doses of BD were effective against high and medium concentrations of prey organisms at the later stage of incubation in 70% ASW, but were not effective against lower concentrations of prey organisms or any concentrations of prey organisms in nutrient medium. Irrespective of the concentrations of prey bacteria, low doses of BD did not show any effect. Varon and Zeigler (1978) have suggested that the probability of a BD's survival becomes smaller as prey density decreases. Hespell *et al.* (1974) calculated that BD could not be maintained in a population of less than 1.5 x 10^5 CFU/mL of the prey organisms. However, Varon *et al.* (1984) reported that in the laboratory environment, survival of BD was possible with concentrations of prey as low as 2-5 x10^4 cells per mL.

5.4.9 Effect of prey : predator ratios on the reduction of *P. phosphoreum* numbers

The present findings (Table 5.6 and Fig. 5.14) suggested that at high concentrations of prey cells (8.43 $\log_{10}$ CFU/mL), the lower the ratio of prey to predator, the more rapid is the decline in viable *P. phosphoreum* cells. Although not as rapid as at ratios of 1.3 and 2.3, prey : predator ratios of 5.4, 10 and 10^5 showed a prolonged and consistent effect throughout the trial period. However, there was no notable reduction of *P. phosphoreum* numbers at the prey : predator ratio of 10^6. The results with the prey : predator ratios of 5.4 and 10 are similar to each other, but vastly different from the lower ratios (1.3 and 2.3). Shemesh *et al.* (2003) observed more predation effect at the predator : prey ratio of 1:1 compare to the ratio of 10:1. The reason for the re-growth of *P. phosphoreum* at the ratios of 1.3 and 2.3 is not clearly understood, but is possibly related to the unavailability of an appropriate amount of host population needed for the survival of BD. As low numbers of host were also present in the experiments using ratios of 5.4 and 10, it is likely that, if the experiment had been extended, host numbers would also have begun to increase here.

Previous researchers have demonstrated that reduction of host populations by BD can vary widely between host organisms. Fratamico and Whiting (1995) showed that *B. bacteriovorus* 109J exhibited only 0.1 – 1.2 $\log_{10}$ reductions of *Salmonella spp*, while a 7 $\log_{10}$ reduction of *Shigella spp* was observed during a similar 7 h incubation period. Although Jackson and Whiting

(1992) also reported a reduction of 7 $\log_{10}$ values in numbers of *E. coli* after 7 h of incubation, the lesser reduction of *P. phosphoreum* numbers (4.5 $\log_{10}$ unit) in the present study may be attributed partly to the use of different species of BD and host organisms.

It is concluded that dense suspensions of pure BD can be stored for relatively long period (2 – 3 weeks) at 4° – 20°C with minimal reduction in numbers. Also, under specific conditions such as at salinity 1.5 – 3.0%(w/v); pH 6.0 – 8.2; prey concentrations of >4 $\log_{10}$ CFU/mL; predator concentrations of 3.4 – 7.3 $\log_{10}$ PFU/mL and prey : predator ratios of >5, the viability and predation characteristics of BD are potentially capable of being applied against the important spoilage organism, *P. phosphoreum*, in the preservation of seafood. However, prey concentration is the most important parameter in the predation of *P. phosphoreum* by BD. The prey concentration required was at least 4 $\log_{10}$ CFU/mL for minimal predation and maximum predation was achieved at the prey concentrations of 8 $\log_{10}$ CFU/mL.

In the present study, the optimum predation conditions were determined in liquid cultures but the data do not provide enough detail to allow firm conclusions about the effectiveness of BD in reducing host bacteria in food, which is a surface bathed in liquid where the nutrient level is high and initial bacterial numbers are low. Solid medium is a type of hygroscopic capillary porous medium made up with appropriate nutritional and biophysical environment which becomes solidified due to the addition of an inert solid e.g., agar, a hydrocolloid that cannot be metabolized by most bacteria but can hold nutrients that are in aqueous solution.

Nevertheless, the current study confirmed that in high nutrient media, high doses of BD showed an effect against medium and low concentrations of *P. phosphoreum* at an early stage of incubation (Section 5.4.8) and Chapter 4 confirmed the effectiveness of BD against spoilage organisms in solid media (Section 4.3.1). An overseas study has claimed the presence of higher numbers of BD occurred on surfaces than in water columns because of the accumulation of a high number of potential prey organisms for BD on surfaces, which supports the new concept of surface-associated existence of BD in nature (Markelova and Gairev, 2005). In solid media, mobility of host bacteria is limited and higher numbers of host bacteria are located in a certain area compared to liquid media. Some bacteria grow better at the interface of liquid and solid

media. Therefore BD may have more opportunity to obtain enough bacteria for predation on solids. Markelova and Gairev, (2005) observed a two to three times higher effect of BD against *Pseudomonas fluorescens* in solid media compared to liquid media in the presence of hazardous contaminants such as cadmium chloride. With regard to an environment of solid food, e.g. fish, the situation is likely to be more similar to a solid rather than liquid media. However, this situation is likely to be more complex due to variations in the local environment, e.g. water activity, type of nutrient, as well as having a mixed rather than pure culture of prey. The fact that, during surface growth, the individual prey cells are in much closer contact with each other than in a liquid culture may lead to more effective predation since there will be increased contact between predator and prey cells.

In the present work, no quantitative work was performed on solid media, and care must be taken in extrapolating from liquid media to solid food where initial nutrient concentration will be high and bacterial numbers low.

Chapter 6

Effect of BD on the reduction of numbers of spoilage and pathogenic organisms in King Salmon

6.1 Introduction

New Zealand exported fish and fish portions worth NZ$1,351.14 million in 2006, of which the New Zealand King Salmon Company exported King salmon worth about $28.5 million (New Zealand Seafood Industry Council, 2007). The majority of fish and fish portions are exported frozen and only 3.3% are exported as fresh fish. With increasing demand for good quality fresh fish in overseas markets, globalization of the market for these products and increasing awareness of customers, the export industry in fresh fish and fish products is growing. However, there are a number of factors limiting the export of fresh fish, particularly its short natural shelf life. The shelf life mainly depends on storage temperature, the location of fishing ground, the season, physical damage and intrinsic factors such as the shape, size and fat content of the flesh and skin. It has been well documented that fish is a highly perishable food and microbial activity is mainly responsible for limiting the shelf life of packed and unpacked fresh fish (Dalgaard, 1995). Cross contamination from various sources can cause rapid decline in the hygienic quality of fresh fish and fish products (Gram and Huss, 1996). The microflora responsible for spoilage of fresh fish depends on the storage temperature. At low temperatures (0-5°C), *Shewanella putrefaciens, Photobacterium phosphoreum, Aeromonas* spp. and *Pseudomonas* spp. cause spoilage. However, at high storage temperatures (15-30°C) different species of Vibrionaceae, Enterobacteriaceae and Gram-positive organisms are responsible for spoilage (Gram *et al.*, 1987; Dalgaard, 1995). One option to control spoilage is biopreservation: the use of microorganisms which are antagonistic to spoilage bacteria. Biopreservation of fish would particularly have to control the Gram-negative spoilage bacteria but this has been explored to only a limited degree.

Most studies about 'biopreservation' deal with the application of lactic acid bacteria against Gram-positive bacteria and have focussed mainly on meat products (Wessels and Huss, 1996; Jacobsen *et al.* 2003; Vermeiren *et al.*, 2004; Castellano and Vignolo, 2006; Vermeiren *et al.*, 2006). Very little work has been performed on the use of biopreservatives on fish products and

available results are contradictory. Altieri *et al.* (2005) used bifidobacteria and thymol as biopreservatives to extend the shelf life of fresh packed plaice fillets at 4°C and 12°C and observed a synergistically extended lag phase of *Pseudomonas* spp. In contrast, Al-Dagal and Bazaraa (1999) used bifidobacteria and potassium sorbate as biopreservatives on whole shrimps, but they noticed no statistically significant differences in psychrotrophic total viable counts after 9 days storage.

Some researchers have suggested terrestrial BD as a potential biopreservation agent to extend the shelf life of food (Jackson and Whiting, 1992; Fratamico and Whiting, 1995), as a means to control *E. coli* biofilms (Kadouri and O'Toole, 2005) and phytopathogens (Uematsu, 1980; Epton *et al.*, 1989). Takaaki *et al.* (2000) patented the mass pure culture of marine BD in Japan, and Takaaki (1994) also patented marine BD as a therapeutic and prophylactic agent for *Vibrio* infectious diseases. However, to date no study has been carried out on the potential application of marine BD to biopreservation. The determination of the *in vitro* efficacy of marine BD against food-borne pathogenic and spoilage bacteria in the present study (Chapter 5) showed up to 4.8 $\log_{10}$ reductions of spoilage and pathogenic bacteria such as *P. phosphoreum* and some other Gram–negative bacteria. However the effectiveness of BD against mixed populations of bacteria was not studied. Hence, the aim of this study was to investigate the effect of marine BD in reducing the growth of spoilage organisms on king salmon. Such reductions may play a role in extending the shelf life.

6.2 Experimental procedure

The fish processing, sampling positions, microbiological analysis and sensory evaluations used methodologies described by Fletcher *et al.* (2002) with some modifications. King salmon (*Oncorhynchus tshawytscha*) were supplied by the New Zealand King Salmon Company, Nelson. Two trials coded as Trial 1 and Trial 2 were involved in this study. One fish was used in Trial 1 and 3 fish were used in Trial 2. The fish were packed in ice in a bin and transferred to the Crop & Food Research Laboratory in Auckland within 24 h of harvest. The temperature on arrival was 2.8 – 4.7°C. The fish weights were recorded using a two decimal place balance. The fish were skinned and filleted to 2 or 6 paired fillets which were further trimmed to loins of at least 2 cm thick. The loins were trimmed to fit into labeled boxes (1.9 x 3.8 x 3.8 cm^3). The portions were then quartered and the weights were recorded using the same balance. The fish pieces were randomly divided into two groups – a treatment group and the control group.

The BD isolate used was OT2 which was purified, propagated, and challenge inocula were prepared as described in Sections 2.2 and 2.5. The BD doses were chosen to ~10 log_{10} PFU/mL for the proven effectiveness in Sections 5.3.7 and 5.4.8, where only high doses of BD (~7.2 log_{10} PFU/mL) showed an effect against medium and low concentrations of *P. phosphoreum* in nutrient media at an early stage of incubation. In Trial 1, twenty-one pieces of treatment groups were dipped into a dense OT2 suspension (10.3 log_{10} PFU/mL) for 1 min. The twenty one pieces of control sample (not dipped into BD suspension) and BD-treated samples were then individually packaged in highly oxygen permeable Cryovac® 10K OTR bags (Sealed Air Corporation, USA). The oxygen transmission rate (OTR) of the bag was specified as greater than 10,000-cc/m^2/24 h. The bags were heat-sealed prior to storage (sPs Sealer, Scot – Turner Plastic Sealers Ltd, Auckland) and incubated at 20°C.

In Trial 2, eighty-five pieces of treatment samples were dipped into a dense OT2 suspension (final concentration 10.0 log_{10} PFU/mL) for 1 min. Eighty five pieces of control samples (not dipped into BD solution) as well as the BD treated samples were then packaged as described for Trial 1. Thirty pieces of each BD treated and control samples were incubated at 20°C and fifty-five pieces of each were incubated at 10°C. The storage temperatures were chosen to determine,

firstly, the effectiveness of BD at relatively high temperatures (20°C; Trial 1 and 2) to prevent spoilage if the cold chain failed during transport, retail marketing, restaurant or local fish shop display, and secondly, to prevent spoilage at lower temperatures (10°C; Trial 2) which are still considerably higher than that of the commercial seafood distribution chain where temperatures of 0-2°C are recommended.

6. 2.1 Microbiological analysis

The samples were homogenized (2 min) in a stomacher bag in salt-peptone water (1% NaCl, 0.1% peptone (Difco)) at a ratio of 1:10 in a Stomacher Lab Blender (Masticator, IUL S.A, Spain). Further serial dilutions of the homogenates were made in salt-peptone water and the spread plate method, using 100μL from each dilution, was used for total aerobic counts (plate count agar (Difco) with 1% NaCl - sPCA). Plates were incubated aerobically for 3 d at 20°C and colonies were counted and recorded as colony forming units per g (CFU / g).

6. 2.2 Sensory evaluation

Trial 2 samples were homogenized after 48 h of incubation at 20°C and were assessed by a five member panel for odour characteristics on a 5-point scale (described by Fletcher *et al.*, 2004 where 0 = characteristic salmon; 1 = neutral; 2 = neutral, slight other odour; 3 = fishy, oily, fruity, sweet, meaty, nutty; 4 = rancid, metallic; and 5 = cheese, rotten, sour). The sensory scores were statistically analysed by analysis of variance (ANOVA) using the mean score per fish and a t-test was performed to compare the five BD-treated and five control samples.

6.3 Results

Figs 6.1 and 6.2 show the total viable counts of microbial growth on salmon stored at 20°C for Trial 1 and Trial 2 respectively. The total number of bacteria in both BD-treated and control samples in both trials increased in a log-linear fashion. The lag phase of the microbial growth was slightly longer (8 – 12 h) in BD-treated samples. The total number of bacteria in the BD-treated group remained at relatively low levels throughout the period of study, but the rate of increase of bacteria in the exponential phase was similar in both samples. In both trials the total viable count reached 7 $\log_{10}$ CFU/g after 30 and 40-42 h of storage time in the control and BD-treated samples respectively.

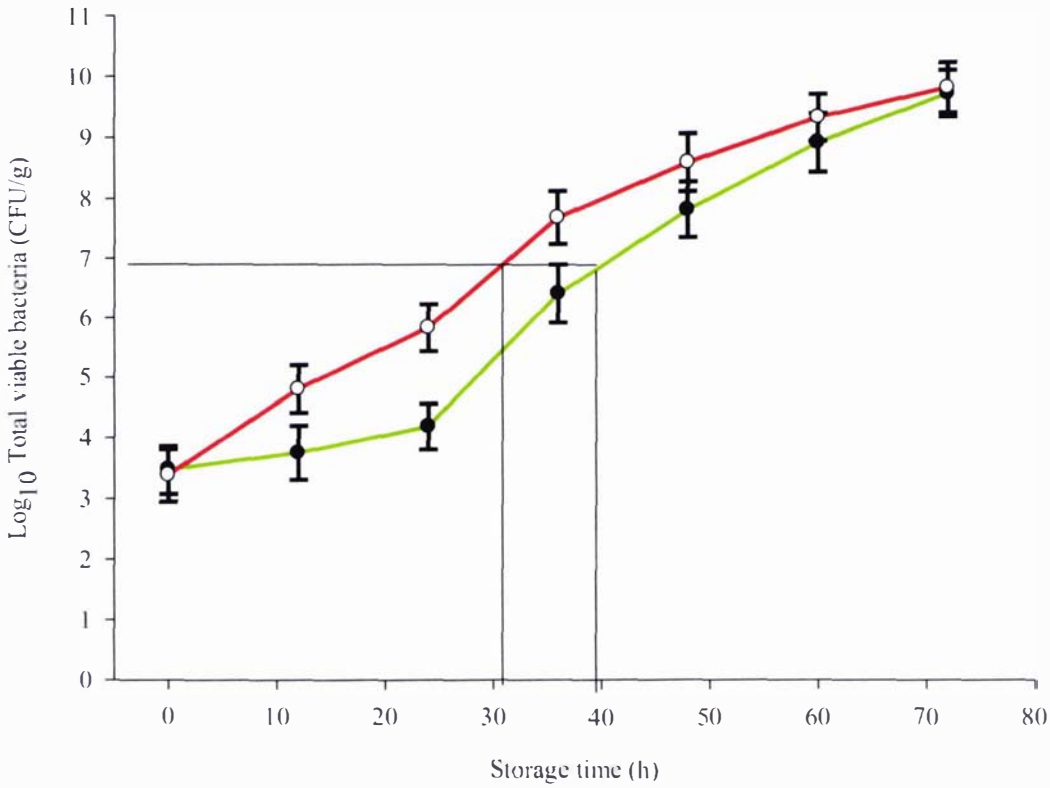


Fig. 6.1 Total viable counts of bacteria on sPCA plates (20°C for 3 days) from salmon samples stored at 20°C for trial 1. Error bars depict standard deviations of the means. Each point represents the mean of 3 samples. ○, untreated controls; •, BD treated salmon. The lines at 7 $\log_{10}$ CFU/g show the estimated times when sensory spoilage occurs due to spoilage bacteria (e.g., *P. phosphoreum*) (Dalgaard *et al.*, 1997).

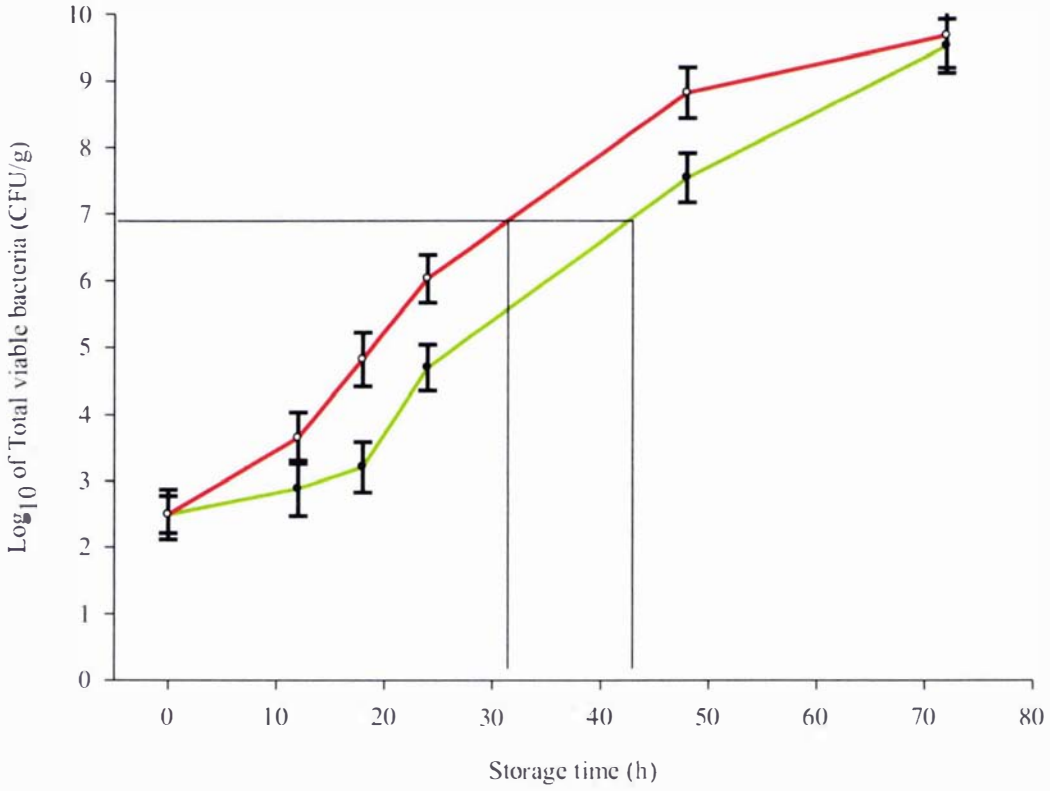


Fig. 6.2 Total viable counts of bacteria on sPCA plates (20°C for 3 days) from salmon samples stored at 20°C for trial 2. Error bars depict standard deviations of the means. Each point represents the mean of 5 samples. ○, controls; ●, BD-treated. The lines at 7 $\log_{10}$ CFU/g show the estimated times when sensory spoilage occurs due to spoilage bacteria (e.g., *P. phosphoreum*) (Dalgaard *et al.*, 1997).

Table 6.1 shows the sensory evaluation results obtained using 5 panellists in Trial 2. The results provide evidence for BD's ability to slow spoilage, as the BD-treated group was given scores of 0-2 by most of the panellist, except for 2 samples which were given a score of 3 by panellist no. 5. In contrast, the control group had scores ranging from 0-4, with the dominant score being 3. The statistical evidence shows a significant difference between the BD-treated and control fish samples ($t = 2.62$ on 8 df, $p = 0.031$).

Table 6.1 Results of sensory evaluation of King Salmon samples after 48 h of incubation at 20°C.

BD treated samples					
Sample No.	Panellist 1	Panellist 2	Panellist 3	Panellist 4	Panellist 5
1	0	0	0	1	1
2	2	2	0	1	2
3	0	0	1	1	3
4	2	2	2	0	2
5	2	2	1	2	3

Control samples					
Sample No.	Panellist 1	Panellist 2	Panellist 3	Panellist 4	Panellist 5
1	2	2	1	0	2
2	2	1	1	2	4
3	2	3	3	1	3
4	3	3	3	2	4
5	3	4	3	2	4

The 5-point sensory scales are 0 = characteristic salmon; 1 = neutral; 2 = neutral, slight other odour; 3 = fishy, oily, fruity, sweet, meaty, nutty; 4 = rancid, metallic; 5 = cheese, rotten, sour.

Fig. 6.3 shows the total viable count of microbial growth on sPCA plates for the salmon samples stored at 10°C. As expected, the lag phase of the microbial growth was longer followed by a less rapid exponential phase in both BD-treated and control samples compared to 20°C storage. The total viable bacteria reached 7 $\log_{10}$ CFU/g after 5 days of storage time in both BD-treated and control samples. There was no detectable effect of BD on the population of total viable bacteria at 10°C. Time and resources did not permit sensory evaluation of the salmon samples stored at 10°C.

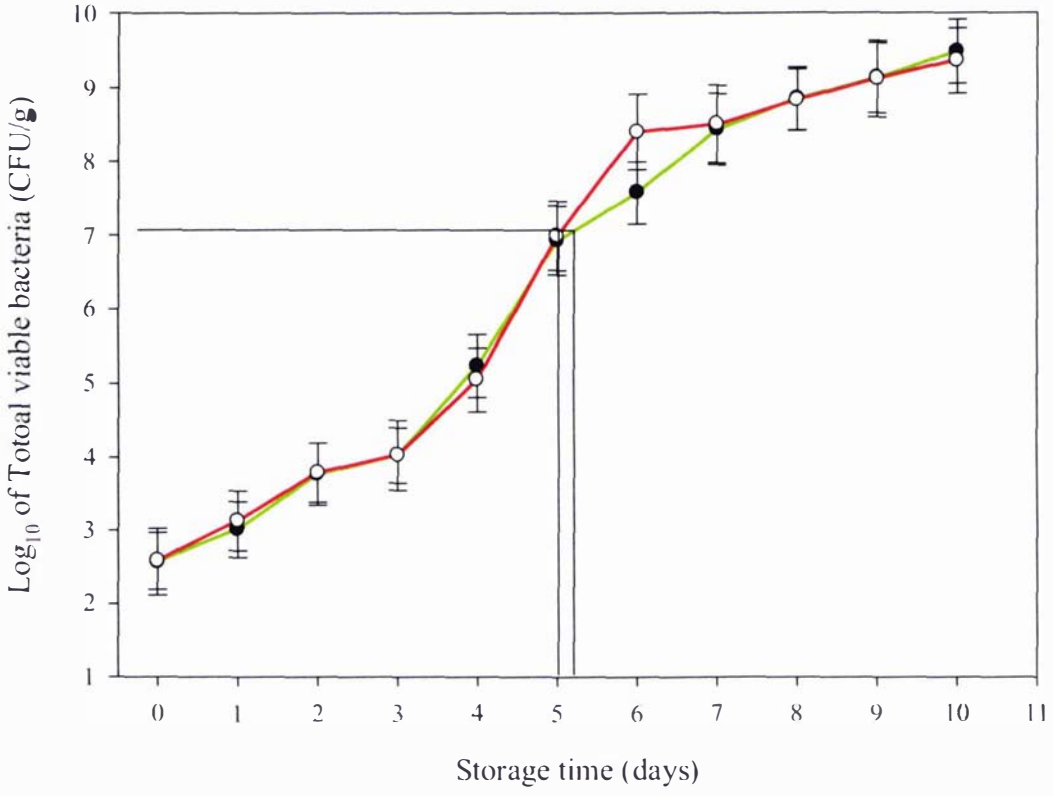


Fig. 6.3 Total viable counts of bacteria on sPCA plates (20°C for 3 days) for salmon samples stored at 10°C. Error bars depict standard deviations of the means. Each point represents the mean of 5 samples. ○, controls; ●, BD-treated. The lines at 7 $\log_{10}$ CFU/g show the estimated times when sensory spoilage occurs due to spoilage bacteria (e.g., *P. phosphoreum*) (Dalgaard *et al.*, 1997).

6.4 Discussion

The environment on the surface of salmon is vastly different to that in liquid media, e.g. high nutrient concentration, mixed cultures of prey and non-prey bacteria, so it is difficult to compare the two situations. Nevertheless, the present study, the first reported *in vivo* study on the use of BD as a biopreservative for fresh packaged fish, gave encouraging results. The initial microbial populations of the tested samples were consistent with the literature data about the microbiological content of King Salmon (Fletcher *et al.*, 2002; 2004). The results obtained indicate that at 20°C BD can effectively extend the lag phase of bacterial growth and lower the total number of viable bacteria for up to 70 h storage (Figs. 6.1 and 6.2). This could lengthen the shelf life of King salmon, particularly in an ineffective cold-chain during transport, to retail or to home. The sensory evaluation report also provided evidence of the ability of BD to extend the shelf life (Table 6.1). Dalgaard *et al.* (1997) reported that signs of sensory spoilage occur only when the number of spoilage bacteria (e.g., *P. phosphoreum*) exceeded $\log_{10}$ 7 CFU/g. In the present study the estimated spoilage line was exceeded after ~43 h in BD-treated samples and ~30 h in non-BD- treated samples (Figs. 6.1 and 6.2). The sensory evaluation report confirmed a significant difference in spoilage (low spoilage) after 48 h in BD-treated samples compared to non-BD- treated samples in Trial 2 (Table 6.1). However, the sensory evaluation data were collected on only one occasion in only one trial (Trial 2; 48 h); therefore the conclusions must be restricted. Further investigation of daily sensory evaluation and comparison with bacterial data is warranted. However, no effect of BD was observed in reducing the rate of bacterial growth during the exponential phase. This observation is in agreement with the *in vitro* study of BD against *P. phosphoreum* in nutrient medium, where the effect of BD was only minimal at higher concentrations of prey bacteria (Chapter 5). Since only one BD isolate (OT2) was used as challenge inoculum in this study, the evidence of these findings must be restricted to this particular isolate. It might be of interest to know whether multiple BD challenge doses would have a synergistic action. There has been no research in this field and the results of these trials suggest further investigation. It is reasonable to assume that because the NZ-BD isolates are taxonomically and host specifically very similar (Chapter 3 and Chapter 4) and they have a certain predation limit and threshold (Section 4.4.2), they will probably behave in a similar

manner. However, future biological control studies require further confirmation with trials using taxonomically and host-specifically different multiple BD isolates.

Not surprisingly given its slow growth at low temperatures (chapter 4), BD did not have any effect on the growth of total viable bacteria at 10°C (Fig. 6.3). This observation reflects the mesophilic nature of the New Zealand marine BD isolates. Hence, the commercial application of mesophilic BD to seafood quality is limited, and, for further advances, there is clearly a need to isolate BD strains which can grow in the range of 0 – 4°C.

In terms of future work, to optimize the use of BD as a biopreservative in fish, it may be appropriate to perform experiments *in vitro* using solid media specifically designed to simulate the *in vivo* situation, for example, nutrient content, water activity, populations of prey and non-prey bacteria and mixed BD cultures, could all be studied to gain a more thorough theoretical understanding of the environmental situation. This would then allow better identification of appropriate conditions for *in vivo* applications. For example, in a mixed culture of prey and non-prey bacteria, reduction in numbers of prey by BD could allow increased growth of non-prey organisms, possibly pathogens, which may worsen the microbiological environment of the fish.

Chapter 7

Final Discussion

This investigation has succeeded in isolating thirteen isolates of Bdellovibrionaceae (BD) from seawater from thirteen different coastal sites around New Zealand, in the spring of 2004. No BD isolates were recovered in winter months of 2004. Although this study was limited in scope and concentrated in only two seasons in the North Island of New Zealand, the results obtained provide evidence to suggest an abundance of these predator bacteria in the warmer months. In the isolation investigation, the overall prevalence of BD in the present study (6,000 – 40,000 /litre of seawater) ranks New Zealand among those countries reported to have high recovery rates in the isolation of BD. The present study shows that routine absorbance measurement techniques could be utilized as important parameters for the isolation and enumeration of BD in natural samples as well as revival of BD from storage conditions. This is an effective alternative to the "by eye" estimation of the degree of clearing by the suspected BD samples after incubation.

A major criticism, which could be made is the use of just one host bacteria *V. parahaemolyticus*, possibly resulting in a lack of accuracy in the recovery of BD isolates. Overseas workers (Schoeffield and Williams, 1990; Pineiro *et al.*, 2004; 2007) reported *V. parahaemolyticus* as the most efficient host in the isolation of widely diversified BD isolates. However a more intense investigation using different host bacteria might be necessary to accurately map the isolation and diversification of BD in North and South Island of New Zealand throughout the year. The thirteen isolates of BD were characterized and then, by evaluating ecological factors affecting the survival and efficacy of BD, examined for their potential to inhibit spoilage of seafood in industry by means of their predation on Gram-negative spoilage bacteria.

In the characterization investigation (Chapter 3), fluorescence microscopy, transmission electron microscopy (TEM), API ZYM enzymic reactions, antibiogram profiles, partial and complete 16S rDNA sequencing, phylogenetic analysis, SDS-PAGE and pulsed field gel electrophoresis established the identity of BD. Through the use of these appropriate and proven techniques, the New Zealand BD isolates have been positively placed in the *Bacteriovorax* genus of the family Bdellovibrionaceae. The present study confirmed that all the isolates are phylogenetically and

pulsotypically (PFGE analysis) similar to each other. They are different from overseas isolates, but very closely related to some of the US isolates (cluster III). It is important to recognise that the diversification reported in this study may not reflect the total New Zealand picture. Only fourteen coastal sites in the North Island of New Zealand were included in this study and only one host bacteria (*V. parahaemolyticus*) was used for the recovery of BD isolates. This is in broad agreement with the findings of overseas study (Pineiro *et al*., 2007), where they confirmed that all the New Zealand BD isolates (8 NZ-BD isolates including MNZ1 of the present study) were situated in the same cluster with some other Great Salt Lake, Atlantic Ocean, Chesapeake Bay and gills of aquarium fish, although they identified different clusters of BD isolates from one isolation station or ocean. A possible reason for the presence of similar clusters of BD isolates in thirteen different sites is the presence of lesser number of host or other viable bacteria in the seawater of New Zealand compared to other regions of seawater (personal work experience with Piñeiro and Williams), however a detailed isolation and characterization study including some other molecular techniques such as MLST (Multilocus sequence typing) using the amplifications of housekeeping genes is being warranted.

The predation pattern of the BD isolates (Chapter 4) demonstrated that the variation in predation efficiencies of the New Zealand isolates against selected Gram-negative host species was consistent with those of overseas studies. Notably, BD was found to be effective against *P. phosphoreum*, *V. parahaemolyticus*, *V. vulnificus*, *E. cloacae*, *P. fluorescens*, *P. cepacia*, *P. aeruginosa*, *M. morganeii* and *P. vulgaris*, but not against marine spoilage organisms such as, *P. mendocina*, *P. pseudomallei* or *S. putrefaciens* or the terrestrial organisms *L. monocytogenes*, *E. coli* B, *E. coli* ML35 or *S.* Typhimurium. However, the speed of predation varied: BD quickly attacked *P. phosphoreum*, *V. parahaemolyticus*, *V. vulnificus* and *E. cloacae* but only slowly attacked *P. fluorescens*, *P. cepacia*, *P. aeruginosa*, *M. morganii* and *P. vulgaris*. It is speculated that the reasons for this variation in efficacy may lie with the specificity of receptors and interaction of cell wall components with different strains of BD.

BD are obligate aerobes (microaerophilic) with an optimal growth temperature of 25-35°C (Varon and Shilo, 1980), although some marine strains grow at 37°C (Richardson, 1990). Little is known about the viability of pure BD cultures stored at different temperatures. The current

method of storage of BD such as maintenance with host cells as bdelloplasts or bdellocycsts or as freeze dried cultures, all have complications. From this study (Chapter 5), it is concluded that large stocks of pure BD cultures can be stored at 4°C to 20°C for relatively long periods with little likelihood of change in character and this is therefore recommended as the preferred method.

With no published work on the efficacy of seawater BD against spoilage and pathogenic organisms, despite evidence shown of the potential of freshwater BD in this role, this study undertook to examine the potential of using seawater BD as a tool for inhibiting the contamination and spoilage of fresh fish and fish products by Gram-negative spoilage organisms. Notwithstanding the close taxonomic relationship between the thirteen isolates of BD from different coastal New Zealand sites, there was considerable variation in the rate at which the isolates reduced numbers of *P. phosphoreum* after ten hours of incubation, although by twenty four hours, the degree of reduction was similar amongst all isolates (Chapter 5). This finding was consistent with studies in related fields and laid the grounds for trials to be undertaken where the optimal conditions for a selected isolate had to be established for viability and maximum efficiency. The results of these trials indicated that optimal conditions exist for the maximum viability of BD and its efficiency in significant reduction of spoilage organism numbers and accordingly, for application in industry as a biopreservation technique. It is concluded that optimal conditions exist for salinity, pH, ratio of prey and predators and nutrition, for the maximum viability of BD and its efficiency in significant reduction in spoilage organism numbers. The present study (Chapter 5.4.7) confirmed that despite of the importance of predator concentration, media and prey to predator ratio, prey concentration is the most important factor in the reduction of spoilage organism, *P. phosphoreum*. The study of the prey : predator interaction model concluded that the prey and predators co-exist together; the predators can consume a proportion of prey, but cannot eliminate the prey and the predator population will starve when the prey population is low. These conditions would restrict its application in industry as a biopreservation technique.

Bacterial spoilage of saltwater fish is caused by several Gram-negative bacteria and BD is a proven predator of Gram-negative bacteria. However, despite the growing awareness of the

potential for the use of biopreservation of fresh and packed foods with host specific predatory microbial activity, little has been done to examine the potential of this technique with seawater fish. This study is the first in this field of research. The potential for use of seawater BD in biopreservation was examined in cultured samples of fresh King salmon. At 20°C the shelf life was extended through extension of the lag phase of growth of the prey bacteria and reduction in total numbers. Sensory evaluation of the salmon product being tested confirmed that the shelf life was extended. However, at 10°C there was no reduction in prey organisms, which suggested that the organisms are ineffective at refrigeration temperatures. Thus the mesophilic nature of the New Zealand seawater BD isolates limits the commercial application of this strain in biopreservation in seafood. While 20°C may be representative of conditions where the cold chain fails, low temperatures of < 10°C are the normal conditions of the commercial seafood distribution chain. Hence effectiveness at low temperature is critical for the success of any biopreservation method used in fish storage. For success in this field, there is clearly a need to isolate BD strains which function at temperatures of 0-4°C. Furthermore, the *in vitro* trials carried out in this study focused on the efficiency of BD on a pure culture of one organism, *P. phosphoreum* and not on mixed cultures of Gram-negative spoilage bacteria, the normal condition observed in saltwater fish. There has been very little research in this field and the results of these trials suggest further investigation into the effect of BD on mixed cultures of Gram-negative spoilage organisms is warranted.

It is concluded that the possibility of industrial application of BD as an agent in the control of Gram-negative spoilage and contaminant organisms has been demonstrated in this study. However, the benefits of further investigations into the potential of BD in the fresh fish industry will depend on finding BD that can have a beneficial effect under refrigerated conditions.

References

Abram D and Davis BK. (1970). Structural properties and features of parasitic *Bdellovibrio bacteriovorus*. Journal of Bacteriology. 104, 948-965.

Abram D and Shilo M. (1967). Structural features of *Bdellovibrio bacteriovorus* in cultures and in host-parasite mixtures. Bacteriology Proceedings. 41-42.

Abram D, Castro e Melo J and Chou D. (1974). Penetration of *Bdellovibrio bacteriovorus* into host cells. Journal of Bacteriology. 118, 663-680.

Al-Dagal MM and Bazaraa WA. (1999). Extension of shelf life of whole and peeled shrimp with organic acid salts and bifidobacteria. Journal of Food Protection. 62, 51-56.

Althauser M, Samsonoff WA, Anderson C and Conti SF. (1972). Isolation and preliminary characterisation of bacteriophages for *Bdellovibrio bacteriovorus*. Journal of Virology. 10, 516-523.

Altieri C, Speranza B, Del Nobile MA and Sinigaglia M. (2005). Suitability of bifidobacteria and thymol as biopreservatives in extending the shelf life of fresh packed plaice fillets. Journal of Applied microbiology. 99, 1294-1302.

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W and Lipman DJ. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research. 25, 3389-3402.

Baer ML, Ravel J, Chun J, Hill RT and Williams HN. (2000). A proposal for the reclassification of *Bdellovibrio stolpii* and *Bdellovibrio starrii* into a new genus, *Bacteriovorax* gen. Nov. as *Bacteriovorax stolpii* comb. Nov. and *Bacteriovorax starrii* comb. Nov., respectively. International Journal of Systematic and Evolutionary Microbiology. 50, 219-224.

Baer ML, Ravel J, Piñeiro SA, Guether-Borg D and Williams HN. (2004). Reclassification of salt-water *Bdellovibrio* sp. as *Bacteriovorax marinus* sp. nov. and *Bacteriovorax litoralis* sp. nov. International Journal of Systematic and Evolutionary Microbiology. 54, 1011-1016.

Beck S, Schwudke D, Strauch E, Appel B and Linscheid M. (2004). *Bdellovibrio bacteriovorus* strains produce a novel major outer membrane protein during predacious growth in the periplasm of prey bacteria. Journal of Bacteriology. 186, 2766-2773.

Bradford, MM. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. 72, 248-254.

Buckmire FLA. (1971). A protective role for a cell wall protein layer of *Spirillum serpens* against infection by *Bdellovibrio bacteriovorus*. Bacteriology Proceedings. G122, p. 48.

Burger A, Drews G and Ladwig R. (1968). Host range and infection cycle of a newly isolated strain of *Bdellovibrio bacteriovorus*. Archives of Microbiology. 61, 261-279.

Burnham JC and Conti SF. (1984). Genus *Bdellovibrio* Stolp and Starr 1963, 243^{AL} *In* Bergey's manual of systematic bacteriology. Edited by Krieg NR and Holt JG. Baltimore: Williams and Wilkins. Volume 1, 118-124.

Burnham JC, Hashimoto T and Conti SF. (1968). Electron microscopic observations on the penetration of *Bdellovibrio bacteriovorus* into Gram-negative bacterial hosts. Journal of Bacteriology. 96, 1366-1381.

Burnham JC and Robinson J. (1974). Genus *Bdellovibrio* Stolp and Starr 1963. *In* Bergey's manual of determinative bacteriology. Edited by Buchanan, RE, Gibbons NE. Baltimore: Williams & Wilkins. 8^{th} ed, 212-216.

Castellano P and Vignolo G. (2006). Inhibition of *Listeria innocua* and *Brochothrix thermosphacta* in vacuum-packaged meat by addition of bacteriocinogenic *Lactobacillus curvatus* CRL705 and its bacteriocins. Letters in Applied Microbiology. 43, 194-199.

Chauhan A and Williams HN. (2006). Response of *Bdellovibrio* and like organisms (BALOs) to the migration of naturally occurring bacteria to chemoattractants. Current Microbiology. 53, 516-522.

Cover WH and Rittenberg SC. (1984). Change in the surface hydrophobicity of substrate cells during bdelloplast formation by *Bdellovibrio bacteriovorus* 109J. Journal of Bacteriology. 157, 391-397.

Crothers SF and Robinson J. (1971). Changes in the permeability of *Escherichia coli* during parasitization by *Bdellovibrio bacteriovorus*. Canadian Journal of Microbiology. 17, 689-697.

Dalgaard P (1995). Modelling of microbial activity and prediction of shelf life for packed fresh fish. International Journal of food Microbiology. 26, 305-317.

Dalgaard P, Mejlholm O, Christiansen TJ and Huss HH. (1997). Importance of *Photobacterium phosphoreum* in relation to spoilage of modified atmosphere-packed fish products. Letters in Applied Microbiology. 24, 373-378.

Davidov Y, Friedjung A and Jurkevitch E. (2006). Structure analysis of a soil community of predatory bacteria using culture-dependent and culture-independent methods reveals a hitherto undetected diversity of *Bdellovibrio*-and-like organisms. Environmental Microbiology. 8, 1667-1673.

Davidov Y and Jurkevitch E. (2004). Diversity and evolution of *Bdellovibrio*-and-like organisms (BALOs), reclassification of *Bacteriovorax starrii* as *Peredibacter starii* gen. nov., comb. nov., and description of the *Bacteriovorax-Peredibacter* clade as Bacteriovoracaceae fam. nov. International Journal of Systematic and Evolutionary Microbiology. 54, 1439-1452.

Dawson RMC, Elliott DC, Elliott WH and Jones KM. (1986). PH, buffers, and physiological media; *In* Data for biochemical research (third edition). Oxford University Press Inc., New York. 417-448.

Dias FF and Bhat JV. (1965). Microbial ecology of activated sludge II. Bacteriophages, *Bdellovibrio*, Coliforms and other organisms. Applied Microbiology. 13, 257-261.

Diedrich DL, Duran CP and Conti SF. (1984). Acquisition of *Escherichia coli* outer membrane proteins by *Bdellovibrio* sp. strain 109D. Journal of Bacteriology. 159, 329-334.

Donze D, Mayo JA and Diedrich DL. (1991). Relationships among the bdellovibrios revealed by partial sequences of 16S ribosomal RNA. Current Microbiology. 23, 115-119.

Engelking HM and Seidler RJ. (1974). The involvement of extracellular enzymes in the metabolism of *Bdellovibrio*. Archives of Microbiology. 95, 293-304.

Epton HAS, Walker NM and Sigee DC. (1989). *Bdellovibrio*: a potential control agent for soft rot and black leg of potato. *In* Plant pathogenic bacteria: part A. Edited by Klement Z. Proceedings of the 7th International conference of Plant Pathology and Bacteriology, Budapest, Hungary. 207-212.

Fackrell HB and Robinson J. (1973). Purification and characterization of a lytic peptidase produced by *Bdellovibrio bacteriovorus* 6-5-S. Canadian Journal of Microbiology. 19, 659-666.

Fenwick SG. (1997). *Yersinia enterocolitica* infections in people and other animals: A New Zealand study. Ph.D. Thesis, Massey University, Palmerston North, New Zealand (unpublished work).

Filip Z, Schmelz P and Smed-Hildmann R. (1991). *Bdellovibrio* sp. – A predator under groundwater conditions? A short communication. Water Science Technology. 24, 321-324.

Fletcher GC, Summers G, Corrigan VK, Cumarasamy S, Dufour J. (2002). Spoilage of King salmon (*Oncorhynchus tshawystscha*) fillets stored under different atmospheres. Journal of Food Science. 67, 2362-2374.

Fletcher GC, Summers G, Corrigan VK, Johanson MR and Hedderley D (2004). Optimizing gas mixtures for modified atmosphere packaging of fresh King salmon (*Oncorhynchus tshawystscha*). Journal of Aquatic Food Product Technology. 13, 5-28.

Fratamico PM and Cooke PH. (1996). Isolation of bdellovibrios that prey on *Escherichia coli* 0157:H7 and *Salmonella* species and application for removal of prey from stainless steel surfaces. Journal of Food Safety. 16, 161-173.

Fratamico PM and Whiting RC. (1995). Ability of *Bdellovibrio bacteriovorus* 109J to lyse Gram-negative food-borne pathogenic and spoilage bacteria. Journal of Food Protection. 58, 160-164.

Fry JC and Staples DG. (1974). The occurrence and role of *Bdellovibrio bacteriovorus* in a polluted river. Water Research. 8, 1029-1035.

Fry JC and Staples DG. (1976). Distribution of *Bdellovibrio bacteriovorus* in sewage works, river water and sediments. Applied and Environmental Microbiology. 31, 469-474.

Gadkari D and Stolp H. (1975). Energy metabolism of *Bdellovibrio bacteriovorus*. Archives of Microbiology. 102, 179-185.

Gram L and Huss HH. (1996). Microbiological spoilage of fish and fish products. International Journal of Food Microbiology. 33, 121-137.

Gram L, Trolle G and Huss HH. (1987). Detection of specific spoilage bacteria from fish stored at low (0° and 5°C) and high (20° and 30°C) temperatures. International Journal of Food Microbiology. 4, 65-72.

Hespell RB, Miozzari GF and Rittenberg SC. (1975). Ribonucleic acid destruction and synthesis during intraperiplasmic growth of *Bdellovibrio bacteriovorus*. Journal of Bacteriology. 123, 481-491.

Hespell RB, Rosson RA, Thomashow MF and Rittenberg SC. (1973). Respiration of *Bdellovibrio bacteriovorus* strain 109 J and its energy substrates for intraperiplasmic growth. Journal of Bacteriology. 113, 1280-1288.

Hespell RB, Thomashow MF and Rittenberg SC. (1974). Changes in cell composition and viability of *Bdellovibrio bacteriovorus* during starvation. Archives of Microbiology. 97, 313-327.

Hespell RB. (1976). Glycolytic and tricarboxylic acid cycle enzyme activities during intraperiplasmic growth of *Bdellovibrio bacteriovorus* on *Escherichia coli*. Journal of Bacteriology. 128, 677-680.

Hespell RB. (1978). Intraperiplasmic growth of *Bdellovibrio bacteriovorus* on heat-treated *Escherichia coli*. Journal of Bacteriology. 133, 1156-1162.

Hobley L, King JR and Sockett RE. (2006). *Bdellovibrio* predation in the presence of decoys: three- way bacterial interactions revealed by mathematical and experimental analyses. Applied and Environmental Microbiology. 72, 6757-6765.

Hoeniger JFM, Ladwig R and Moor H. (1972). The fine structure of 'resting bodies' of *Bdellovibrio* sp. strain W developed in *Rhodospirillum rubrum*. Canadian Journal of Microbiology. 18, 87-92.

Huang JCC and Starr MP. (1973). Effects of calcium and magnesium ions and host viability on growth of bdellovibrios. Antonie van Leeuwenhoek. 39, 151-167.

Ibragimov FKH. (1980). Dissemination of *Bdellovibrio bacteriovorus* in animals and their interaction with the agents of acute intestinal infections. Zhurnal Mikrobiologii, Epidemiologii and Immunobiologii. 5, 97-99.

Ishiguro EE. (1973). A growth initiation factor for host-independent derivatives of *Bdellovibrio bacteriovorus*. Journal of Bacteriology. 115, 243-252.

Jackson L and Whiting RC. (1992). Reduction of an *Escherichia coli* K12 population by *Bdellovibrio bacteriovorus* under various in vitro conditions of parasite:host ratio, temperature, or pH. Journal of Food Protection. 55, 859-861.

Jacobsen T, Budde BB and Koch AG. (2003). Application of *Leuconostoc carnosum* for biopreservation of cooked meat products. Journal of Applied Microbiology. 95, 242-249.

Jayasimhulu K, Hunt SM, Kaneshiro ES, Watanabe Y and Giner JL. (2007). Detection and identification of *Bacteriovorax stolpii* Uki2 sphingophosphonolipid molecular species. Journal of the American Society for Mass Spectrometry. 18, 394-403.

Jurkevitch E, Minz D, Ramati B and Barel G. (2000). Prey range characterization, ribotyping, and diversity of soil and rhizosphere *Bdellovibrio* spp. isolated on phytopathogenic bacteria. Applied and Environmental Microbiology. 66, 2365-2371.

Kadouri D and O'Toole GA. (2005). Susceptibility of biofilms to *Bdellovibrio bacteriovorus* attack. Applied and Environmental Microbiology. 71, 4044-4051.

Kelley JI and Williams HN. (1992). Bdellovibrios in *Callinectus sapidus*, the blue crab. Applied and Environmental Microbiology. 58, 1408-1410.

Kelley JI, Turng B, Williams HN and Baer ML. (1997). Effects of temperature, salinity, and substrate on the colonization of surfaces in situ by aquatic bdellovibrios. Applied and Environmental Microbiology. 63, 84-90.

Keya SO and Alexander M. (1975). Factors affecting growth of *Bdellovibrio* on *Rhizobium*. Archives of Microbiology. 103, 37-43.

Klein DA and Casida LE Jr. (1967). Occurrence and enumeration of *Bdellovibrio bacteriovorus* in soil capable of parasitizing *Escherichia coli* and indigenous soil bacteria. Canadian Journal of Microbiology. 13, 1235-1241.

Koval SF and Hynes SH. (1991). Effect of paracrystalline protein surface layers on predation by *Bdellovibrio bacteriovorus*. Journal of Bacteriology. 173, 2244-2249.

Kramer TT and Westergaard JM. (1977). Antigenicity of bdellovibrios. Applied and Environmental Microbiology. 33, 967-970.

LaMarre AG, Straley SC and Conti SF. (1977). Chemotaxis toward amino acids by *Bdellovibrio bacteriovorus*. Journal of Bacteriology. 131, 201-207.

Lambert C, Smith MCM and Sockett RE. (2003). A novel assay to monitor predator-prey interactions for *Bdellovibrio bacteriovorus* 109J reveals a role for methyl-accepting chemotaxis proteins in predation. Environmental Microbiology. 5, 127-132.

Lambert C, Evans KJ, Till R, Hobley L, Capeness M, Rendulic S, Schuster SC, Aizawa SI and Sockett RE. (2006a). Characterizing the flagellar filament and the role of motility in bacterial prey-penetration by *Bdellovibrio bacteriovorus*. Molecular Microbiology. 60, 274-286.

Lambert C, Morehouse KA, Chang CY and Sockett RE. (2006b). Bdellovibrio: growth and development during the predatory cycle. Current Opinion in Microbiology. 9, 639-644.

Luderitz O, Staub AM and Westphal O. (1966). Immunochemistry of O and R antigens of Salmonella and related Enterobacteriaceae. Bacteriological Reviews. 30, 192-255.

McCann MP, Solimeo HT, Cusick F Jr, Panunti B and McCullen C. (1998). Developmentally regulated protein synthesis during intraperiplasmic growth of *Bdellovibrio bacteriovorus* 109J. Canadian Journal of Microbiology. 44, 50-55.

Macedo AJ, Kuhlicke U, Neu TR, Timmis KN and Abraham WR. (2005). Three stages of a biofilm community developing at the liquid-liquid interface between polychlorinated biphenyls and water. Applied and Environmental Microbiology. 71, 7301-7309.

Marbach A, Varon M and Shilo M. (1976). Properties of marine bdellovibrios. Microbial Ecology. 2, 284-295.

Markelova NY and Gariev IA. (2005). Predatory bacteria *Bdellovibrio*: survival strategy. Process Biochemistry. 40, 1089-1094.

Martin MO. (2002). Predatory prokaryotes: An emerging research opportunity. Journal of Molecular Microbiology and Biotechnology. 4, 467-477.

Matin A and Rittenberg SC. (1972). Kinetics of deoxyribonucleic acid destruction and synthesis during growth of *Bdellovibrio bacteriovorus* strain 109D on *Pseudomonas putida* and *Escherichia coli*. Journal of Bacteriology. 111, 664-673.

Mejlholm O and Dalgaard P. (2002). Antimicrobial effect of essential oils on the seafood spoilage micro-organism *Photobacterium phosphoreum* in liquid media and fish products. Letters in Applied Microbiology. 34, 27-31.

Mitchell R, Yankofsky S and Jannasch HW. (1967). Lysis of *Escherichia coli* by marine micro-organisms. Nature. 215, 891-893.

Miyamoto S and Kuroda K. (1975). Lethal effect of fresh seawater on *Vibrio parahaemolyticus* and isolation of bdellovibrios parasitic against the organism. Japanese Journal of Microbiology. 19, 309-317.

Muyzer G, De Waal EC, Uitterlinden AG. (1993). Profiling of complex microbial populations by denaturing gel electrophoresis analysis of polymerase chain reaction-amplified genes coding 16S rRNA. Applied and Environmental Microbiology. 59, 695-700.

Nakamura M. (1972). Alteration of *Shigella* pathogenicity by other bacteria. American Journal of Clinical Nutrition. 25, 1441-1451.

Nelson DR and Rittenberg SC. (1981). Incorporation of substrate cell lipid A components into the lipopolysaccharide of intraperiplasmically grown *Bdellovibrio bacteriovorus*. Journal of Bacteriology. 147, 860-868.

Neuhoff V, Arnold N, Taube D., Ehrhardt, W. (1988). Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. Electrophoresis. 9, 255-262.

New Zealand Seafood Industry Council. (2007). Export of seafood produce for 12 months ending December 2006. http://intranet.crop.cri.nz/online-resources/Library/NZSeafoodExports.htm. Report 7, December 2006, 1-91.

Núñez ME, Martin MO, Chan PH and Spain EM. (2005). Predation, death, and survival in a biofilm: *Bdellovibrio* investigated by atomic force microscopy. Colloids and Surfaces B: Biointerfaces. 42, 263-271.

Núñez ME, Martin MO, Duong LK, Ly E and Spain EM. (2003). Investigations into the life cycle of the bacterial predator *Bdellovibrio bacteriovorus* 109J at an interface by atomic force microscopy. Biophysical Journal. 84, 3379-3388.

Pan C-L, Hsu Y-L, Tsai G-J, Kuo H-J, Chang C-M, Wang F-J and Wu C-S. (1997) Isolation and identification of *Bdellovibrio* from coastal areas of Taiwan. Fisheries Science. 63, 52-59.

Parker CA and Grove PL. (1970). *Bdellovibrio bacteriovorus* parasitizing *Rhizobium* in Western Australia. Journal of Applied Bacteriology. 33, 253-255.

Piñeiro SA, Sahaniuk GE, Romberg E and Williams HN. (2004). Predation pattern and phylogenetic analysis of *Bdellovibrionaceae* from the Great salt lake, Utah. Current Microbiology. 48, 113-117.

Piñeiro SA, Stine OC, Chauhan A, Steyert SR, Smith R and Williams HN. (2007). Global survey of diversity among environmental saltwater *Bacteriovoracaceae*. Environmental Microbiology. 9, 2441- 2450.

Reichelt JL and Baumann P. (1974). Effect of sodium chloride on growth of heterotrophic marine bacteria. Archives of Microbiology. 97, 329-345.

Rendulic S, Jagtap P, Rosinus A, Eppinger M, Baar C, Lanz C, Keller H, Lambert C, Evans KJ, Goesmann A, Meyer F, Sockett RE and Schuster S. (2004). A predator unmasked: Life cycle of *Bdellovibrio bacteriovorus* from a genomic perspective. Science. 303, 689-692.

Rice TD, Williams HN and Turng BF. (1998). Susceptibility of bacteria in estuarine environments to autochthonous bdellovibrios. Microbial Ecology. 35, 256-264.

Richardson IR. (1990). The incidence of *Bdellovibrio* spp. in man-made water systems: coexistence with legionellas. Journal of Applied Bacteriology. 69, 134-140.

Rittenberg SC and Shilo M. (1970). Early host damage in the infection cycle of *Bdellovibrio bacteriovorus*. Journal of Bacteriology. 102, 149-160.

Rittenberg SC and Thomashow MF. (1979). Intraperiplasmic growth – life in a cozy environment. *In* 'Microbiology-1979'. Edited by Schlessinger D. American Society for Microbiology. Washington DC. 80-86.

Rittenberg SC. (1982). Bdellovibrios-Intraperiplasmic growth. *In* Experimental Microbial ecology. Edited by. Burns RG and Slater JH. Blackwell Scientific Publications, Oxford. 379-390.

Rogosky AM, Moak PL and Emmert EAB. (2006). Differential Predation by *Bdellovibrio bacteriovorus* 109J. Current Microbiology. 52, 81-85.

Rosson RA and Rittenberg SC. (1979). Regulated breakdown of *Escherichia coli* deoxyribonucleic acid during intraperiplasmic growth of *Bdellovibrio bacteriovorus* 109J. Journal of Bacteriology. 140, 620-633.

Ruby EG. (1992). The Genus *Bdellovibrio*. *In* 'The Prokaryotes' 2nd edition. Edited by Balows A, Truper HG, Dworkin M, Harder W and Schliefer KH. Springer-Verlag, New York. 3400-3415.

Ryan SN, Laing WA and McManus MT. (1998). A cysteine proteinase inhibitor purified from apple fruit. Phytochemistry. 49, 957-963.

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB and Erlich HA. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science. 239, 487-491.

Saint Joseph's University. (2006). Dr. John Tudor's personal web page. http://www.sju.edu/~jtudor/pages/research_interests.html. Philadelphia, USA.

Schelling ME and Conti SF. (1983). Se otyping of bdellovibrios by agglutination and indirect immunofluorescence. International Journal of Systematic Bacteriology. 33, 816-821.

Scherff RH, DeVay JE and Carroll TW. (1966). Ultrastructure of host-parasite relationships involving reproduction of *Bdellovibrio bacteriovorus* in host bacteria. Phytopathology. 56, 627-632.

Scherff RH. (1973). Control of bacterial blight of soybean by *Bdellovibrio bacteriovorus*. Phytopathology. 63, 400-402.

Schoeffield AJ and Williams HN. (1990). Efficiencies of recovery of bdellovibrios from brackish-water environments by using various bacterial species as prey. Applied and Environmental Microbiology. 56, 230-236.

Schoeffield AJ, Falkler WA Jr, Desai D and Williams HN. (1991). Serogrouping of halophilic bdellovibrios from Chesapeake Bay and environs by immunodiffusion and immunoelectrophoresis. Applied and Environmental Microbiology. 57, 3470-3475.

Schwartz DC and Cantor CR. (1984). Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. Cell. 37, 67-75.

Schwudke D, Bernhardt A, Beck S, Madela K, Linscheid MW, Appel B and Strauch E. (2005). Transcriptional activity of the host-interaction locus and a putative pilin gene of *Bdellovibrio bacteriovorus* in the predatory life cycle. Current Microbiology. 51, 310-316.

Schwudke D, Linscheid M, Strauch E, Appel B, Zahringer U, Moll H, Muller M, Brecker L, Gronow S and Linder B. (2003). The obligate predatory *Bdellovibrio bacteriovorus* possesses a neutral lipid A containing alpha-D-Mannoses that replace phosphate residues: similarities and differences between the lipid As and the lipopolysaccharides of the wild type strain *B. bacteriovorus* HD100 and its host-independent derivative H1100. Journal of Biological Chemistry. 278, 27502-27512.

Schwudke D, Strauch E, Krueger M and Appel B. (2001). Taxonomic studies of predatory bdellovibrios based on 16S rRNA analysis, ribotyping and the *hit* locus and characterization of isolates from the gut of animals. Systematic and Applied Microbiology. 24, 385-394.

Seidler RJ and Mandel M. (1971). Quantitative aspects of deoxyribonucleic acid renaturation: base composition, state of chromosome replication, and polynucleotide homologies. Journal of Bacteriology. 106, 608-614.

Seidler RJ, Mandel M and Baptist JN. (1972). Molecular heterogeneity of the bdellovibrios: evidence of two new species. Journal of Bacteriology. 109, 209-217.

Seidler RJ and Starr MP. (1968). Structure of the flagellum of *Bdellovibrio bacteriovorus*. Journal of Bacteriology. 95, 1952-1955.

Seidler RJ and Starr MP. (1969). Isolation and characterisation of host-independent bdellovibrios. Journal of Bacteriology. 100, 769-785.

Seidler RJ, Starr MP and Mandel M. (1969). Deoxyribonucleic acid characterisation of bdellovibrios. Journal of Bacteriology. 100, 786-790.

Shemesh Y, Davidov Y, Koval S and Jurkevitch E. (2003). Small eats big: ecology and diversity of *Bdellovibrio* and like organisms, and their dynamics in predator-prey interactions. Agronomie. 23, 433-439.

Shilo M and Bruff B. (1965). Lysis of Gram-negative bacteria by host-independent ectoparasitic *Bdellovibrio bacteriovorus* isolates. Journal of General Microbiology. 40, 317-328.

Shilo M. (1966). Predatory bacteria. Journal of Science. 2, 33-37.

Shilo M. (1969). Morphological and physiological aspects of the interaction of *Bdellovibrio* with host bacteria. Current Topics in Microbiology and Immunology. 50, 174-204.

Simpson FJ and Robinson J. (1968). Some energy-producing systems in *Bdellovibrio bacteriovorus*, strain 6-5-S. Canadian Journal of Biochemistry. 46, 865-873.

Sleytr UB and Messner P. (1983). Crystalline surface layers on bacteria. Annual Review of Microbiology. 37, 311-339.

Snyder AR, Williams HN, Baer ML, Walker KE and Stine OC. (2002). 16S rDNA sequence analysis of environmental *Bdellovibrio*-and-like organisms (BALO) reveals extensive diversity. International Journal of Systematic and Evolutionary Microbiology. 52, 2089-2094.

Song ZP, Cai JP, Wang Z and Han Y. (2005). Study of bacteria-lysis abilities and growth conditions of 4 *Bdellovibrio*. Wei Sheng Wu Xue Bao. 45, 571-575.

Staples DG and Fry JC. (1973). Factors which influence the enumeration of *Bdellovibrio bacteriovorus* in sewage and river water. Journal of Applied Bacteriology. 36, 1-11.

Starr MP and Baigent NL. (1966). Parasitic interaction of *Bdellovibrio bacteriovorus* with other bacteria. Journal of Bacteriology. 91, 2006-2017.

Starr MP and Huang JCC. (1972). Physiology of the bdellovibrios. Advances in Microbial Physiology. 8, 215-265.

Starr MP and Seidler RJ. (1971). The bdellovibrios. Annual Review of. Microbiology. 25, 649-678.

Starr MP and Stolp H. (1976). *Bdellovibrio* methodology, *In* Methods in Microbiology. Edited by Norris JR. Volume 9, Academic Press, London. 217-244.

Steiner S, Conti SF and Lester RL. (1973). Occurrence of phosphonosphingolipids in *Bdellovibrio bacteriovorus* strain Uki2. Journal of Bacteriology. 116, 1199-1211.

Stolp H and Petzold H. (1962). Untersuchungenuber einen obligat parasitischen mikroorganismus mit lytischer aktivat fur *Pseudomonas*-bakterien. Phytopathology. Zeit. 45, 364-390.

Stolp H and Starr MP. (1963). *Bdellovibrio bacteriovorus*. gen. et. sp. n., a predatory, ectoparasitic, and bacteriolytic microorganism. Antonie van Leeuwenhoek. 29, 217-248.

Stolp H. (1981). The genus *Bdellovibrio*. *In* The Prokaryotes. Edited by Starr MP, Stolp H, Truper HG, Balows A and Schlegel HG. Springer-Verlag, Berlin. 618-629.

Straley SC and Conti SF. (1974). Chemotaxis in *Bdellovibrio bacteriovorus*. Journal of Bacteriology. 120, 549-551.

Straley SC and Conti SF. (1977). Chemotaxis by *Bdellovibrio bacteriovorus* toward prey. Journal of Bacteriology. 132, 628-640.

Straley SC, LaMarre AG, Lawrence LJ and Conti SF. (1979). Chemotaxis of *Bdellovibrio bacteriovorus* toward pure compounds. Journal of Bacteriology. 140, 634-642.

Sutton DC and Besant PJ. (1994). Ecology and characteristics of bdellovibrios from three tropical marine habitats. Marine Biology. 119, 313-320.

Swofford DL. (1998). PAUP: Phylogenetic analysis using parsimony (and other methods), version 4.0. Sinauer Associates, Sunderland, MA, USA.

Takaaki M. (1994). Therapeutic agent for *Vibrio* infectious disease and method for treating and preventing the disease using the same. Patent number: JP6237665.

Takaaki M, Kohel K and Takashi S. (2000). Mass pure culture of *Bdellovibrio*. Patent number: JP2000325074.

Talley BG, McDade RL Jr and Diedrich DL. (1987). Verification of the protein in the outer membrane of *Bdellovibrio bacteriovorus* as the OmpF protein of its *Escherichia coli* prey. Journal of Bacteriology. 169, 694-698.

Taylor VI, Baumann P, Reichelt JL and Allen RD. (1974). Isolation, enumeration, and host range of marine bdellovibrios. Archives of Microbiology. 98, 101-114.

Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH and Swaminathan B. (1995) Interpreting chromosomal DNA restriction patterns produced by pulsed field gel electrophoresis: criteria for bacterial strain typing. Journal of Clinical Microbiology. 33, 2233-2239.

Thomashow LS and Rittenberg SC. (1985). Waveform analysis and structure of flagella and basal complexes from *Bdellovibrio bacteriovorus* 109J. Journal of Bacteriology. 163, 1038-1046.

Thomashow MF and Rittenberg SC. (1978a). Penicillin-induced formation of osmotically stable spheroplasts in nongrowing *Bdellovibrio bacteriovorus*. Journal of Bacteriology. 133, 1484-1491.

Thomashow MF and Rittenberg SC. (1978b). Intraperiplasmic growth of *Bdellovibrio bacteriovorus* 109J: solubilisation of *Escherichia coli* peptidoglycan. Journal of Bacteriology. 135, 998-1007.

Thomashow MF and Rittenberg SC. (1978c). Intraperiplasmic growth of *Bdellovibrio bacteriovorus* 109J: N-deacetylation of *Escherichia coli* peptidoglycan amino sugars. Journal of Bacteriology. 135, 1008-1014.

Thomashow MF and Rittenberg SC. (1978d). Intraperiplasmic growth of *Bdellovibrio bacteriovorus* 109J: attachment of long-chain fatty acids to *Escherichia coli* peptidoglycan. Journal of Bacteriology. 135, 1015-1023.

Torrella F, Guerrero R and Seidler RJ. (1978). Further taxonomic characterisation of the genus *Bdellovibrio*. Canadian Journal of Microbiology. 24, 1387-1394.

Tudor JJ and Conti SF. (1977). Characterization of bdellocysts of *Bdellovibrio* sp. Journal of Bacteriology. 131, 314-322.

Tudor JJ and Karp MA. (1994). Translocation of an outer membrane protein into prey cytoplasmic membranes by bdellovibrios. Journal of Bacteriology. 176, 948-952.

Uematsu T. (1980). Ecology of *Bdellovibrio* parasitic to rice bacterial leaf blight pathogen, *Xanthomonas oryzae*. Review of Plant Protection Research. 13, 12-26.

U.S. Food and Drug Administration. (2006). Foodborne pathogenic microorganisms and natural toxins handbook, *Vibrio parahaemolyticus*. http://www.cfsan.fda.gov/~mow/chap9., 1-5.

Varon M and Shilo M (1970). Methods for separation of *Bdellovibrio* from mixed bacterial population by filtration through Millipore filters or by gradient differential centrifugation. Revue Internationale d'Ocâeanographie Mâedicale, 18-19: 145-152.

Varon M and Shilo M. (1968). Interaction of *Bdellovibrio bacteriovorus* and host bacteria 1. Kinetic studies of attachment and invasion of *Escherichia coli* B by *Bdellovibrio bacteriovirus*. Journal of Bacteriology. 95, 744-753.

Varon M and Shilo M. (1969a). Interaction of *Bdellovibrio bacteriovirus* and host bacteria 11. Intracellular growth and development of *Bdellovibrio bacteriovorus* in liquid cultures. Journal of Bacteriology. 99, 136-141.

Varon M and Shilo M. (1969b). Attachment of *Bdellovibrio bacteriovorus* to cell wall mutants of *Salmonella* spp. and *Escherichia coli*. Journal of Bacteriology. 97, 977-979.

Varon M and Shilo M. (1980). Ecology of aquatic bdellovibrios. Advance in Aquatic Microbiology. 2, 1-48.

Varon M and Zeigler BP. (1978). Bacterial predator-prey interaction at low prey density. Applied and Environmental Microbiology. 36, 11-17.

Varon M, Fine M and Stein A. (1984). The maintenance of *Bdellovibrio* at low prey density. Microbial Ecology. 10, 95-98.

Varon M. (1981). Interaction of *Bdellovibrio* with its prey in mixed microbial populations. Microbial Ecology. 7, 97-105.

Vermeiren L, Devlieghere F and Debevere J. (2004). Evaluation of meat born lactic acid bacteria as protective cultures for the biopreservation of cooked meat products. International Journal of Food Microbiology. 96, 149-164.

Vermeiren L, Devlieghere F, Vandekinderen I and Debevere J. (2006). The interaction of the non-bacteriocinogenic *Lactobacillus sakei* 10A and lactocin S producing *Lactobacillus sakei* 148 towards *Listeria monocytogenes* on a model cooked ham. Journal of Food Microbiology. 23, 511-518.

Weisburg WG, Barns SM, Pelletier DA and Lane DJ. (1991). 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology. 173, 697-703.

Wessels S and Huss HH. (1996). Suitability of *Lactococcus lactis* subsp. *Lactis* ATCC 11454 as a protective culture for lightly preserved fish products. Food Microbiology. 13, 323-332.

Westergaard JM and Kramer TT. (1977). *Bdellovibrio* and the intestinal flora of vertebrates. Applied and Environmental Microbiology. 34, 506-511.

Williams HN and Falkler Jr WA. (1984). Distribution of bdellovibrios in the water column of an estuary. Canadian Journal of Microbiology. 30, 971-974.

Williams HN, Baer ML and Tudor JJ (2003). Genus I. *Bdellovibrio* Stolp and Starr 1963, 243^{AL}. *In* Bergey's Manual® of Systematic Bacteriology, The Proteobacteria, Part C The Alpha-, Beta-, Delta-, and Epsilonproteobacteria. Edited by G. M. Garrity. 2nd Edition, Volume 2, Springer-Verlag, New York, NY. 1040 – 1058.

Williams HN, Falkler Jr WA and Shay DE. (1980). Incidence of marine bdellovibrios lytic against *Vibrio parahaemolyticus* in Chesapeake Bay. Applied and Environmental Microbiology. 40, 970-972.

Williams HN, Falkler Jr WA and Shay DE. (1982). Seasonal distribution of bdellovibrios at the mouth of the Patuxent river in the Chesapeake Bay. Canadian Journal of Microbiology. 28, 111-116.

Williams HN, Kelley JI, Baer ML and Turng BF. (1995). The association of bdellovibrios with surfaces in the aquatic environment. Canadian Journal of Microbiology. 41, 1142-1147.

Williams HN. (1988). A study of the occurrence and distribution of bdellovibrios in estuarine sediment over an annual cycle. Microbial Ecology. 15, 9-20.

Woese CR. (1987). Bacterial evolution. Microbiology Reviews. 51, 221-271.

Appendices

Appendix 1. Media used for BD experiment

1.A Standard SWYE broth

Peptone (Merck, Germany) ----------10.0 g

Yeast Extract (Becton Dickinson, USA) ----------3.0 g

70% ASW to make ----------1 L

Adjust pH to 7.8. Mix thoroughly in a magnetic stirrer and autoclave at 121°C for 15 min

1.B SWYE agar

Peptone (Merck, Germany) ----------10.0 g

Yeast Extract (Becton Dickinson, USA) ----------3.0 g

Microbiology agar-agar (Merck, Germany) ----------15.0 g

70% ASW to make ----------1 L

Adjust pH to 7.8. Mix thoroughly in a magnetic stirrer and autoclave at 121°C for 15 min

1.C Pp20 top agar tubes (0.7% agar)

Polypeptone peptone (Becton Dickinson, USA) ----------0.1 g

Microbiology agar-agar (Merck, Germany)----------0.7 g

70% ASW to make ----------100 mL

Dissolve and boil, then dispense 3.5 mL per test tube and autoclave at 121°C for 15 min

1.D Pp20 bottom agar plate (1.5% agar)

Polypeptone peptone (Becton Dickinson, USA) ----------1.0 g

Microbiology agar-agar (Merck, Germany) ----------15 g

70% ASW to make ----------1 L

Mix thoroughly and boil or steam to dissolve. Autoclave at 121°C for 15 min.

Cool to 50°C and pour a thin layer (~10 mL) over the bottom of petri dishes. Allow to cool until solid.

1.E 0% ASW (Artificial Sea Water)

Distilled water ----------500 mL

Filter and autoclave at 121°C for 15 min

1.F 12.5% ASW

Artificial Sea Water (Instant Ocean; Aquarium system; USA) ----------2.08 g

Distilled water ----------500 mL

Mix thoroughly in a magnetic stirrer for 15 min. Filter and autoclave at 121°C for 15 min

1.G 25% ASW

Artificial Sea Water (Instant Ocean; Aquarium system; USA) ----------4.16 g

Distilled water ----------500 mL

Mix thoroughly in a magnetic stirrer for 15 min. Filter and autoclave at 121°C for 15 min

1.H 50% ASW

Artificial Sea Water (Instant Ocean; Aquarium system; USA) ----------8.33 g

Distilled water ----------500 mL

Mix thoroughly in a magnetic stirrer for 15 min. Filter and autoclave at 121°C for 15 min

1.I 70% ASW

Artificial Sea Water (Instant Ocean; Aquarium system; USA) ----------11.65 g

Distilled water ----------500 mL

Mix thoroughly in a magnetic stirrer for 15 min. Filter and autoclave at 121°C for 15 min

1.J 100% ASW

Artificial Sea Water (Instant Ocean; Aquarium system; USA) ----------16.65 g

Distilled water ----------500 mL

Mix thoroughly in a magnetic stirrer for 15 min. Filter and autoclave at 121°C for 15 min

1.K 150% ASW

Artificial Sea Water (Instant Ocean; Aquarium system; USA) ----------25 g

Distilled water ----------500 mL

Mix thoroughly in a magnetic stirrer for 15 min. Filter and autoclave at 121°C for 15 min

1.L 6 x gel loading dye

Bromophenol blue (Sigma-Aldrich, USA)----------0.25 g

Xylene cyanol FF (Sigma-Aldrich, USA)----------0.25 g

Ficoll Type 4000 (Sigma-Aldrich, USA)----------15 g

0.5 M EDTA (Scharlau Chemie, Spain)----------24 mL

Distilled water added to make----------100 mL

Appendix 2. Cultures

2.A. Storage of BD at -80°C

30% glycerol solution was made in distilled water. The solution was autoclaved for 15 min at 121°C, then cooled and stored at 4°C until required. A single, pure BD plaque was inoculated into a sterile 250 mL Erlenmeyer flask containing 50 mL of 70% sterile ASW and 1 mL of 10^9 host cells (*V. parahaemolyticus*).The initial absorbance of the dual culture was measured by spectrophotometer (OD of c.a. 0.300 at 610 nm). The flask was incubated aerobically at 25°C on the orbital shaker at 130 rpm for 10 – 12 h. One mL of the dual cultures of lag phase BD and host cell (spectrophotometric readings of c.a. 0.18) was mixed with 30 µL of 30% glycerol solution. The suspension was placed in a cryo-resistant plastic vial in ice and precooled by placing it at –20°C for 1 to 2 h. The vials then stored at –80°C for long term storage.

The frozen specimens were revived by direct plating onto Pp20 plate as described in Section 2.2 or diluted by 10-fold serial dilution. One mL of each dilution was added into a 250 mL Erlenmeyer flask containing 50 mL of 70% ASW and 1 mL of 10^9 CFU/mL of *V. parahaemolyticus*. The flasks were incubated at 25°C in an orbital shaker (130 rpm) and plaques were selected, purified, propagated and cultivated as described in Section 2.2.

2.B Names and sources of the spoilage and pathogenic organisms used in Section 4.2.1

Spoilage and pathogenic bacteria	Culture ID	Isolated from
Acinetobacter johnsonii	03A07	Tuna
Aeromonas hydrophilia	05B01	King Salmon
Enterobacter cloacae	01D04	Kahawai
Escherichia coli B	M 1	ATCC 11303
Escherichia coli ML 35	M 1	ATCC 43827
Listeria monocytogenes	06B03	King salmon
Morganella morganii	03A11	Kahawai
Photobacterium phosphoreum	08B10	Cod from Denmark
Proteus vulgaris	11A07	King Salmon
Pseudomonas aeruginosa	01E10	Mackerel
Pseudomonas cepacia	01D02	Tuna
Pseudomonas fluorescens	10A05	King Salmon
Pseudomonas mendocina	03D03	King salmon
Pseudomonas pseudomallei	03D04	King salmon
Salmonella Typhimurium	●S001	ATCC 1772
Shewanella putrefaciens	05B10	King Salmon
Vibrio parahaemolyticus	05B03	ESR, Porirua, Wellington
Vibrio vulnificus	10D01	King Salmon

2.C Names and sources of the spoilage and pathogenic organisms used in Section 4.2.2

Spoilage and pathogenic bacteria	Culture ID	Isolated from
Enterobacter cloacae	01D04	Kahawai
Morganella morganii	03A11	Kahawai
Pseudomonas aeruginosa	01E10	Mackerel
Pseudomonas cepacia	01D02	Tuna
Pseudomonas fluorescens	10A05	King Salmon
Pseudomonas mendocina	03D03	King salmon
Photobacterium phosphoreum	08B10	Cod from Denmark
Pseudomonas pseudomallei	03D04	King salmon
Proteus vulgaris	11A07	King Salmon
Shewanella putrefaciens	05B10	King Salmon
Vibrio parahaemolyticus	05B03	ESR, Porirua, Wellington
Vibrio vulnificus	10D01	King Salmon

2.D BD isolates used in the characterization and *in vitro* studies

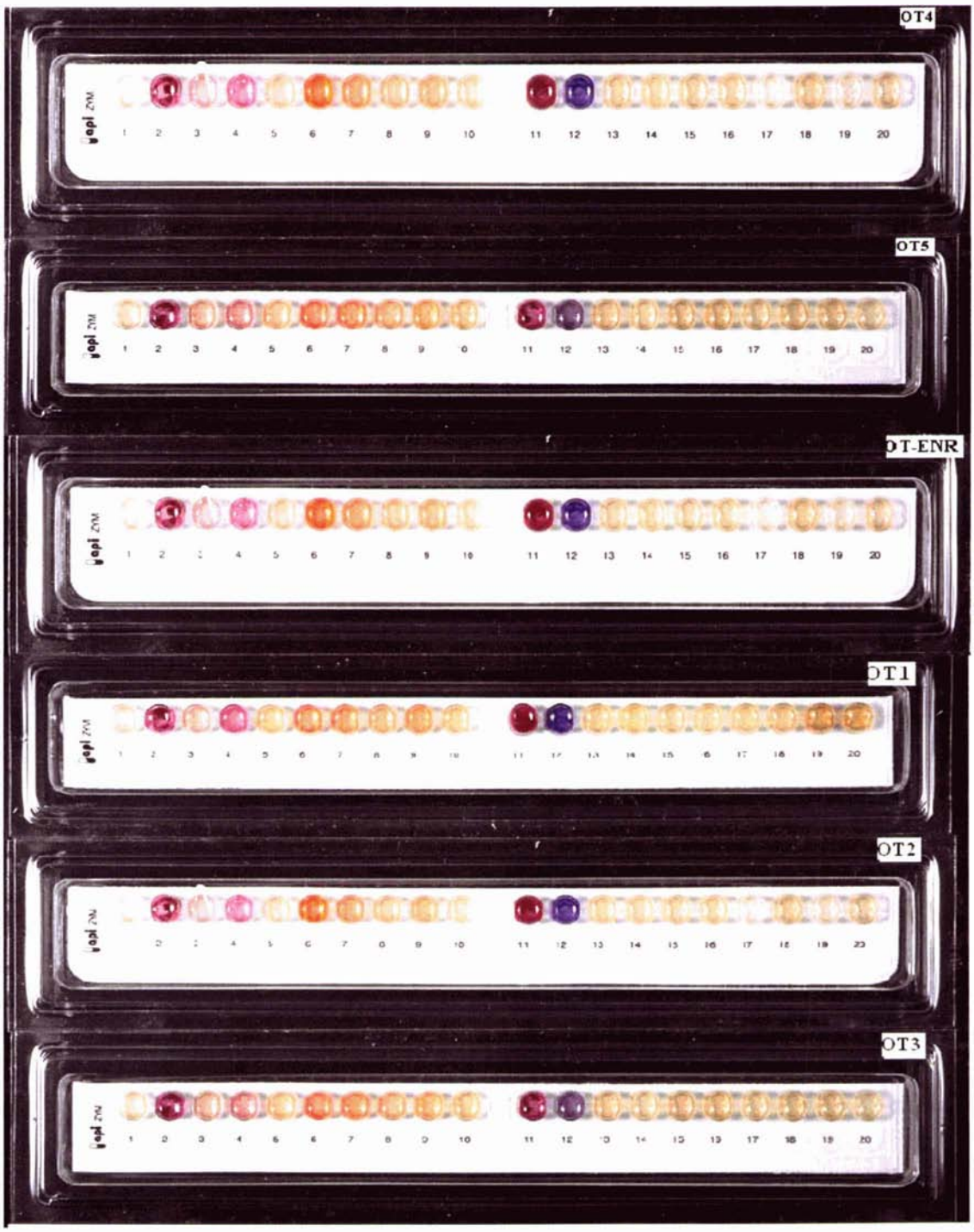
Name of BD isolates	Location of isolation
OT1	One Tree Point, Whangarei Harbour
OT2	One Tree Point, Whangarei Harbour
OT3	One Tree Point, Whangarei Harbour
OT4	One Tree Point, Whangarei Harbour
OT5	One Tree Point, Whangarei Harbour
OT-enr	One Tree Point, Whangarei Harbour
TB1	Moturiki Island, Tauranga
TB2	Pilot Bay, Tauranga
TB-enr	Moturiki Island, Tauranga
MCB	Cox's Bay, Auckland
SP	South Piha, Waitakere, Auckland
MNA	Napier
MNZ1	Ferry terminal, Auckland

2.E BD isolates used for PFGE study

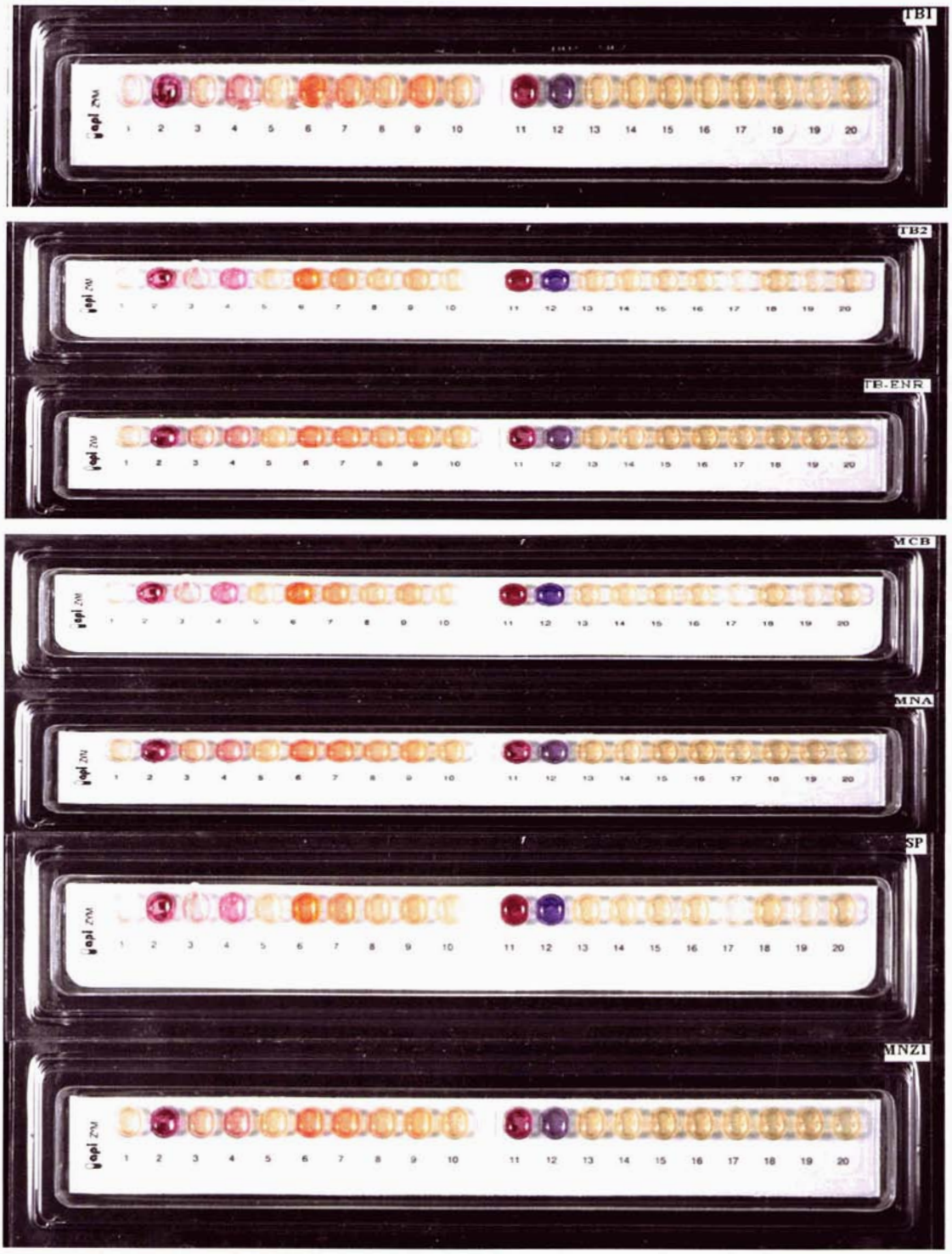
Name of BD isolates	Location of isolation
OT1	One Tree Point, Whangarei Harbour
OT2	One Tree Point, Whangarei Harbour
OT3	One Tree Point, Whangarei Harbour
OT4	One Tree Point, Whangarei Harbour
OT5	One Tree Point, Whangarei Harbour
OT-enr	One Tree Point, Whangarei Harbour
TB1	Moturiki Island, Tauranga
TB2	Pilot Bay, Tauranga
TB-enr	Moturiki Island, Tauranga
MCB	Cox's Bay, Auckland
MCB-enr	Cox's Bay, Auckland
SP	South Piha, Waitakere, Auckland
SP-enr	South Piha, Waitakere, Auckland
MNA	Napier
MNZ1	Ferry terminal, Auckland

Appendix 3. Isolation and characterisation of NZ-BD isolates

3.A.1 Enzymic reactions of BD isolates OT1, OT2, OT3, OT4, OT5 and OT-enr using API ZYM test system (bioMe'rieux).



3.A.2 Enzymic reactions of BD isolates TB1, TB2, TB-enr, MCB, MNA, SP and MNZ1 using API ZYM test system (bioMe'rieux).



3.B Contigs of the BD isolates obtained from complete 16S rDNA sequences

3. B.1 OT1 16S contig

TAGACGCTCCCCTCCAAAGGTTAGGGCCACGGCTTCAGGTAAGGTCAACTCCCATGGTGTGACGGGCGGTGTGTAC
AAGGCCCGGGAACGTATTCACCGCAGCGTGCTGATCTGCGATTACTAGCGATTCCAACTTCATGGAGTCGAGTTGC
AGACTCCAATCCGGACTGAGATGCACTTTTTGAGATTTGCTAACACTCGCGTGCTCGCGTCCCTTTGTATGCACCAT
TGTATTACGTGTGTAGCCCTGGACATAAGGGCCATGAGGACTTGACGTCATCCCCACCTTCCTCCTGGTTAACCCA
GGCAGTCTCTCTAGAGTGCCCAACTTAATGCTGGCAACTAAAGATAGGGGTTGCGCTCGTTGCGAGACTTAACCCA
ACATCTCACGACACGAGCTGACGACAGCCATGCAGCGCCTGTCACCGAGTTCTCCGAAGAGCACAATCAATTTTGG
TCGATCTTCTCGGGATTTCAAGCCCAGGTAAGGTTCTGCGCGTTGCTTCGAATTAAACCACATAATCCACCGCTTGT
GCGGGCCCCCGTCAATTCCTTTGAGTTTTAGTCTTGCGACCGTACTTCCCAGGCGGAGTACTTAATGCGTTAGCTTT
GGCACTGAGAGGGTCAAACCCCCAATACCTAGTACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGT
TTGCTCCCCACGCTTTCGCGCCTCAGCGTCAGTTGATGGCCAGAGAGCCCCCTTCGGCTCTGGTGTTCCTTCGCATC
TCTACGGATTTTACCCCTACATGCGAAATTCCGCTCTCCCCTCCATAACTCTAGATTACCAGTTCCAGACGCAGTTT
CGGGGTTGAGCCCCGAGATTTCACATCTGGCTTAATAATCCGCCTGCGCGCGCTTTACGCCCAGTAAATCCGAACA
ACGCTTGCACCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGTGCTTCCTTTGATGGTACCGTCACTCCA
GACACCTATTAGATATCTGGCAATTCTTCCCATCTGACAGAGCTTTACAACCCGAAGGCCTTCCTCACTCACGCGG
CATTGCTGCGTCAGGCTTTCGCCCATTGCGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCA
GTTCCAGTGTGGCTGATCGTCCTCTCAGACCAGCTATGCATCGTCGCCTTGGTAGGCCGTTACCCCACCAACAAGC
TAATGCAACGCAGGCTCATCTCATAGTAAATGCTTCCAAGGAGAGGCATTCTTTCATCTACTTTTTCCATGTCGTAG
ATCGTATGCGGTATTAGCCAGAATTTCTCCTGGTTATCCCCCGGTATGAGGCAGATTACCTACGCGTTACTCACCCG
TGCGCCACTCTACTCACACCCGAAGGTGCTTTCTCGTTCGACTTGCATGTGTAAAGCAT

3. B.2 OT2 16S contig

ACCGACCGTAGACGCTCCCCTCCAAAGGTTAGGGCCACGGCTTCAGGTAAGGTCAACTCCCATGGTGTGACGGGC
GGTGTGTACAAGGCCCGGGAACGTATTCACCGCAGCGTGCTGATCTGCGATTACTAGCGATTCCAACTTCATGGAG
TCGAGTTGCAGACTCCAATCCGGACTGAGATGCACTTTTTGAGATTTGCTAACACTCGCGTGCTCGCGTCCCTTTGT
ATGCACCATTGTATTACGTGTGTAGCCCTGGACATAAGGGCCATGAGGACTTGACGTCATCCCCACCTTCCTCCTG
GTTAACCCAGGCAGTCTCTCTAGAGTGCCCAACTTAATGCTGGCAACTAAAGATAGGGGTTGCGCTCGTTGCGAGA
CTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCGCCTGTCACCGAGTTCTCCGAAGAGCACAA
TCAATTTTGGTCGATCTTCTCGGGATTTCAAGCCCAGGTAAGGTTCTGCGCGTTGCTTCGAATTAAACCACATAATC
CACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTTAGTCTTGCGACCGTACTTCCCAGGCGGAGTACTTAATG
CGTTAGCTTTGGCACTGAGAGGGTCAAACCCCCAATACCTAGTACTCATCGTTTACGGCGTGGACTACCAGGGTAT
CTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGCGTCAGTTGATGGCCAGAGAGCCCCCTTCGGCTCTGGTGTT
CCTTCGCATCTCTACGGATTTTACCCCTACATGCGAAATTCCGCTCTCCCCTCCATAACTCTAGATTACCAGTTCCA
GACGCAGTTTCGGGGTTGAGCCCCGAGATTTCACATCTGGCTTAATAATCCGCCTGCGCGCGCTTTACGCCCAGTA
AATCCGAACAACGCTTGCACCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGTGCTTCCTTTGATGGTA
CCGTCACTCCAGACACCTATTAGATATCTGGCAATTCTTCCCATCTGACAGAGCTTTACAACCCGAAGGCCTTCCTC
ACTCACGCGGCATTGCTGCGTCAGGCTTTCGCCCATTGCGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGA
CCGTGTCTCAGTTCCAGTGTGGCTGATCGTCCTCTCAGACCAGCTATGCATCGTCGCCTTGGTAGGCCGTTACCCCA
CCAACAAGCTAATGCAACGCAGCTCATCTCATAGTAAATGCTTCCAAGGAGAGGCATTCTTTCATCTACTTTTTCCA
TGTCGTAGATCGTATGCGGTATTAGCCAGAATTTCTCCTGGTATCCCCGCTATGAGGCAGATTACCTACGCGTACTC
ACCCGTGCTCACTCTACTCACACCCGAGTGCTATCTCGTTCGACTGCATGTGTTAGCATGCGCAGCGTTCGTCT

3. B.3 OT3 16S contig

AACGCTCCCCTCCAAAAGGTTAGGGCCACGGCTTCAGGTAAGGTCAACTCCCATGGTGTGACGGGCGGTGTGTAC
AAGGCCCGGGAACGTATTCACCGCAGCGTGCTGATCTGCGATTACTAGCGATTCCAACTTCATGGAGTCGAGTTGC
AGACTCCAATCCGGACTGAGATGCACTTTTTGAGATTTGCTAACACTCGCGTGCTCGCGTCCCTTTGTATGCACCAT
TGTATTACGTGTGTAGCCCTGGACATAAGGGCCATGAGGACTTGACGTCATCCCCACCTTCCTCCTGGTTAACCCA
GGCAGTCTCTCTAGAGTGCCCAACTTAATGCTGGCAACTAAAGATAGGGGTTGCGCTCGTTGCGAGACTTAACCCA
ACATCTCACGACACGAGCTGACGACAGCCATGCAGCGCCTGTCACCGAGTTCTCCGAAGAGCACAATCAATTTTGG
TCGATCTTCTCGGGATTTCAAGCCCAGGTAAGGTTCTGCGCGTTGCTTCGAATTAAACCACATAATCCACCGCTTGT
GCGGGCCCCCCGTCAATTCCTTTGAGTTTTAGTCTTGCGACCGTACTTCCCAGGCGGAGTACTTAATGCGTTAGCTTT
GGCACTGAGAGGGTCAAACCCCCAATACCTAGTACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGT
TTGCTCCCCACGCTTTCGCGCCTCAGCGTCAGTTGATGGCCAGAGAGCCCCCTTCGGCTCTGGTGTTCCTTCGCATC
TCTACGGATTTTACCCCTACATGCGAAATTCCGCTCTCCCCTCCATAACTCTAGATTACCAGTTCCAGACGCAGTTT
CGGGGTTGAGCCCCCGAGATTTCACATCTGGCTTAATAATCCGCCTGCGCGCGCTTTACGCCCAGTAAATCCGAACA
ACGCTTGCACCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGTGCTTCCTTTGATGGTACCGTCACTCCA
GACACCTATTAGATATCTGGCAATTCTTCCCATCTGACAGAGCTTTACAACCCGAAGGCCTTCCTCACTCACGCGG
CATTGCTGCGTCAGGCTTTCGCCCATTGCGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCA
GTTCCAGTGTGGCTGATCGTCCTCTCAGACCAGCTATGCATCGTCGCCTTGGTAGGCCGTTACCCCCACCAACAAG
CTAATGCAACGCAGGCTCATCTCATAGTAAATGCTTCCAAGGAGAGGCATTCTTTCATCTACTTTTTCCATGTCGTA
GATCGTATGCGGTATTAGCCAGAATTTCTCCTGGTTATCCCCCGCTATGAGGCAGATTACCTACGCGTTACTCACCG
TGCGCCACTCTACTCACACCCGAAGGTGCTTTCTCGTTCGACTG

3. B.4 SP1 16S contig

GCATGCTTACACATGCAAGTCGAACGAGAAAGCACCTTCGGGTGTGAGTAGAGTGGCGCACGGTGAG
TAACGCGTAGGTAATCTGCCTCATAGCGGGGGATAACCAGGAGAAATTCTGGCTAATACCGCATACGA
TCTACGACATGGAAAAAGTAGATGAAAGAATGCCTCTCCTTGGAAGCATTTACTATGAGATGAGCCTG
CGTTGCATTAGCTTGTTGGTGGGGTAACGGCCTACCAAGGCGACGATGCATAGCTGGTCTGAGAGGAC
GATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCG
CAATGGGCGAAAGCCTGACGCAGCAATGCCGCGTGAGTGAGGAAGGCCTTCGGGTTGTAAAGCTCTG
TCAGATGGGAAGAATTGCCAGATATCTAATAGGTGTCTGGAGTGACGGTACCATCAAAGGAAGCACC
GGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTGTTCGGATTTACTGGGCGTA
AAGCGCGCGCAGGCGGATTATTAAGCCAGATGTGAAATCTCGGGGCTCAACCCCGAAACTGCGTCTGG
AACTGGTAATCTAGAGTTATGGAGGGGAGAGCGGAATTTCGCATGTAGGGGTAAAATCCGTAGAGAT
GCGAAGGAACACCAGAGCCGAAGGGGGCTCTCTGGCCATCAACTGACGCTGAGGCGCGAAAGCGTGG
GGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAGGTATTGGGGGTTT
GACCCTCTCAGTGCCAAAGCTAACGCATTAAGTACTCCGCCTGGGAAGTACGGTCGCAAGACTAAAAC
TCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGATTATGTGGTTTAATTCGAAGCAACGCGCAGA
ACCTTACCTGGGCTTGAAATCCCGAGAAGATCGACCAAAATTGATTGTGCTCTTCGGAGAACTCGGTG
ACAGGCGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCTCGCAACGAGCGCAA
CCCCTATCTTTAGTTGCCAGCATTAAGTTGGGCACTCTAGAGAGACTGCCTGGGTTAACCAGGAGGAA
GGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGTCCAGGGCTACACACGTAATACAATGGTGCATA
CAAAGGGACGCGAGCACGCGAGTGTTAGCAAATCTCAAAAAGTGCATCTCAGTCCGGATTGGAGTCT
GCAACTCGACTCCATGAAGTTGGAATCGCTAGTAATCGCAGATCAGCACGCTGCGGTGAATACGTTCC
CGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGACCTTACCTGAAGCCGTGGCCCTAACCTTT
GGAGGGGAGCGTCTACGGTCGG

3. B.5 TB2 16S contig

CAAGTCGAACGAGAAAGCACCTTCGGGTGTGAGTAGAGTGGCGCACGGGTGAGTAACGCGTAGGTAATCTGCCTC
ATAGCGGGGGATAACCAGGAGAAATTCTGGCTAATACCGCATACGATCTACGACATGGAAAAAGTAGATGAAAG
AATGCCTCTCCTTGGAAGCATTTACTATGAGATGAGCCTGCGTTGCATTAGCTTGTTGGTGGGGTAACGGCCTACC
AAGGCGACGATGCATAGCTGGTCTGAGAGGACGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGG
GAGGCAGCAGTGGGGAATATTGCGCAATGGGCGAAAGCCTGACGCAGCAATGCCGCGTGAGTGAGGAAGGCCTT
CGGGTTGTAAAGCTCTGTCAGATGGGAAGAATTGCCAGATATCTAATAGGTGTCTGGAGTGACGGTACCATCAAA
GGAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTGTTCGGATTTACTGGGCG
TAAAGCGCGCGCAGGCGGATTATTAAGCCAGATGTGAAATCTCGGGGCTCAACCCCGAAACTGCGTCTGGAACTG
GTAATCTAGAGTTATGGAGGGGAGAGCGGAATTTCGCATGTAGGGGTAAAATCCGTAGAGATGCGAAGGAACACC
AGAGCCGAAGGGGGCTCTCTGGCCATCAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATA
CCCTGGTAGTCCACGCCGTAAACGATGAGTACTAGGTATTGGGGGTTTGACCCTCTCAGTGCCAAAGCTAACGCAT
TAAGTACTCCGCCTGGGAAGTACGGTCGCAAGACTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGG
ATTATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCTGGGCTTGAAATCCCGAGAAGATCGACCAAAATTGA
TTGTGCTCTTCGGAGAACTCGGTGACAGGCGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT
CTCGCAACGAGCGCAACCCCTATCTTTAGTTGCCAGCATTAAGTTGGGCACTCTAGAGAGACTGCCTGGGTTAACC
AGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGTCCAGGGCTACACACGTAATACAATGGTGCAT
ACAAAGGGACGCGAGCACGCGAGTGTTAGCAAATCTCAAAAAGTGCATCTCAGTCCGGATTGGAGTCTGCAACTC
GACTCCATGAAGTTGGAATCGCTAGTAATCGCAGATCAGCACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACA
CCGCCCGTCACACCATGGGAGTTGACCTTACCTGAAGCCGTGGCCCTAACCTTTTGGAGGGGAGCGTCTAC

3. B.6 MCB 16S contig

TCCCCCCTCCAAAAGGTTAGGGCCACGGCTTCAGGTAAGGTCAACTCCCATGGTGTGACGGGCGGTGTGTACAAG
GCCCGGAACGTATTCACCGCAGCGTGCTGATCTGCGATTACTAGCGATTCCAACTTCATGGAGTCGAGTTGCAGAC
TCCAATCCGGACTGAGATGCACTTTTTGAGATTTGCTAACACTCGCGTGCTCGCGTCCCTTTGTATGCACCATTGTA
TTACGTGTGTAGCCCTGGACATAAGGGCCATGAGGACTTGACGTCATCCCCACCTTCCTCCTGGTTAACCCAGGCA
GTCTCTCTAGAGTGCCCAACTTAATGCTGGCAACTAAAGATAGGGGTTGCGCTCGTTGCGAGACTTAACCCAACAT
CTCACGACACGAGCTGACGACAGCCATGCAGCGCCTGTCACCGAGTTCTCCGAAGAGCACAATCAATTTTGGTCGA
TCTTCTCGGGATTTCAAGCCCAGGTAAGGTTCTGCGCGTTGCTTCGAATTAAACCACATAATCCACCGCTTGTGCGG
GCCCCCCGTCAATTCCTTTGAGTTTTAGTCTTGCGACCGTACTTCCCAGGCGGAGTACTTAATGCGTTAGCTTTGGCA
CTGAGAGGGTCAAACCCCCAATACCTAGTACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGC
TCCCCACGCTTTCGCGCCTCAGCGTCAGTTGATGGCCAGAGAGCCCCCTTCGGCTCTGGTGTTCCTTCGCATCTCTA
CGGATTTTACCCCTACATGCGAAATTCCGCTCTCCCCTCCATAACTCTAGATTACCAGTTCCAGACGCAGTTTCGGG
GTTGAGCCCCGAGATTTCACATCTGGCTTAATAATCCGCCTGCGCGCGCTTTACGCCCAGTAAATCCGAACAACGC
TTGCACCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGTGCTTCCTTTGATGGTACCGTCACTCCAGACA
CCTATTAGATATCTGGCAATTCTTCCCATCTGACAGAGCTTTACAACCCGAAGGCCTTCCTCACTCACGCGGCATTG
CTGCGTCAGGCTTTCGCCCATTGCGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCC
AGTGTGGCTGATCGTCCTCTCAGACCAGCTATGCATCGTCGCCTTGGTAGGCCGTTACCCCACCAACAAGCTAATG
CAACGCAGGCTCATCTCATAGTAAATGCTTCCAAGGAGAGGCATTCTTTCATCTACTTTTTCCATGTCGTAGATCGT
ATGCGGTATTAGCCAGAATTTCTCCTGGTTATCCCCCGCTATGAGGCAGATTACCTACGCGTTACTCACCCGTGCGC
CACTCTACTCACACCCGAAGGTGCTTTCTCGTTCGAACCT

3. B.7 MNA 16S contig

ACCGTAGACGCTCCCCTCCAAAGGTAGGGCCACGGCTTCAGGTAAGGTCAACTCCCATGGTGTGACGGGCGGTGT
GTACAAGGCCCGGGAACGTATTCACCGCAGCGTGCTGATCTGCGATTACTAGCGATTCCAACTTCATGGAGTCGAG
TTGCAGACTCCAATCCGGACTGAGATGCACTTTTTGAGATTTGCTAACACTCGCGTGCTCGCGTCCCTTTGTATGCA
CCATTGTATTACGTGTGTAGCCCTGGACATAAGGGCCATGAGGACTTGACGTCATCCCCACCTTCCTCCTGGTTAAC
CCAGGCAGTCTCTCTAGAGTGCCCAACTTAATGCTGGCAACTAAAGATAGGGGTTGCGCTCGTTGCGAGACTTAAC
CCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCGCCTGTCACCGAGTTCTCCGAAGAGCACAATCAATTT
TGGTCGATCTTCTCGGGATTTCAAGCCCAGGTAAGGTTCTGCGCGTTGCTTCGAATTAAACCACATAATCCACCGCT
TGTGCGGGCCCCCGTCAATTCCTTTGAGTTTTAGTCTTGCGACCGTACTTCCCAGGCGGAGTACTTAATGCGTTAGC
TTTGGCACTGAGAGGGTCAAACCCCCAATACCTAGTACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCC
TGTTTGCTCCCCACGCTTTCGCGCCTCAGCGTCAGTTGATGGCCAGAGAGCCCCCTTCGGCTCTGGTGTTCCTTCGC
ATCTCTACGGATTTTACCCCTACATGCGAAATTCCGCTCTCCCCTCCATAACTCTAGATTACCAGTTCCAGACGCAG
TTTCGGGGTTGAGCCCCGAGATTTCACATCTGGCTTAATAATCCGCCTGCGCGCGCTTTACGCCCAGTAAATCCGA
ACAACGCTTGCACCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGTGCTTCCTTTGATGGTACCGTCACT
CCAGACACCTATTAGATATCTGGCAATTCTTCCCATCTGACAGAGCTTTACAACCCGAAGGCCTTCCTCACTCACGC
GGCATTGCTGCGTCAGGCTTTCGCCCATTGCGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCT
CAGTTCCAGTGTGGCTGATCGTCCTCTCAGACCAGCTATGCATCGTCGCCTTGGTAGGCCGTTACCCCACCAACAA
GCTAATGCAACGCAGGCTCATCTCATAGTAAATGCTTCCAAGGAGAGGCATTCTTTCATCTACTTTTTCCATGTCGT
AGATCGTATGCGGTATTAGCCAGAATTTCTCCTGGTTATCCCCCCGCTATGAGGCAGATTACCTACGCGTTACTCACC
CGTGCGCCACTCTACTCACACCCGAAGGTGCTTTCTCGTTCGACTTGCATGTGTTAAGCATGCC

3. B.8 MNZ1 16S contig

TGCAAGTCGAACGAGAAAGCACCTTCGGGTGTGAGTAGAGTGGCGCACGGGTGAGTAACGCGTAGGTAATCTGCC
TCATAGCGGGGGATAACCAGGAGAAATTCTGGCTAATACCGCATACGATCTACGACATGGAAAAAGTAGATGAAA
GAATGCCTCTCCTTGGAAGCATTTACTATGAGATGAGCCTGCGTTGCATTAGCTTGTTGGTGGGGTAACGGCCTAC
CAAGGCGACGATGCATAGCTGGTCTGAGAGGACGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACG
GGAGGCAGCAGTGGGGAATATTGCGCAATGGGCGAAAGCCTGACGCAGCAATGCCGCGTGAGTGAGGAAGGCCT
TCGGGTTGTAAAGCTCTGTCAGATGGGAAGAATTGCCAGATATCTAATAGGTGTCTGGAGTGACGGTACCATCAAA
GGAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTGTTCGGATTTACTGGGCG
TAAAGCGCGCGCAGGCGGATTATTAAGCCAGATGTGAAATCTCGGGGCTCAACCCCGAAACTGCGTCTGGAACTG
GTAATCTAGAGTTATGGAGGGGAGAGCGGAATTTCGCATGTAGGGGTAAAATCCGTAGAGATGCGAAGGAACACC
AGAGCCGAAGGGGGCTCTCTGGCCATCAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATA
CCCTGGTAGTCCACGCCGTAAACGATGAGTACTAGGTATTGGGGGTTTGACCCTCTCAGTGCCAAAGCTAACGCAT
TAAGTACTCCGCCTGGGAAGTACGGTCGCAAGACTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGG
ATTATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCTGGGCTTGAAATCCCGAGAAGATCGACCAAAATTGA
TTGTGCTCTTCGGAGAACTCGGTGACAGGCGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT
CTCGCAACGAGCGCAACCCCTATCTTTAGTTGCCAGCATTAAGTTGGGCACTCTAGAGAGACTGCCTGGGTTAACC
AGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGTCCAGGGCTACACACGTAATACAATGGTGCAT
ACAAAGGGACGCGAGCACGCGAGTGTTAGCAAATCTCAAAAAGTGCATCTCAGTCCGGATTGGAGTCTGCAACTC
GACTCCATGAAGTTGGAATCGCTAGTAATCGCAGATCAGCACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACA
CCGCCCGTCACACCATGGGAGTTGACCTTACCTGAAGCCGTGGCCCTAACCTTTTGGAGGGGAGCCGTT

3.C Partial 16S rDNA sequences using primers 63F and 842R

3.C.1 OT4

CGTAGGTGATCTACCATTTggcGGGGGATAACCAGAAGAAATTCTGGCTAATACCGCATACGTACTGCAATTTTGAA
AGTAGCAGTAGAAAGAGTGCCTCTCCTTGGAAGCACTTATCAAATGATGAGCCTGCGTAGCATTAGTTAGATGGTG
GGGTAATGGCTTACCATGACTACGATGCTTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGG
TCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCGCAATGGGGGAAACCCTGACGCAGCAATGCCGAGTGA
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AGGTACCTTCAAAGGAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTGTTCG
GATTTATTGGGCGTAAAGCGCGCGCAGGCGGATTGTTAAGTCAGATGTGAAATCTCGGGGCTCAACCCCGAAACT
GCGTCTGAAACTGATAATCTAGAATCTCGGAGAGGGAAGGGGAATTTCGCATGTAGGGGTAAAATCCGTAGAGAT
GCGAAGGAACACCAGAGGCGAAGGCGCCTTCCTGGACGAGTATTGACGCTGAGGCGCGAAAGCGTGGGGTAGCAA
ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTGCACTAGATATTGGAGGTTTGACCCCTTCAGTGTC
GTAGCTAACGCATTAAGTGCACCGCCTGGGGAGTACGGTCGCAAGACTAAAACTCAAAGGAATTGACGGGGGCCC
GCACAAGCGGTGGATTATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCTAGGCTTGAAATCCTGAGAATCT
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TGTTGGGTTAAGTCTCGCAACGAGCGCAACCCCTATCCTTAGTTGCCAGCATTAAGTTGGGCACTCTAGGGAGACT
GCCCGGGCTAACCGGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGTCTAGGGCTACACACGTAA
TACAATGGTGCATACAAAGGGAAGCGACCTGGCAACAGTGAGCAAATCTCAAAAAGTGCATCTCAGTCCGGATTG
GAGTCTGCAACTCGACTCCATGAAGTTGGAATCGCTAGTAATCGGAGATCAGCACGCTCCGGTGAATACGTTCCCG
GGCCTTGTACACACCGCCCGTCACaCcacgGGAGTTGGTCTTACCTGAAGTCGTGGCCCTAACTGCTTG

3.C.2 OT5

TAGGTGATCTACCATTTggcGGGGGATaccctgAAGAAATTCTGGCTAATACCGCATACGTACTGCAATTTTGAAAGTA
GCAGTAGAAAGAGTGCCTCTCCTTGGAAGCACTTATCAAATGATGAGCCTGCGTAGCATTAGTTAGATGGTGGGGGT
AATGGCTTACCATGACTACGATGCTTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCA
GACTCCTACGGGAGGCAGCAGTGGGGAATATTGCGCAATGGGGGAAACCCTGACGCAGCAATGCCGAGTGAGTG
AGGAAGGCCTTCGGGTTGTAAAGCTCTGTCAGAAGGGAATAATGGTATAGGGTCCAATAGGCCTTATATTTGAAG
GTACCTTCAAAGGAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTGTTCGGA
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GTCTGAAACTGATAATCTAGAATCTCGGAGAGGGAAGGGGAATTTCGCATGTAGGGGTAAAATCCGTAGAGATGC
GAAGGAACACCAGAGGCGAAGGCGCCTTCCTGGACGAGTATTGACGCTGAGGCGCGAAAGCGTGGGGTAGCAAAC
AGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTGCACTAGATATTGGAGGTTTGACCCCTTCAGTGTCGT
AGCTAACGCATTAAGTGCACCGCCTGGGGAGTACGGTCGCAAGACTAAAACTCAAAGGAATTGACGGGGGCCCGC
ACAAGCGGTGGATTATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCTAGGCTTGAAATCCTGAGAATCTGA
TGGAAACATCGGAGTGCTCTTCGGAGAATTCAGTGACAGGCGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATG
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CCGGGCTAACCGGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGTCTAGGGCTACACACGTAATA
CAATGGTGCATACAAAGGGAAGCGACCTGGCAACAGTGAGCAAATCTCAAAAAGTGCATCTCAGTCCGGATTGGA
GTCTGCAACTCGACTCCATGAAGTTGGAATCGCTAGTAATCGGAGATCAGCACGCTCCGGTGAATACGTTCCCGGG
CCTTGTACACACCGCCCGTCAcaccacgGGAGTTGGTCTTACCTGAAGTCGTGGCCCTAACTGCttgcagagGGGAG

3.C.3 OT-enr

CGTAGGTGATCTACCATTTggcGGGGGGATaaccataaGAAATTCTGGCTAATACCGCATACGTACTGCAATTTTGAAAGT
AGCAGTAGAAAGAGTGCCTCTCCTTGGAAGCACTTATCAAATGATGAGCCTGCGTAGCATTAGTTAGATGGTGGG
GTAATGGCTTACCATGACTACGATGCTTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCC
AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCGCAATGGGGGAAACCCTGACGCAGCAATGCCGAGTGAGT
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GGTACCTTCAAAGGAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTGTTCGG
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CGTCTGAAACTGATAATCTAGAATCTCGGAGAGGGAAGGGGAATTTCGCATGTAGGGGTAAAATCCGTAGAGATG
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CAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTGCACTAGATATTGGAGGTTTGACCCCTTCAGTGTCG
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CACAAGCGGTGGATTATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCTAGGCTTGAAATCCTGAGAATCTG
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AGTCTGCAACTCGACTCCATGAAGTTGGAATCGCTAGTAATCGGAGATCAGCACGCTCCGGTGAATACGTTCCCGG
GCCTTGTACACACCGCCCGTCaCACcacgGGAGTTGGTCTTACCTGAAGTCGTGGCCCTAACTGC

3. C.4 TB1

CGTAGGTGATCTACCATTTggcGGGGGGATaacctgAAGAAATTCTGGCTAATACCGCATACGTACTGCAATTTTGAAAG
TAGCAGTAGAAAGAGTGCCTCTCCTTGGAAGCACTTATCAAATGATGAGCCTGCGTAGCATTAGTTAGATGGTGGG
GTAATGGCTTACCATGACTACGATGCTTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCC
AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCGCAATGGGGGAAACCCTGACGCAGCAATGCCGAGTGAGT
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GGTACCTTCAAAGGAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTGTTCGG
ATTTATTGGGCGTAAAGCGCGCGCAGGCGGATTGTTAAGTCAGATGTGAAATCTCGGGGCTCAACCCCGAAACTG
CGTCTGAAACTGATAATCTAGAATCTCGGAGAGGGAAGGGGAATTTCGCATGTAGGGGTAAAATCCGTAGAGATG
CGAAGGAACACCAGAGGCGAAGGCGCCTTCCTGGACGAGTATTGACGCTGAGGCGCGAAAGCGTGGGTAGCAAA
CAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTGCACTAGATATTGGAGGTTTGACCCCTTCAGTGTCG
TAGCTAACGCATTAAGTGCACCGCCTGGGGAGTACGGTCGCAAGACTAAAACTCAAAGGAATTGACGGGGGCCCG
CACAAGCGGTGGATTATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCTAGGCTTGAAATCCTGAGAATCTG
ATGGAAACATCGGAGTGCTCTTCGGAGAATTCAGTGACAGGCGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGAT
GTTGGGTTAAGTCTCGCAACGAGCGCAACCCCTATCCTTAGTTGCCAGCATTAAGTTGGGCACTCTAGGGAGACTG
CCCGGGGCTAACCGGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGTCTAGGGCTACACACGTAAT
ACAATGGTGCATACAAAGGGAAGCGACCTGGCAACAGTGAGCAAATCTCAAAAAGTGCATCTCAGTCCGGATTGG
AGTCTGCAACTCGACTCCATGAAGTTGGAATCGCTAGTAATCGGAGATCAGCACGCTCCGGTGAATACGTTCCCGG
GCCTTGTACACACCGCCCGTcacaaaggggGAGTTGGTCTTACCTGAAGTCGTGGCCCTAACTgC

3. C.5 TB-enr

CGTAGGTGATCTACCATTTggcGGGGGATaacctgAAGAAATTCTGGCTAATACCGCATACGTACTGCAATTTTGAAAG
TAGCAGTAGAAAGAGTGCCTCTCCTTGGAAGCACTTATCAAATGATGAGCCTGCGTAGCATTAGTTAGATGGTGGG
GTAATGGCTTACCATGACTACGATGCTTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCC
AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCGCAATGGGGGAAACCCTGACGCAGCAATGCCGAGTGAGT
GAGGAAGGCCTTCGGGTTGTAAAGCTCTGTCAGAAGGGAATAATGGTATAGGGTCCAATAGGCCTTATATTTGAA
GGTACCTTCAAAGGAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTGTTCGG
ATTTATTGGGCGTAAAGCGCGCGCAGGCGGATTGTTAAGTCAGATGTGAAATCTCGGGGCTCAACCCCGAAACTG
CGTCTGAAACTGATAATCTAGAATCTCGGAGAGGGAAGGGGAATTTCGCATGTAGGGGTAAAATCCGTAGAGATG
CGAAGGAACACCAGAGGCGAAGGCGCCTTCCTGGACGAGTATTGACGCTGAGGCGCGAAAGCGTGGGTAGCAAA
CAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTGCACTAGATATTGGAGGTTTGACCCCTTCAGTGTCG
TAGCTAACGCATTAAGTGCACCGCCTGGGGAGTACGGTCGCAAGACTAAAACTCAAAGGAATTGACGGGGGCCCG
CACAAGCGGTGGATTATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCTAGGCTTGAAATCCTGAGAATCTG
ATGGAAACATCGGAGTGCTCTTCGGAGAATTCAGTGACAGGCGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGAT
GTTGGGTTAAGTCTCGCAACGAGCGCAACCCCTATCCTTAGTTGCCAGCATTAAGTTGGGCACTCTAGGGAGACTG
CCCGGGCTAACCGGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGTCTAGGGCTACACACGTAAT
ACAATGGTGCATACAAAGGGAAGCGACCTGGCAACAGTGAGCAAATCTCAAAAAGTGCATCTCAGTCCGGATTGG
AGTCTGCAACTCGACTCCATGAAGTTGGAATCGCTAGTAATCGGAGAAGATCAGCACGCTCCGGTGAATACGTTCC
CGGGCCTTGTACACACCGCCCGTCACACcacgGGAGTTGGTCTTACCTGAAGTCGTGGCCCTAACTGCTTG

3.D The closest phylogenetic relatives of different BD isolates

3.D.1 The closest phylogenetic relatives of OT1 isolate.

Closest phylogenetic relative	Score (bits)	Identities / Similarity (%)	Accession number
Bacteriovorax sp. DA5	2730	1423 / 1437 (99%)	EF092435
Bacteriovorax sp. OC71	2724	1377 / 1378 (99%)	DQ536436
Bacteriovorax sp. NB2	2706	1420 / 1437 (98%)	EF092436
Bdellovibrio sp. JS2	2202	1353 / 1431 (94%)	AF084856
Bdellovibrio sp. JS7	2194	1352 / 1431 (94%)	AF084861
Bdellovibrio sp. JS4	2194	1352 / 1431 (94%),	AF084858
Bdellovibrio sp. JS7	2187	1335 / 1411 (94%)	AF084862
Bacteriovorax sp. NE1	1859	965 / 974 (99%)	EF092445
Bacteriovorax sp. DD1	1852	977 / 990 (98%),	EF092444
Bacteriovorax sp. GSL41	1729	914 / 928 (98%),	DQ536440

3.D.2 The closest phylogenetic relatives of isolate OT3.

Closest phylogenetic relative	Score (bits)	Identities / Similarity (%)	Accession number
Bacteriovorax sp. OC71	2702	1377/1379 (99%)	DQ536436
Bacteriovorax sp. DA5	2694	1406/1419 (99%)	EF092435
Bacteriovorax sp. NB2	2670	1403/1419 (98%)	EF092436
Bdellovibrio sp. JS2	2157	1340/1420 (94%)	AF084856
Bdellovibrio sp. JS7	2149	1339/1420 (94%)	AF084861
Bdellovibrio sp. JS4	2149	1339/1420 (94%)	AF084858
Bdellovibrio sp. JS7	2147	1326/1403 (94%)	AF084862
Bacteriovorax sp. NE1	1873	972/981 (99%)	EF092445
Bacteriovorax sp. DD1	1861	975/987 (98%)	EF092444
Bacteriovorax sp. GSL41	1729	914/928 (98%)	DQ536440
Bdellovibrio sp. JS10	1673	953/988 (96%)	AF084863
Bdellovibrio sp. JS6	1673	953/988 (96%)	AF084860

3.D.3 The closest phylogenetic relatives of isolate SP.

Closest phylogenetic relative	Score (bits)	Identities / Similarity (%)	Accession number
Bacteriovorax sp. DA5	2732	1418/1440 (98%)	EF092435
Bacteriovorax sp. OC71	2716	1359/1365 (99%)	DQ536436
Bacteriovorax sp. NB2	2708	1414/1439 (98%)	EF092436
Bdellovibrio sp. JS2	2208	1354/1441 (93%)	AF084856
Bdellovibrio sp. JS7	2200	1353/1441 (93%)	AF084861
Bdellovibrio sp. JS4	2200	1353/1441 (93%)	AF084858
Bdellovibrio sp. JS7	2192	1352/1441 (93%)	AF084862
Bacteriovorax sp. DD1	1865	984/997 (98%)	EF092444
Bacteriovorax sp. NE1	1861	984/997 (98%)	EF092445
Bacteriovorax sp. GSL41	1729	914/928 (98%)	DQ536440
Bdellovibrio sp. JS10	1693	964/998 (96%)	AF084863
Bdellovibrio sp. JS6	1693	964/998 (96%)	AF084860

3.D.4 The closest phylogenetic relatives of isolate TB2.

Closest phylogenetic relative	Score (bits)	Identities / Similarity (%)	Accession number
Bacteriovorax sp. DA5	2736	1413/1424 (99%)	EF092435
Bacteriovorax sp. OC71	2732	1378/1378 (99%)	DQ536436
Bacteriovorax sp. NB2	2712	1410/1424 (99%)	EF092436
Bdellovibrio sp. JS2	2198	1347/1425 (94%)	AF084856
Bdellovibrio sp. JS4	2190	1346/1425 (94%)	AF084858
Bdellovibrio sp. JS7	2190	1346/1425 (94%)	AF084861
Bdellovibrio sp. JS7	2183	1345/1425 (94%)	AF084862
Bacteriovorax sp. NE1	1873	972/981 (99%)	EF092445
Bacteriovorax sp. DD1	1869	979/991 (98%)	EF092444
Bacteriovorax sp. GSL41	1729	914/928 (98%)	DQ536440
Bdellovibrio sp. JS10	1681	957/992 (96%)	AF084863
Bdellovibrio sp. JS6	1681	957/992 (96%)	AF084860

3.D.5 The closest phylogenetic relatives of isolate MCB.

Closest phylogenetic relative	Score (bits)	Identities / Similarity (%)	Accession number
Bacteriovorax sp. OC71	2716	1377/1378 (99%)	DQ536436
Bacteriovorax sp. DA5	2694	1399/1411 (99%)	EF092435
Bacteriovorax sp. NB2	2670	1396/1411 (98%)	EF092436
Bdellovibrio sp. JS2	2165	1325/1400 (94%)	AF084856
Bdellovibrio sp. JS7	2157	1324/1400 (94%)	AF084862
Bdellovibrio sp. JS4	2157	1324/1400 (94%)	AF084858
Bacteriovorax sp. NE1	1857	1324/1400 (94%)	EF092445.1
Bacteriovorax sp. DD1	1836	971/981 (98%)	EF092444
Bacteriovorax sp. GSL41	1713	969/982 (98%)	DQ536440
Bdellovibrio sp. JS10	1655	913/928 (98%)	AF084863
Bdellovibrio sp. JS6	1655	939/971 (96%)	AF084860

3.D.6 The closest phylogenetic relatives of isolate MNA.

Closest phylogenetic relative	Score (bits)	Identities / Similarity (%)	Accession number
Bacteriovorax sp. DA5	2746	1430/1444 (99%)	EF092435
Bacteriovorax sp. OC71	2732	1378/1378 (100%)	DQ536436
Bacteriovorax sp. NB2	2722	1427/1444 (98%)	EF092436
Bdellovibrio sp. JS2	2224	1366/1445 (94%)	AF084856
Bdellovibrio sp. JS7	2216	1365/1445 (94%)	AF084861
Bdellovibrio sp. JS4	2216	1365/1445 (94%)	AF084858
Bdellovibrio sp. JS7	2208	1364/1445 (94%)	AF084862
Bacteriovorax sp. NE1	1848	959/968 (99%)	EF092445
Bacteriovorax sp. DD1	1846	979/994 (98%)	EF092444
Bacteriovorax sp. GSL41	1729	914/928 (98%)	DQ536440
Bdellovibrio sp. JS10	1681	960/995 (96%)	AF084863
Bdellovibrio sp. JS6	1681	960/995 (96%)	AF084860

3.D.7 The closest phylogenetic relatives of isolate MNZ1.

Closest phylogenetic relative	Score (bits)	Identities / Similarity (%)	Accession number
Bacteriovorax sp. OC71	2732	1378/1378 (100%)	DQ536436
Bacteriovorax sp. DA5	2728	1409/1420 (99%)	EF092435
Bacteriovorax sp. NB2	2704	1406/1420 (99%)	EF092436
Bdellovibrio sp. JS2	2192	1332/1406 (94%)	AF084856
Bdellovibrio sp. JS7	2185	1331/1406 (94%)	AF084861
Bdellovibrio sp. JS7	2185	1331/1406 (94%)	AF084862
Bdellovibrio sp. JS4	2185	1331/1406 (94%)	AF084858
Bacteriovorax sp. NE1	1873	972/981 (99%)	EF092445
Bacteriovorax sp. DD1	1857	973/985 (98%)	EF092444
Bacteriovorax sp. GSL41	1729	914/928 (98%)	DQ536440
Bdellovibrio sp. JS10	1671	940/971 (96%)	AF084863
Bdellovibrio sp. JS6	1671	940/971 (96%)	AF084860

Appendix 4. Predation patterns of BD isolates against some pathogenic and spoilage organisms in solid and liquid media

4. A Predation ability of BD isolates in solid media at 20°C after 24 h of incubation.

Host bacteria	OT1	OT2	OT3	OT4	OT5	OT-enr	TB1	TB2	TB-enr	MCB	MNA	SP	MNZ1
Acinetobacter johnsonii	v	v	v	v	v	v	v	v	v	v	v	v	v
Aeromonas hydrophilia	v	v	v	v	v	v	v	v	v	v	v	v	v
Enterobacter cloacae	v	v	v	v	v	v	v	v	v	v	v	v	v
Escherichia coli B	-	-	-	-	-	-	-	-	-	-	-	-	-
Escherichia coli ML 35	-	-	-	-	-	-	-	-	-	-	-	-	-
Listeria monocytogenes	-	-	-	-	-	-	-	-	-	-	-	-	-
Morganella morganii	v	v	v	v	v	v	v	v	v	v	v	v	v
Photobacterium phosphoreum	+	+	+	+	+	+	+	+	+	+	+	+	+
Proteus vulgaris	v	v	v	v	v	v	v	v	v	v	v	v	v
Pseudomonas aeruginosa	v	v	v	v	v	v	v	v	v	v	v	v	v
Pseudomonas cepacia	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas fluorescens	v	v	v	v	v	v	v	v	v	v	v	v	v
Pseudomonas mendocina	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas pseudomallei	-	-	-	-	-	-	-	-	-	-	-	-	-
Salmonella Typhimurium	-	-	-	-	-	-	-	-	-	-	-	-	-
Shewanella putrefaciens	-	-	-	-	-	-	-	-	-	-	-	-	-
Vibrio parahaemolyticus	+	+	+	+	+	+	+	+	+	+	+	+	+
Vibrio vulnificus	+	+	+	+	+	+	+	+	+	+	+	+	+

'+' means positive predation in all 3 replicate experiments, 'v' means variable predation between 3 replicate experiments, '-' means no predation observed in any of the 3 replicate experiments.

4.B Predation ability of BD isolates against some pathogenic and spoilage organisms in solid media at 20°C after 48 h of incubation.

Host bacteria	OT1	OT2	OT3	OT4	OT5	OT-enr	TB1	TB2	TB-enr	MCB	MNA	SP	MNZ1
Acinetobacter johnsonii	+	+	+	+	+	+	+	+	+	+	+	+	+
Aeromonas hydrophilia	+	+	+	+	+	+	+	+	+	+	+	+	+
Enterobacter cloacae	+	+	+	+	+	+	+	+	+	+	+	+	+
Escherichia coli B	-	-	-	-	-	-	-	-	-	-	-	-	-
Escherichia coli ML 35	-	-	-	-	-	-	-	-	-	-	-	-	-
Listeria monocytogenes	-	-	-	-	-	-	-	-	-	-	-	-	-
Morganella morganii	+	+	+	+	+	+	+	+	+	+	+	+	+
Photobacterium phosphoreum	+	+	+	+	+	+	+	+	+	+	+	+	+
Proteus vulgaris	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas aeruginosa	v	v	v	v	v	v	v	v	v	v	v	v	v
Pseudomonas cepacia	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas fluorescens	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas mendocina	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas pseudomallei	-	-	-	-	-	-	-	-	-	-	-	-	-
Salmonella Typhimurium	-	-	-	-	-	-	-	-	-	-	-	-	-
Shewanella putrefaciens	-	-	-	-	-	-	-	-	-	-	-	-	-
Vibrio parahaemolyticus	+	+	+	+	+	+	+	+	+	+	+	+	+
Vibrio vulnificus	+	+	+	+	+	+	+	+	+	+	+	+	+

'+' means positive predation in all 3 replicate experiments, 'v' means variable predation between 3 replicate experiments, '-' means no predation observed in any of the 3 replicate experiments.

4.C Predation ability of BD isolates in solid media at 20°C after 72 h of incubation.

Host bacteria	OT1	OT2	OT3	OT4	OT5	OT-enr	TB1	TB2	TB-enr	MCB	MNA	SP	MNZ1
Acinetobacter johnsonii	+	+	+	+	+	+	+	+	+	+	+	+	+
Aeromonas hydrophilia	+	+	+	+	+	+	+	+	+	+	+	+	+
Enterobacter cloacae	+	+	+	+	+	+	+	+	+	+	+	+	+
Escherichia coli B	-	-	-	-	-	-	-	-	-	-	-	-	-
Escherichia coli ML 35	-	-	-	-	-	-	-	-	-	-	-	-	-
Listeria monocytogenes	-	-	-	-	-	-	-	-	-	-	-	-	-
Morganella morganii	+	+	+	+	+	+	+	+	+	+	+	+	+
Photobacterium phosphoreum	+	+	+	+	+	+	+	+	+	+	+	+	+
Proteus vulgaris	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas aeruginosa	v	v	v	v	v	v	v	v	v	v	v	v	v
Pseudomonas cepacia	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas fluorescens	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas mendocina	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas pseudomallei	-	-	-	-	-	-	-	-	-	-	-	-	-
Salmonella Typhimurium	-	-	-	-	-	-	-	-	-	-	-	-	-
Shewanella putrefaciens	-	-	-	-	-	-	-	-	-	-	-	-	-
Vibrio parahaemolyticus	+	+	+	+	+	+	+	+	+	+	+	+	+
Vibrio vulnificus	+	+	+	+	+	+	+	+	+	+	+	+	+

'+' means positive predation in all 3 replicate experiments, 'v' means variable predation between 3 replicate experiments, '-' means no predation observed in any of the 3 replicate experiments.

4.D Predation ability of BD isolates in solid media at 25°C after 24 h of incubation.

Host bacteria	OT1	OT2	OT3	OT4	OT5	OT-enr	TB1	TB2	TB-enr	MCB	MNA	SP	MNZ1
Acinetobacter johnsonii	+	+	+	+	+	+	+	+	+	+	+	+	+
Aeromonas hydrophilia	+	+	+	+	+	+	+	+	+	+	+	+	+
Enterobacter cloacae	+	+	+	+	+	+	+	+	+	+	+	+	+
Escherichia coli B	-	-	-	-	-	-	-	-	-	-	-	-	-
Escherichia coli ML 35	-	-	-	-	-	-	-	-	-	-	-	-	-
Listeria monocytogenes	-	-	-	-	-	-	-	-	-	-	-	-	-
Morganella morganii	+	+	+	+	+	+	+	+	+	+	+	+	+
Photobacterium phosphoreum	+	+	+	+	+	+	+	+	+	+	+	+	+
Proteus vulgaris	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas aeruginosa	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas cepacia	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas fluorescens	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas mendocina	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas pseudomallei	-	-	-	-	-	-	-	-	-	-	-	-	-
Salmonella Typhimurium	-	-	-	-	-	-	-	-	-	-	-	-	-
Shewanella putrefaciens	-	-	-	-	-	-	-	-	-	-	-	-	-
Vibrio parahaemolyticus	+	+	+	+	+	+	+	+	+	+	+	+	+
Vibrio vulnificus	+	+	+	+	+	+	+	+	+	+	+	+	+

'+' means positive predation in all 3 replicate experiments, 'v' means variable predation between 3 replicate experiments, '-' means no predation observed in any of the 3 replicate experiments.

4.E Predation ability of BD isolates in solid media at 25°C after 48 h of incubation.

Host bacteria	OT1	OT2	OT3	OT4	OT5	OT-enr	TB1	TB2	TB-enr	MCB	MNA	SP	MNZ1
Acinetobacter johnsonii	+	+	+	+	+	+	+	+	+	+	+	+	+
Aeromonas hydrophilia	+	+	+	+	+	+	+	+	+	+	+	+	+
Enterobacter cloacae	+	+	+	+	+	+	+	+	+	+	+	+	+
Escherichia coli B	-	-	-	-	-	-	-	-	-	-	-	-	-
Escherichia coli ML 35	-	-	-	-	-	-	-	-	-	-	-	-	-
Listeria monocytogenes	-	-	-	-	-	-	-	-	-	-	-	-	-
Morganella morganii	+	+	+	+	+	+	+	+	+	+	+	+	+
Photobacterium phosphoreum	+	+	+	+	+	+	+	+	+	+	+	+	+
Proteus vulgaris	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas aeruginosa	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas cepacia	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas fluorescens	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas mendocina	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas pseudomallei	-	-	-	-	-	-	-	-	-	-	-	-	-
Salmonella Typhimurium	v	v	v	v	v	v	v	v	v	v	v	v	v
Shewanella putrefaciens	-	-	-	-	-	-	-	-	-	-	-	-	-
Vibrio parahaemolyticus	+	+	+	+	+	+	+	+	+	+	+	+	+
Vibrio vulnificus	+	+	+	+	+	+	+	+	+	+	+	+	+

'+' means positive predation in all 3 replicate experiments, 'v' means variable predation between 3 replicate experiments, '-' means no predation observed in any of the 3 replicate experiments.

4.F Predation ability of BD isolates in solid media at 25°C after 72 h of incubation.

Host bacteria	OT1	OT2	OT3	OT4	OT5	OT-enr	TB1	TB2	TB-enr	MCB	MNA	SP	MNZ1
Acinetobacter johnsonii	+	+	+	+	+	+	+	+	+	+	+	+	+
Aeromonas hydrophilia	+	+	+	+	+	+	+	+	+	+	+	+	+
Enterobacter cloacae	+	+	+	+	+	+	+	+	+	+	+	+	+
Escherichia coli B	v	v	v	v	v	v	v	v	v	v	v	v	v
Escherichia coli ML 35	v	v	v	v	v	v	v	v	v	v	v	v	v
Listeria monocytogenes	-	-	-	-	-	-	-	-	-	-	-	-	-
Morganella morganii	+	+	+	+	+	+	+	+	+	+	+	+	+
Photobacterium phosphoreum	+	+	+	+	+	+	+	+	+	+	+	+	+
Proteus vulgaris	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas aeruginosa	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas cepacia	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas fluorescens	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas mendocina	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas pseudomallei	-	-	-	-	-	-	-	-	-	-	-	-	-
Salmonella Typhimurium	-	-	-	-	-	-	-	-	-	-	-	-	-
Shewanella putrefaciens	-	-	-	-	-	-	-	-	-	-	-	-	-
Vibrio parahaemolyticus	+	+	+	+	+	+	+	+	+	+	+	+	+
Vibrio vulnificus	+	+	+	+	+	+	+	+	+	+	+	+	+

'+' means positive predation in all 3 replicate experiments, 'v' means variable predation between 3 replicate experiments, '-' means no predation observed in any of the 3 replicate experiments.

4.G Predation ability of BD isolates in solid media at 30°C after 24 h of incubation.

Host bacteria	OT1	OT2	OT3	OT4	OT5	OT-enr	TB1	TB2	TB-enr	MCB	MNA	SP	MNZ1
Acinetobacter johnsonii	+	+	+	+	+	+	+	+	+	+	+	+	+
Aeromonas hydrophilia	+	+	+	+	+	+	+	+	+	+	+	+	+
Enterobacter cloacae	+	+	+	+	+	+	+	+	+	+	+	+	+
Escherichia coli B	-	-	-	-	-	-	-	-	-	-	-	-	-
Escherichia coli ML 35	-	-	-	-	-	-	-	-	-	-	-	-	-
Listeria monocytogenes	-	-	-	-	-	-	-	-	-	-	-	-	-
Morganella morganii	+	+	+	+	+	+	+	+	+	+	+	+	+
Photobacterium phosphoreum	+	+	+	+	+	+	+	+	+	+	+	+	+
Proteus vulgaris	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas aeruginosa	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas cepacia	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas fluorescens	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas mendocina	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas pseudomallei	-	-	-	-	-	-	-	-	-	-	-	-	-
Salmonella Typhimurium	-	-	-	-	-	-	-	-	-	-	-	-	-
Shewanella putrefaciens	-	-	-	-	-	-	-	-	-	-	-	-	-
Vibrio parahaemolyticus	+	+	+	+	+	+	+	+	+	+	+	+	+
Vibrio vulnificus	+	+	+	+	+	+	+	+	+	+	+	+	+

'+' means positive predation in all 3 replicate experiments, 'v' means variable predation between 3 replicate experiments, '-' means no predation observed in any of the 3 replicate experiments.

4.H Predation ability of BD isolates in solid media at 30°C after 48 h of incubation.

Host bacteria	OT1	OT2	OT3	OT4	OT5	OT-enr	TB1	TB2	TB-enr	MCB	MNA	SP	MNZ1
Acinetobacter johnsonii	+	+	+	+	+	+	+	+	+	+	+	+	+
Aeromonas hydrophilia	+	+	+	+	+	+	+	+	+	+	+	+	+
Enterobacter cloacae	+	+	+	+	+	+	+	+	+	+	+	+	+
Escherichia coli B	-	-	-	-	-	-	-	-	-	-	-	-	-
Escherichia coli ML 35	-	-	-	-	-	-	-	-	-	-	-	-	-
Listeria monocytogenes	-	-	-	-	-	-	-	-	-	-	-	-	-
Morganella morganii	+	+	+	+	+	+	+	+	+	+	+	+	+
Photobacterium phosphoreum	+	+	+	+	+	+	+	+	+	+	+	+	+
Proteus vulgaris	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas aeruginosa	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas cepacia	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas fluorescens	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas mendocina	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas pseudomallei	-	-	-	-	-	-	-	-	-	-	-	-	-
Salmonella Typhimurium	v	v	v	v	v	v	v	v	v	v	v	v	v
Shewanella putrefaciens	-	-	-	-	-	-	-	-	-	-	-	-	-
Vibrio parahaemolyticus	+	+	+	+	+	+	+	+	+	+	+	+	+
Vibrio vulnificus	+	+	+	+	+	+	+	+	+	+	+	+	+

'+' means positive predation in all 3 replicate experiments, 'v' means variable predation between 3 replicate experiments, '-' means no predation observed any of the 3 replicate experiments.

4.1 Predation ability of BD isolates in solid media at 30°C after 72 h of incubation.

Host bacteria	OT1	OT2	OT3	OT4	OT5	OT-enr	TB1	TB2	TB-enr	MCB	MNA	SP	MNZ1
Acinetobacter johnsonii	+	+	+	+	+	+	+	+	+	+	+	+	+
Aeromonas hydrophilia	+	+	+	+	+	+	+	+	+	+	+	+	+
Enterobacter cloacae	+	+	+	+	+	+	+	+	+	+	+	+	+
Escherichia coli B	v	v	v	v	v	v	v	v	v	v	v	v	v
Escherichia coli ML 35	v	v	v	v	v	v	v	v	v	v	v	v	v
Listeria monocytogenes	-	-	-	-	-	-	-	-	-	-	-	-	-
Morganella morganii	+	+	+	+	+	+	+	+	+	+	+	+	+
Photobacterium phosphoreum	+	+	+	+	+	+	+	+	+	+	+	+	+
Proteus vulgaris	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas aeruginosa	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas cepacia	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas fluorescens	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas mendocina	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas pseudomallei	-	-	-	-	-	-	-	-	-	-	-	-	-
Salmonella Typhimurium	v	v	v	v	v	v	v	v	v	v	v	v	v
Shewanella putrefaciens	-	-	-	-	-	-	-	-	-	-	-	-	-
Vibrio parahaemolyticus	+	+	+	+	+	+	+	+	+	+	+	+	+
Vibrio vulnificus	+	+	+	+	+	+	+	+	+	+	+	+	+

'+' means positive predation in all 3 replicate experiments, 'v' means variable predation between 3 replicate experiments, '-' means no predation observed in any of the 3 replicate experiments.

4.J Predation ability of BD isolates in solid media at 37°C after 24 h of incubation.

Host bacteria	OT1	OT2	OT3	OT4	OT5	OT-enr	TB1	TB2	TB-enr	MCB	MNA	SP	MNZ1
Acinetobacter johnsonii	-	-	-	-	-	-	-	-	-	-	-	-	-
Aeromonas hydrophilia	v	v	v	v	v	v	v	v	v	v	v	v	v
Enterobacter cloacae	v	v	v	v	v	v	v	v	v	v	v	v	v
Escherichia coli B	-	-	-	-	-	-	-	-	-	-	-	-	-
Escherichia coli ML 35	-	-	-	-	-	-	-	-	-	-	-	-	-
Listeria monocytogenes	-	-	-	-	-	-	-	-	-	-	-	-	-
Morganella morganii	v	v	v	v	v	v	v	v	v	v	v	v	v
Photobacterium phosphoreum	v	v	v	v	v	v	v	v	v	v	v	v	v
Proteus vulgaris	v	v	v	v	v	v	v	v	v	v	v	v	v
Pseudomonas aeruginosa	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas cepacia	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas fluorescens	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas mendocina	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas pseudomallei	-	-	-	-	-	-	-	-	-	-	-	-	-
Salmonella Typhimurium	-	-	-	-	-	-	-	-	-	-	-	-	-
Shewanella putrefaciens	-	-	-	-	-	-	-	-	-	-	-	-	-
Vibrio parahaemolyticus	v	v	v	v	v	v	v	v	v	v	v	v	v
Vibrio vulnificus	-	-	-	-	-	-	-	-	-	-	-	-	-

'+' means positive predation in all 3 replicate experiments, 'v' means variable predation between 3 replicate experiments, '-' means no predation observed in any of the 3 replicate experiments.

4.K Predation ability of BD isolates in solid media at 37°C after 48 h of incubation.

Host bacteria	OT1	OT2	OT3	OT4	OT5	OT-enr	TB1	TB2	TB-enr	MCB	MNA	SP	MNZ1
Acinetobacter johnsonii	v	v	v	v	v	v	v	v	v	v	v	v	v
Aeromonas hydrophilia	v	v	v	v	v	v	v	v	v	v	v	v	v
Enterobacter cloacae	v	v	v	v	v	v	v	v	v	v	v	v	v
Escherichia coli B	v	v	v	v	v	v	v	v	v	v	v	v	v
Escherichia coli ML 35	v	v	v	v	v	v	v	v	v	v	v	v	v
Listeria monocytogenes	-	-	-	-	-	-	-	-	-	-	-	-	-
Morganella morganii	v	v	v	v	v	v	v	v	v	v	v	v	v
Photobacterium phosphoreum	+	+	+	+	+	+	+	+	+	+	+	+	+
Proteus vulgaris	v	v	v	v	v	v	v	v	v	v	v	v	v
Pseudomonas aeruginosa	v	v	v	v	v	v	v	v	v	v	v	v	v
Pseudomonas cepacia	v	v	v	v	v	v	v	v	v	v	v	v	v
Pseudomonas fluorescens	v	v	v	v	v	v	v	v	v	v	v	v	v
Pseudomonas mendocina	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas pseudomallei	-	-	-	-	-	-	-	-	-	-	-	-	-
Salmonella Typhimurium	v	v	v	v	v	v	v	v	v	v	v	v	v
Shewanella putrefaciens	-	-	-	-	-	-	-	-	-	-	-	-	-
Vibrio parahaemolyticus	v	v	v	v	v	v	v	v	v	v	v	v	v
Vibrio vulnificus	-	-	-	-	-	-	-	-	-	-	-	-	-

'+' means positive predation in all 3 replicate experiments, 'v' means variable predation between 3 replicate experiments, '-' means no predation observed in any of the 3 replicate experiments.

4.L Predation ability of BD isolates in solid media at 37°C after 72 h of incubation.

Host bacteria	OT1	OT2	OT3	OT4	OT5	OT-enr	TB1	TB2	TB-enr	MCB	MNA	SP	MNZ1
Acinetobacter johnsonii	v	v	v	v	v	v	v	v	v	v	v	v	v
Aeromonas hydrophilia	v	v	v	v	v	v	v	v	v	v	v	v	v
Enterobacter cloacae	v	v	v	v	v	v	v	v	v	v	v	v	v
Escherichia coli B	v	v	v	v	v	v	v	v	v	v	v	v	v
Escherichia coli ML 35	v	v	v	v	v	v	v	v	v	v	v	v	v
Listeria monocytogenes	-	-	-	-	-	-	-	-	-	-	-	-	-
Morganella morganii	v	v	v	v	v	v	v	v	v	v	v	v	v
Photobacterium phosphoreum	+	+	+	+	+	+	+	+	+	+	+	+	+
Proteus vulgaris	v	v	v	v	v	v	v	v	v	v	v	v	v
Pseudomonas aeruginosa	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas cepacia	v	v	v	v	v	v	v	v	v	v	v	v	v
Pseudomonas fluorescens	v	v	v	v	v	v	v	v	v	v	v	v	v
Pseudomonas mendocina	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas pseudomallei	-	-	-	-	-	-	-	-	-	-	-	-	-
Salmonella Typhimurium	v	v	v	v	v	v	v	v	v	v	v	v	v
Shewanella putrefaciens	-	-	-	-	-	-	-	-	-	-	-	-	-
Vibrio parahaemolyticus	v	v	v	v	v	v	v	v	v	v	v	v	v
Vibrio vulnificus	-	-	-	-	-	-	-	-	-	-	-	-	-

'+' means positive predation in all 3 replicate experiments, 'v' means variable predation between 3 replicate experiments, '-' means no predation observed in any of the 3 replicate experiments.

4.M1 The effectiveness of isolate OT2 (8.6 Log_{10} PFU/mL) in reducing numbers of different spoilage and pathogenic organisms during a challenge in 70% ASW for 24 h at 25°C. Values are presented as mean ± standard error, n= 3.

Organisms	Initial count	Mean log reductions of tested organisms		
	Log_{10} (CFU/mL ± SEM)[a]	log_{10} (CFU/mL ± SEM)[b]		
	0 h	6 h	12 h	24 h
Enterobacter cloacae	8.3 ± 0.09	-0.3 ±0.08	0.0 ± 0.06	-0.3 ± 0.07
E. cloacae + OT2	8.4 ± 0.06	4.0 ± 0.02	4.1 ± 0.02	4.2 ± 0.01
Morganella morganii	8.4 ± 0.08	-0.0 ±0.09	0.0 ± 0.09	0.0 ± 0.01
M. morganii + OT2	8.4 ± 0.06	2.0 ± 0.06	3.1 ± 0.05	3.8 ± 0.05
Pseudomonas aeruginosa	8.4 ± 0.08	0.1 ± 0.14	0.0 ± 0.15	0.0 ± 0.06
P. aeruginosa + OT2	8.3 ± 0.16	2.1 ± 0.02	3.2 ± 0.04	3.8 ± 0.02
Pseudomonas cepacia	8.4 ± 0.07	0.0 ± 0.05	0.0 ± 0.03	0.1 ± 0.05
P. cepacia + OT2	8.5 ± 0.06	2.5 ± 0.02	3.5 ± 0.03	4.2 ± 0.02
Pseudomonas fluorescens	8.3 ± 0.00	-0.0 ±0.02	0.0 ± 0.02	0.0 ± 0.03
P. fluorescens + OT2	8.3 ± 0.03	2.0 ± 0.02	3.1 ± 0.02	3.8 ± 0.01
Pseudomonas mendocina	8.3 ± 0.08	-0.1 ±0.11	0.1 ± 0.03	0.1 ± 0.16
P. mendocina + OT2	8.3 ± 0.09	0.1 ± 0.12	0.1 ± 0.04	0.1 ± 0.16
Photobacterium phosphoreum	8.5 ± 0.06	0.1 ± 0.09	0.0 ± 0.01	0.2 ± 0.14
P. phosphoreum + OT2	8.6 ± 0.09	3.9 ± 0.06	4.2 ± 0.08	4.3 ± 0.03
Pseudomonas pseudomallei	8.3 ± 0.17	0.1 ± 0.11	-0.1 ± 0.10	0.2 ± 0.13
P. pseudomallei + OT2	8.5 ± 0.08	0.1 ±0.11	0.2 ± 0.09	0.2 ± 0.14
Proteus vulgaris	8.5 ± 0.09	0.1 ± 0.06	0.0 ± 0.17	0.0 ± 0.09
P. vulgaris + OT2	8.4 ± 0.18	2.3 ± 0.05	3.4 ± 0.06	4.0 ± 0.04
Shewanella putrefaciens	8.2 ± 0.13	-0.1 ±0.06	-0.0 ± 0.11	0.0 ± 0.07
S. putrefaciens + OT2	8.3 ± 0.11	0.1 ± 0.04	0.1 ± 0.16	0.7 ± 0.06
Vibrio parahaemolyticus	8.3 ± 0.04	0.0 ± 0.08	0.9 ± 0.05	0.1 ± 0.11
V. parahaemolyticus + OT2	8.3 ± 0.07	4.0 ± 0.05	4.1 ± 0.06	4.2 ± 0.05
Vibrio vulnificus	8.3 ± 0.57	-0.3 ±0.02	0.0 ± 0.13	-0.3 ± 0.03
V. vulnificus + OT2	8.2 ± 0.02	4.0 ± 0.02	4.1 ± 0.02	4.2 ± 0.04

[a] *Mean population (n = 3) of tested cultures (log_{10} CFU/mL) at time 0 h*

[c] *Mean population (n = 3) of tested cultures (log_{10} CFU/mL) at 0 h of incubation minus mean population of the cultures at 6, 12 or 24 h of incubation.*

Appendix 5. In vitro study of BD against *Photobacterium phosphoreum*

5.A Survival of dense suspension of a BD culture (MNA) in 70% ASW without host bacteria stored at 4°C, 10°C, 20°C, 25°C, 30°C and 37°C for 13 days. Values presented as mean ● standard deviation, n = 3.

Mean (n = 3) of $\log_{10}$ PFU/mL ± STD of BD-MNA

Days of storage	4°C	10°C	20°C	25°C	30°C	37°C
0	10.3 ± 0.07	10.3 ± 0.07	10.3 ±	10.3 ± 0.07	10.3 ± 0.07	10.3 ± 0.07
1	10.2 ± 0.09	10.0 ± 0.13	9.7 ± 0.17	9.7 ± 0.14	9.6 ± 0.09	8.2 ± 0.08
2	10.2 ± 0.13	10.1 ± 0.10	9.68 ± 0.16	9.6 ± 0.13	7.8 ± 0.09	5.6 ± 0.09
3	10.2 ± 0.10	10.1 ± 0.14	9.7 ± 0.11	9.6 ± 0.09	7.6 ± 0.09	3.4 ± 0.14
4	10.2 ± 0.09	10.3 ± 0.12	9.5 ± 0.14	9.5 ± 0.10	7.3 ± 0.09	3.9 ± 0.11
5	9.9 ± 0.11	10.0 ± 0.14	9.5 ± 0.09	9.4 ± 0.11	7.1 ± 0.13	3.9 ± 0.14
6	9.8 ± 0.10	9.9 ± 0.12	9.5 ± 0.06	9.3 ± 0.10	6.9 ± 0.10	3.8 ± 0.06
7	9.7 ± 0.09	9.9 ± 0.10	9.3 ± 0.16	9.3 ± 0.14	6.7 ± 0.14	3.7 ± 0.09
8	9.6 ± 0.06	9.9 ± 0.09	9.3 ± 0.12	9.0 ± 0.11	5.5 ± 0.11	3.6 ± 0.12
9	9.5 ± 0.16	9.8 ± 0.14	9.2 ± 0.10	8.8 ± 0.09	5.5 ± 0.09	3.3 ± 0.11
10	9.4 ± 0.12	9.7 ± 0.15	9.1 ± 0.14	8.3 ± 0.09	5.1 ± 0.11	< 2.5
11	9.2 ± 0.06	9.7 ± 0.09	9.0 ± 0.12	8.0 ± 0.14	5.0 ± 0.10	< 2.5
12	9.1 ± 0.14	9.5 ± 0.12	8.9 ± 0.09	7.8 ± 0.15	4.8 ± 0.09	< 2.5
13	9.0 ± 0.07	9.4 ± 0.11	8.9 ± 0.10	7.4 ± 0.10	4.4 ± 0.13	< 2.5

5.B Survival of dense suspension of a BD culture (MCB) in 70% ASW without host bacteria stored at -18°C, 0°C, 4°C, 10°C, 20°C, 25°C, 30°C and 37°C for 16 days. Values presented as mean ± standard deviation, n = 3.

	Mean (n = 3) of $\log_{10}$ PFU/mL ± STD of BD-MCB							
Storage days	-18°C	0°C	4°C	10°C	20°C	25°C	30°C	37°C
0	10.4 ± 0.11	10.4 ± 0.11	10.4 ± 0.11	10.4 ± 0.11	10.4 ± 0.11	10.4 ± 0.11	10.4 ± 0.11	10.4 ± 0.11
1	9.5 ± 0.09	10.3 ± 0.10	10.3 ± 0.12	10.3 ± 0.13	10.3 ± 0.11	10.3 ± 0.09	10.1 ± 0.10	7.8 ± 0.14
2	8.9 ± 0.10	10.2 ± 0.13	10.3 ± 0.10	10.3 ± 0.16	10.3 ± 0.14	10.2 ± 0.13	9.9 ± 0.14	7.8 ± 0.12
3	7.2 ± 0.11	9.7 ± 0.09	10.2 ± 0.14	10.2 ± 0.10	10.3 ± 0.10	10.2 ± 0.13	9.6 ± 0.15	6.9 ± 0.11
4	6.9 ± 0.10	9.7 ± 0.16	10.3 ± 0.11	10.2 ± 0.11	10.2 ± 0.11	9.9 ± 0.13	9.6 ± 0.15	5.4 ± 0.13
5	6.4 ± 0.09	9.7 ± 0.14	9.9 ± 0.09	9.9 ± 0.14	10.1 ± 0.09	9.7 ± 0.09	8.1 ±0.10	6.1 ± 0.13
6	6.0 ± 0.11	9.7 ± 0.09	9.9 ± 0.13	9.9 ± 0.11	10.0 ± 0.13	9.5 ± 0.09	7.7 ± 0.11	5.5 ± 0.10
7	5.1 ± 0.11	9.7 ± 0.09	9.9 ± 0.14	9.9 ± 0.10	9.9 ± 0.11	9.3 ± 0.13	7.0 ± 0.09	5.4 ± 0.13
8	4.5 ± 0.09	9.6 ± 0.10	9.8 ± 0.09	9.9 ± 0.14	9.9 ± 0.09	9.3 ± 0.15	6.9 ± 0.09	4.4 ± 0.11
9	3.6 ± 0.14	9.5 ± 0.13	9.9 ± 0.11	9.9 ± 0.10	9.9 ± 0.14	9.2 ± 0.10	6.9 ± 0.13	3.3 ± 0.13
10	3.9 ± 0.13	9.3 ± 0.13	9.8 ± 0.10	9.9 ± 0.09	9.9 ± 0.13	9.1 ± 0.11	6.9 ± 0.13	< 2.52
11	< 2.52	9.2 ± 0.09	9.8 ± 0.13	9.8 ± 0.14	9.8 ± 0.10	8.9 ± 0.16	6.8 ± 0.11	< 2.52
12	< 2.52	9.0 ± 0.12	9.8 ± 0.10	9.8 ± 0.12	9.8 ± 0.13	8.6 ± 0.09	6.8 ± 0.13	< 2.52
13	< 2.52	8.8 ± 0.13	9.8 ± 0.14	9.8 ± 0.11	9.7 ± 0.10	8.4 ± 0.10	6.7 ± 0.09	< 2.52
14	< 2.52	8.7 ± 0.10	9.7 ± 0.10	9.8 ± 0.16	9.7 ± 0.16	7.9 ± 0.14	6.4 ± 0.14	< 2.52
15	< 2.52	8.3 ± 0.10	9.6 ± 0.13	9.7 ± 0.11	9.5 ± 0.14	7.6 ± 0.10	6.1 ± 0.12	< 2.52
16	< 2.52	8.2 ± 0.12	9.6 ± 0.10	9.8 ± 0.12	9.4 ± 0.10	7.3 ± 0.15	5.6 ± 0.11	< 2.52

5.C The effectiveness of different isolates of BD in reducing numbers of *P. phosphoreum* (8.0 log_{10} CFU/mL) in 70% ASW after 10 and 24 h at 25°C. Values presented as mean ± standard deviation, n = 3.

	Initial count (log_{10} CFU/mL ± STD)[a]	Mean reductions of *P. phosphoreum* (log_{10} CFU/mL ± STD)[b]	
BD isolates	0 h	10 h	24 h
OT1 (7.2)	7.7 ± 0.097	4.1 ± 0.15	4.3 ± 0.12
OT2 (7.3)	7.8 ± 0.13	4.4 ± 0.13	4.55 ± 0.13
OT3 (7.2)	7.7 ± 0.14	3.4 ± 0.10	4.3 ± 0.10
OT4 (7.2)	7.7 ± 0.09	3.6 ± 0.12	4.4 ± 0.10
OT5 (7.3)	7.9 ± 0.11	4.0 ± 0.05	4.5 ± 0.11
OTenr (7.3)	7.6 ± 0.12	3.3 ± 0.14	4.2 ± 0.05
TB1 (7.2)	7.8 ± 0.11	3.5 ± 0.09	4.5 ± 0.16
TB2 (7.1)	7.8 ± 0.10	3.5 ± 0.11	4.3 ± 0.13
TBenr (7.3)	7.0 ± 0.10	2.6 ± 0.11	3.7 ± 0.09
MNA (7.3)	7.7 ± 0.12	3.8 ± 0.13	4.2 ± 0.14
MCB (7.2)	7.7 ± 0.13	3.5 ± 0.09	4.2 ± 0.15
SP (7.2)	7.7 ± 0.15	3.2 ± 0.12	4.2 ± 0.13
MNZ1 (7.1)	7.7 ± 0.10	1.8 ± 0.11	4.1 ± 0.09
Control (no BD)	7.7 ± 0.13	-0.0 ± 0.10	-0.1 ± 0.14

[a] *Counting of the mean (n = 3) log_{10} CFU/mL of* P. phosphoreum *in SWYE plate at 0 h of incubation*

[b] *Mean population (n = 3) log_{10} CFU/mL of* P. phosphoreum *at time 0 h of incubation minus mean population (n = 3) of* P. phosphoreum *at 10 or 24 h of incubation.*

5.D Effect of salinity on the population of *P. phosphoreum* after challenging BD isolate OT2 (7.2 log_{10} PFU/mL) against *P. phosphoreum* (8.4 log_{10} CFU/mL) in 70% ASW after 10, 24 and 48 h of incubation at 25°C. Values presented as mean ± standard deviation, n = 3.

	Initial count[b]	Mean log reductions of *P. phosphoreum* [c]		
Salinity %[a]	0 h	10 h	24 h	48 h
0.0 (treatment)	8.5 ± 0.08	0.0 ± 0.03	0.1 ± 0.02	0.0 ± 0.05
0.0 (control)	8.5 ± 0.03	0.1 ± 0.01	0.1 ± 0.08	-0.3 ± 0.01
0.5 (treatment)	8.4 ± 0.03	0.0 ± 0.03	0.1 ± 0.02	0.0 ± 0.02
0.5 (control)	8.5 ± 0.03	0.1 ± 0.01	0.1 ± 0.01	-0.4 ± 0.02
0.9 (treatment)	8.4 ± 0.03	0.1 ± 0.03	1.5 ± 0.03	2.3 ± 0.03
0.9 (control)	8.5 ± 0.02	-0.1± 0.04	-0.1 ± 0.05	-0.1 ± 0.01
1.5 (treatment)	8.4 ± 0.10	2.6 ± 0.04	4.1 ± 0.06	2.8 ± 0.02
1.5 (control)	8.6 ± 0.02	0.2 ± 0.10	0.3 ± 0.06	-0.1 ± 0.04
2.1 (treatment)	8.5 ± 0.10	0.9 ± 0.12	3.8 ± 0.03	2.7 ± 0.05
2.1 (control)	8.5 ± 0.03	0.2 ± 0.05	-0.0 ± 0.02	-0.7 ± 0.03
3.0 (treatment)	8.5 ± 0.05	0.6 ± 0.02	3.7 ± 0.02	2.5 ± 0.02
3.0 (control)	8.5 ± 0.04	0.2 ± 0.06	0.0 ± 0.14	-0.4 ± 0.01
4.5 (treatment)	8.5 ± 0.07	0.2 ± 0.04	0.9 ± 0.02	0.1 ± 0.02
4.5 (control)	8.5 ± 0.07	0.2 ± 0.07	0.1 ± 0.01	0.2 ± 0.02

[a] *Challenge inocula of 1 mL* P. phosphoreum *(8.4 log_{10} CFU/mL) and 1-mL of OT2 (7.2 log_{10} PFU/mL). Controls contained no BD.*

[b] *Mean population (n = 3) of* P. phosphoreum *(log_{10} CFU/mL ± STD) at time 0 h*

[c] *Mean population (n = 3) of* P. phosphoreum *at 0 h of incubation minus mean population of* P. phosphoreum *at 10, 24 or 48 h of incubation (log_{10} CFU/mL ± STD).*

5.E Effect of pH on the population of *P. phosphoreum* after challenging BD isolate OT2 (7.3 log_{10} PFU/mL) against *P. phosphoreum* (8.4 log_{10} CFU/mL) in 70% ASW after 24 and 48 h of incubation at 25°C. Values are presented as mean ± standard deviation, n = 3.

	Initial count [b]	Mean reductions of *P. phosphoreum* [c]	
pH [a]	0 h	24 h	48 h
4.0 (treatment)	8.7 ± 0.10	>6.7	>6.7[d]
4.0 (control)	8.6 ± 0.10	>6.7[d]	>6.7[d]
4.6 (treatment)	8.7 ± 0.08	>6.7[d]	>6.7[d]
4.6 (control)	8.8 ± 0.13	>6.7[d]	>6.7[d]
5.0 (treatment)	8.8 ± 0.10	0.3 ± 0.13	0.4 ± 0.08
5.0 (control)	8.8 ± 0.14	-0.1 ± 0.07	-1.1 ± 0.09
5.6 (treatment)	8.8 ± 0.08	1.4 ± 0.11	1.4 ± 0.14
5.6 (control)	8.7 ± 0.14	-1.0 ± 0.13	-1.1 ± 0.13
6.0 (treatment)	8.8 ± 0.09	3.1 ± 0.10	1.9 ± 0.10
6.0 (control)	8.7 ± 0.10	-1.1 ± 0.12	-1.1 ± 0.10
6.6 (treatment)	8.8 ± 0.15	3.3 ± 0.10	2.2 ± 0.11
6.6 (control)	8.7 ± 0.09	-0.6 ± 0.01	-1.1 ± 0.09
8.2 (treatment)	8.7 ± 0.11	4.3 ± 0.08	2.8 ± 0.09
8.2 (control)	8.6 ± 0.10	-1.1 ± 0.10	-0.9 ± 0.12

[a] *pH buffer 0.1 M Citic acid – 0.2 M Na_2HPO_4 (pH 4.0 to 8.2); challenge inocula of 1 mL* P. phosphoreum *(8.5 log_{10} CFU/mL) and 1-mL of OT2 (7.3 log_{10} PFU/mL). Controls contained no BD.*

[b] *Mean population (n = 3) of* P. phosphoreum *(log_{10} CFU/mL ± STD) at time 0 h*

[c] *Mean population(n = 3) of* P. phosphoreum *at 0 h of incubation minus mean population (n = 3) of* P. phosphoreum *at 24 or 48 h of incubation (log_{10} CFU/mL ± STD)*

[d] *no detectable growth at 10^2 dilution.*

5.F Time course of different doses of BD isolate OT2 (7.8, 4.8 and 3.5 log_{10} PFU/mL) in 70% ASW for 24 h of incubation at 25°C. Values are presented as mean ± standard error, n = 3.

Initial count [a]		**Mean reductions of *P. phosphoreum*** [b]								
BD inoculum[a]	0 h	2 h	3h	4h	5h	6h	8h	9 h	10 h	24 h
7.8 log_{10} PFU/mL of OT2	8.9 ± 0.11	0.0 ± 0.10	-0.1 ± 0.13	0.0 ± 0.10	0.3± 0.09	0.4 ± 0.11	2.2 ± 0.13	4.0 ± 0.13	4.4 ± 0.08	4.8 ± 0.09
4.8 log_{10} PFU/mL of OT2	9.0 ± 0.09	0.0 ± 0.13	0.1 ± 0.08	0.0 ± 0.11	0.1 ± 0.10	0.0 ± 0.09	0.3 ± 0.12	0.5 ± 0.09	1.0 ± 0.10	4.5 ± 0.13
3.5 log_{10} PFU/mL of OT2	8.9± 0.14	0.0 ± 0.09	0.1 ± 0.13	-0.1 ± 0.10	-0.1 ± 0.10	0.0 ± 0.09	0.1 ± 0.09	0.4 ± 0.11	0.6 ± 0.11	4.6 ± 0.11
Control	9.0 ± 0.15	0.1 ± 0.10	0.1 ± 0.14	0.0 ± 0.09	0.1 ± 0.08	0.1 ± 0.11	0.1 ± 0.10	0.0 ± 0.13	0.1 ± 0.10	0.1 ± 0.08

[a] *Mean population (n = 3) of* P. phosphoreum *(log_{10} CFU/mL ± SEM) at time 0 h of incubation*

[b] Mean population (n = 3) of *P. phosphoreum* at time 0 h of incubation minus the mean population at 2, 3, 4, 5, 6, 8, 9, 10 or 24 h of incubation (log_{10} CFU/mL ± SEM).

5.G Effect of different doses of BD isolate OT2 (7.8, 4.8 and 3.5 log_{10} PFU/mL) on its growth in the presence of *P. phosphoreum* (8.6 log_{10} CFU/mL) in 70% ASW for 24 h of incubation at 25°C. Values are presented as mean ± standard error, n = 3.

Initial count [a]	Mean log increase of *Bdellovibrionaceae* [b]								
BD inoculum	0 h	2 h	3 h	4 h	5 h	6 h	8 h	9 h	10 h	24 h
7.8 log_{10} PFU/mL of OT2	7.4 ± 0.10	-0.1 ± 0.08	-0.0 ± 0.10	-0.0 ± 0.08	0.1 ± 0.08	0.3 ± 0.11	0.4 ± 0.08	1.6 ± 0.11	1.9 ± 0.11	2.2 ± 0.11
4.8 log_{10} PFU/mL of OT2	4.5 ± 0.08	-0.0 ± 0.08	-0.0 ± 0.11	-0.0 ± 0.10	-0.0 ± 0.13	0.0 ± 0.09	0.3 ± 0.11	1.8 ± 0.07	1.8 ± 0.08	4.8 ± 0.09
3.5 log_{10} PFU/mL of OT2	3.3 ± 0.11	-0.0 ± 0.11	-0.1 ± 0.09	-0.0 ± 0.08	0.0 ± 0.08	0.0 ± 0.10	0.0 ± 0.13	0.1 ± 0.10	0.6 ± 0.13	6.2 ± 0.13

[a] *Mean population (n = 3) of* Bdellovibrionaceae *(log_{10} PFU/mL ± SEM) at time 0 h of incubation*

[b] Mean population (n = 3) of *Bdellovibrionaceae* at time 0 h of incubation minus mean population at 2, 3, 4, 5, 6, 8, 9, 10 or 24 h of incubation (log_{10} PFU/mL ± SEM).

5.H Effect of different doses of BD isolate OT2 (7.2, 3.9 and 2.1 log_{10} PFU/mL) against a high concentration of *P. phosphoreum* (8.1 log_{10} CFU/mL) in diluted SWYE (16% SWYE broth) at 25°C. Values are presented as mean ± standard deviation, n = 3.

BD inocula (log_{10} PFU/mL)	Prey: predator ratio	Initial count (log_{10} CFU/mL)[a]	Mean log reductions (log_{10} CFU/mL)[b]		
		0 h	8 h	16 h	24 h
7.23	7.0	7.7 ± 0.19	-0.8 ± 0.06	-0.4 ± 0.13	-0.7 ± 0.14
3.91	1.4×10^4	7.8 ± 0.24	-0.9 ± 0.23	-0.8 ± 0.18	-1.7 ± 0.13
2.11	9×10^5	7.8 ± 0.16	- 0.9 ± 0.17	-1.0 ± 0.10	-1.9 ± 0.18
Nil	Control	7.8 ●0.20	- 1.1 ± 0.23	- 1.6 ± 0.14	-2.1 ± 0.09

[a] *Results are presented as mean ± standard deviation, n = 3*
[b] *Mean population of* P. phosphoreum *(log_{10} CFU/mL) at time 0 h of incubation minus mean population at time 8, 16 or 24 h of incubation.*

5.I Effect of different doses of BD isolate OT2 (7.2, 3.9 and 2.1 log_{10} PFU/mL) against a high concentration of *P. phosphoreum* (8.1 log_{10} CFU/mL) in 70% ASW at 25°C. Values are presented as mean ± standard deviation, n = 3.

BD inocula (log_{10} PFU/mL)	Prey: predator ratio	Initial count (log_{10} CFU/mL)[a]	Mean log reductions (log_{10} CFU/mL)[b]	
		0 h	16 h	24 h
7.23	7.0	7.7 ± 0.13	3.7 ± 0.10	4.9 ± 0.12
3.91	1.4×10^4	7.8 ± 0.12	0.2 ± 0.08	3.8 ± 0.22
2.11	9×10^5	7.8 ± 0.09	-0.1 ± 0.15	0.1 ± 0.21
Nil	Control	7.7 ± 0.13	0.0 ± 0.22	-0.0 ± 0.18

[a] *Results are presented as mean ± standard deviation, n = 3*
[b] *Mean population (n = 3) of* P. phosphoreum *(log_{10} CFU/mL) at time 0 h of incubation minus mean population (n = 3) at time 16 or 24 h of incubation.*

5.J Effect of different doses of BD isolate OT2 (7.2, 3.9 and 2.1 log_{10} PFU/mL) against a concentration of *P. phosphoreum* (3.7 log_{10} CFU/mL) in diluted SWYE (16% SWYE broth) at 25°C. Values are presented as mean ± standard deviation, n = 3.

BD inocula (log_{10} PFU/mL)	Prey: predator ratio	Initial count (log_{10} CFU/mL) [a]	Mean log reductions (log_{10} CFU/mL) [b]		
		0 h	8 h	16 h	24 h
7.23	2.7×10^{-3}	3.5 ± 0.24	-0.4 ± 0.06	-0.4 ± 0.20	-2.7 ± 0.14
3.91	5.70	3.5 ± 0.15	-0.9 ± 0.23	-3.9 ± 0.20	-5.9 ± 0.19
2.11	3.6×10^{2}	3.5 ± 0.24	-0.9 ± 0.14	-3.7 ± 0.21	-6.0 ± 0.27
Nil	Control	3.5 ± 0.34	-1.2 ± 0.24	-4.1 ± 0.11	-6.1 ± 0.24

[a] *Results are presented as mean ± standard deviation, n = 3*
[b] *Mean population (n = 3) of* P. phosphoreum *(log_{10} CFU/mL) at time 0 h of incubation minus mean population (n = 3) at time 8, 16 or 24 h of incubation.*

5.K Effect of different doses of BD isolate OT2 (7.2, 3.9 and 2.1 log_{10} PFU/mL) against a concentration of *P. phosphoreum* (3.7 log_{10} CFU/mL) in 70% ASW at 25°C. Values are presented as mean ± standard deviation, n = 3.

BD inoculums (log_{10} PFU/mL)	Prey: predator ratio	Initial count (log_{10} CFU/mL) [a]	Mean log reductions (log_{10} CFU/mL) [b]	
		0 h	16 h	24 h
7.23	2.7×10^{-3}	3.5 ± 0.22	0.6 ± 0.15	0.9 ± 0.12
3.91	5.7	3.5 ● 0.12	0.3 ± 0.13	-0.4± 0.15
2.11	3.6×10^{2}	3.5 ± 0.21	-0.7 ± 0.08	-2.4 ± 0.11
Nil	Control	3.6 ± 0.17	-0.4 ± 0.21	-2.3 ± 0.13

[a] *Results are presented as mean ± standard deviation, n = 3*
[b] *Mean population (n = 3) of* P. phosphoreum *(log_{10} CFU/mL) at time 0 h of incubation minus mean population (n = 3) at time 16 or 24 h of incubation.*

5.L Effect of different doses of BD isolate OT2 (7.2, 3.9 and 2.1 log_{10} PFU/mL) against a low concentration of *P. phosphoreum* (2.8 log_{10} CFU/mL) in diluted SWYE (16% SWYE broth) at 25°C. Values are presented as mean ± standard deviation, n = 3.

BD inocula (log_{10} PFU/mL)	Prey: predator ratio	Initial count (log_{10} CFU/mL)[a]	Mean log reductions (log_{10} CFU/mL)[b]		
		0 h	8 h	16 h	24 h
7.23	9.5×10^{-3}	2.6 ± 0.27	-0.8± 0.22	-0.5 ± 0.15	-2.5 ± 0.19
3.91	0.19	2.5 ± 0.17	-2.0 ± 0.23	-4.3 ± 0.14	-6.5 ± 0.19
2.11	12	2.6 ± 0.19	-1.1 ± 0.13	-3.5 ± 0.24	-6.1 ± 0.30
Nil	Control	2.7 ± 0.13	-0.6 ± 0.18	-2.6 ± 0.14	-7.5 ± 0.32

[a] *Results are presented as mean ± standard deviation, n = 3*
[b] *Mean population (n = 3) of* P. phosphoreum *(log_{10} CFU/mL) at time 0 h of incubation minus mean population (n = 3) at time 8, 16 or 24 h of incubation.*

5.M Effect of different doses of BD isolate OT2 (7.2, 3.9 and 2.1 log_{10} PFU/mL) against a low concentration of *P. phosphoreum* (2.8 log_{10} CFU/mL) in 70% ASW at 25°C. Values are presented as mean ± standard deviation, n = 3.

BD inocula[a] (log_{10} PFU/mL)	Prey: predator ratio	Initial count (log_{10} CFU/mL)[a]	Mean log reductions (log_{10} CFU/mL)[a]	
		0 h	16 h	24 h
7.23	9.5×10^{-3}	2.5 ± 0.15	-0.5 ± 0.20	-1.5 ± 0.18
3.91	0.19	2.5 ± 0.24	-0.5 ± 0.15	-1.6 ± 0.12
2.11	12	2.7 ± 0.15	-0.4 ± 0.11	-1.6 ± 0.11
Nil	Control	2.7 ± 0.16	-0.2 ± 0.11	-1.7 ± 0.13

[a] *Results are presented as mean ± standard deviation, n = 3*
[b] *Mean population of* P. phosphoreum *(log_{10} CFU/mL) at time 0 h of incubation minus mean population (n = 3) at time 16 or 24 h of incubation.*

5.N Effect of predator levels of 8.3, 8.1, 7.7, 7.4, 3.4 and 2.4 log_{10} PFU/mL on reducing high numbers of *P. phosphoreum* (8.3 log_{10} CFU/mL) in 70% ASW. Samples were incubated at 25°C for 24 h and tested on 0, 2, 4, 6, 8, 10, 12 and 24 h. Values are shown as means with the standard error bars.

	Initial count (CFU/mL ± SEM)[b]	Mean log reductions of *P. phosphoreum* (CFU/mL ± SEM)[c]						
Prey : predator ratio[a]	0 h	2 h	4 h	6 h	8 h	10 h	12 h	24 h
1.3 : 1	8.1 ± 0.05	2.1 ± 0.09	4.3 ± 0.14	4.2 ± 0.04	3.1 ± 0.06	3.0 ± 0.03	3.0 ± 0.03	2.2 ± 0.02
2.3 : 1	8.1 ± 0.02	0.1 ± 0.05	4.2 ± 0.07	4.5 ± 0.05	4.6 ± 0.06	4.6 ± 0.11	4.6 ± 0.13	2.2 ± 0.10
5.4 : 1	8.1 ± 0.05	0.1 ± 0.03	0.2 ± 0.02	3.5 ± 0.12	4.4 ± 0.09	4.5 ± 0.08	4.4 ± 0.35	4.5 ± 0.12
10.4 : 1	8.1 ± 0.03	0.1 ± 0.03	0.2 ± 0.04	3.2 ± 0.07	3.4 ± 0.13	4.4 ± 0.08	4.5 ± 0.13	4.5 ± 0.12
10^5 : 1	8.1 ± 0.03	0.0 ± 0.02	0.2 ± 0.04	0.1 ± 0.02	0.0 ± 0.04	0.1 ± 0.04	0.1 ± 0.03	4.0 ± 0.36
10^6 : 1	8.1 ± 0.01	0.0 ± 0.03	0.0 ± 0.03	0.0 ± 0.02	0.1 ± 0.02	0.1 ± 0.02	0.1 ± 0.04	0.1 ± 0.11
Control (no BD added)	8.1 ± 0.02	-0.0 ± 0.05	-0.0 ± 0.03	0.0 ± 0.07	0.0 ± 0.03	-0.0 ± .02	0.0 ± .03	-0.0 ± .04

[a] *Prey: predator ratio prepared as specific amount of BD isolate OT2 (8.32 log_{10} PFU/mL) and P. phosphoreum (8.43 log_{10} CFU/mL) in 50-mL of 70% ASW*

[c] *Mean population (n = 3) of* P. phosphoreum *(log_{10} CFU/mL) at time 0 h of incubation minus the mean population (n = 3) at 2, 4, 6, 8, 10, 12 or 24 h of incubation.*