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Virus- like- particles of *Pseudoalteromonas marina* and their possible medical significance

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Melanie Kappelmann

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	Ao. Prof. Dr. Branko Velimirov

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1 Introduction

1.1 Significance of *Virus- like- particles* produced by some gram- negative bacteria

Virus-like-particles spontaneously produced by some bacteria and released by budding were detected more than ten years ago (Chiura, 1997). Extensive review of literature revealed that Virus- like- particles (VLPs) resemble structures which are also known as membrane vesicles (MVs), Outer Membrane vesicles (OMVs) or Membrane blebs. All this denominations include extracellular vesicles produced by mainly gram- negative bacteria. MVs are proposed to be a result of cell wall turnover and the vesicle formation depends on disturbances in growth or in outer membrane integrity (Zhou et al. 1998). In case of several gram- negative bacteria the maximum rate of vesicle production occurs during the end of log phase growth. The reduction of the number of cross-links between the peptidoglycan (PG) and the outer membrane (OM) may adjust the release of OM vesicles (Kuehn and Kesty, 2005; Figure 1). It was demonstrated that those extracellular membrane vesicles are associated with DNA export. Additionally plasmids and RNA were found associated with blebs (Dorward et al. 1989; Dorward and Garon, 1990). Further investigations of MVs showed that they are produced into culture medium during normal growth of *Pseudomonas aeroginosa* as spherical bilayers with a diameter of 50- 150 nm (Renelli et al. 2004). The MVs contain lipopolysacchrides, several enzymes and DNA and could consequently play an important role as a transporter for DNA and virulence factors within genetic transfer and disease in case of determined septic shock per MVs. Most important enzymes found in MVs involved in the pathogenicity of *Pseudomonas* are phopholipase C, protease, hemolysin and alkaline phosphatase. Alkaline phosphatase is produced by bacteria in times of phosphate starvation for uptake and use (Kadurugamuwa and Beveridge, 1995). It is also well established that MVs can fuse with and anneal into the outer membranes of other gram-negative bacteria and posses bacteriolytic activity due to the autolysins (peptidoglycan hydrolases) within the MVs (Kadurugamuwa and Beveridge, 1996). Some gram- negative bacteria apply vesicle production as an offensive tactic to get a growth advantage over other bacteria; vesicles could serve for interaction with bacteria resulting in lytic processes of

other gram- negative or gram- positive bacteria. So those vesicles are able to secure a niche in a competitive bacterial environment. Moreover, the MVs transfer valuable material between bacteria and contribute to genetic diversity and bacterial survival (Kuehn and Kesty, 2005). In general the genetic flexibility of prokaryotes arises from the natural capability for inducing horizontal gene transfer among bacteria and between bacteria and other organisms (Lengler et al. 1999, Madigan et al. 2000). Studies of MVs from diverse bacterial strains suggest that their function encompasses the delivery of toxic substances to host cells, the transfer of proteins, cell- cell communication, elimination of other bacteria and gene transfer (Lee et al. 2008). Based on the knowledge that DNA is packaged into MVs of *Escherichia, Neisseria* and *Pseudomonas* species (Dorward et al. 1989; Kadurugamuwa and Beveridge, 1995; Kolling and Matthews, 1999) as well as of other gram- negative bacteria it is

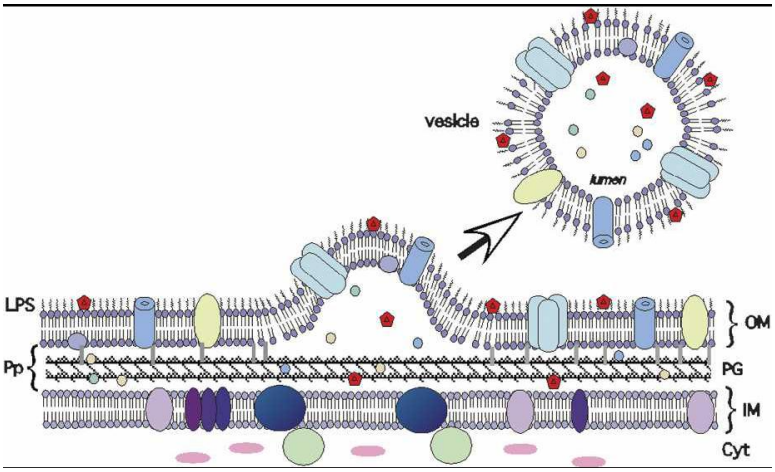


Figure 1: Model of vesicle biogenesis. Outer membrane (OM) vesicles consist of OM phospholipids and Lipopolysaccharides (LPS); Proteins and lipids of the IM and cytosolic content are excluded from OM vesicles; vesicles generate demaged regions of the outer membrane; (LPS) Lipopolysaccharide; (Pp) periplasm; (OM) outer membrane; (PG) peptidoglycan; (IM) inner membrane; (Cyt) cytosol. (Kuehn and Kesty, 2005)

suggested that not only plasmids are packaged but also chromosomal DNA and even bacteriophage DNA. All this consolidated findings resulted in an increasing interest in the genetic diversity of the MVs even though the mechanism behind the DNA packaging is still poorly understood (Renelli et al. 2004). Further investigations showed that vesicles carry luminal DNA and surface DNA, which is localized to the exterior surface of the vesicles and binds in some instances leucotoxin. Moreover they are capable of transforming neighboring bacteria (Kuehn and Kesty, 2005). A study by Maria Nevot et al. (2006) has shown that bacteria of the order *Pseudoalteromonas* also produce OMVs with high protein content. The mechanism how the proteins get into the OMVs and how they are released in the environment is still unclear but analysis of the LPS, phospholipids and proteins from vesicles showed that these structures are derived from bacterial outer membrane.

Gene transfer can be understood as evolutionary machinery for genetic diversity of bacteria and includes 5 mechanisms of genetic exchange (Wilharm, 2008).

- Transformation (uptake of free DNA of the environment)
- Conjugation (DNA transfer from a bacterial donor to a bacterial recipient cell)
- Transduction (phage mediated passage of bacterial genes)
- DNA- transfer via MVs
- DNA-transfer by gene transfer agents (described as bacteriophage- like vehicles)

Marrs (1974) first discovered the phenomenon of a bacteriophage-like vesicle in *Rhodopseudomonas capsulata*. He suggested that these vesicles induce horizontal gene transfer although he could not detect them by electron microscopy. Solioz and Marrs (1977) first introduced the term GTA- particles and found out that those particles contain a random 4.5 kb fragment of bacterial DNA that can be transferred between cells. These GTA particles are not inducible with mitomycin C and do not form plaques although they have a phage-like morphology (Solioz et al. 1975; Biers et al. 2008).

Chiura (1997) detected in the course of his exploration of *Ahrensia kieliense* and *Flavobacterim sp.* that marine isolates start to produce VLPs in a state of starvation. Within this study he addressed the hypothesis that VLPs may have the capability to mediate generalized gene transfer. Drake et al. (1998) confirmed the occurrence of viruses and virus-like- particles in aquatic systems and compared the virus-bacteria interactions in sediments with those in water columns. There is no reason to believe that those VLPs can be characterized by comparing them with traditional viruses associated with bacteria (T4 or lambda phages for Escherichia coli), but it is well established that some marine bacteria spontaneously release VLPs, which contain DNA and are morphologically similar to viruses. The VLPs focused within this work range in size from 30 to 320 nm in diameter (Chiura and Umitsu, 2004). However, they seem to be different from the well-known types of lysogenic or virulent phages (Chiura et al. 2002). During further investigations Chiura found out that VLPs are able to mediate gene transfer, similar to the mechanism observed with GTAs (Chiura, 2002). He showed that the capability of amino acid synthesis of *E. coli AB1157,* which is deficient for several aminoacid synthetic pathways, was restored by VLPs from

other bacteria and proposed that VLPs are capable of transferring genes to recipients by generalized transduction (Chiura, 1997; Chiura and Umitsu, 2004; Chiura et al. 2009). The feature of inducing horizontal gene transfer was never recorded for classical MVs, thus this seems to be a crucial difference between VLPs and MVs.

1.2 Alteromonadales- a marine bacteria order

Alteromonadales inhabit all known non- geothermal marine biomes. The order *Alteromonadales* includes marine gram- negative bacteria and forms a phylogenetic clade within the *γ-Proteobacteria.* Bacteria belonging to this order form straight or curved rods, are motile by means of a single polar flagellum and do not form endospores or cysts. They are chemoheterotrophs, either facultative anaerobes or strictly aerobic; the anaerobic phenotypes are able to grow by respiration and fermentation. Most strains are catalase and oxidase positive and require Na^+ ions for growth. The best growing conditions are provided in seawater- based media. Many species are able to utilize ammonia as a nitrogen source and some have a requirement for amino acids. Bacteria of this order are usually nonpathogenic (Bowman et al. 2005).

Members of the order *Alteromonadales* have been evolving in the oceans for at least 500-600 million years, as estimated by the molecular clock calculating the divergence of the 16S rDNA (Bowman, 2007).

1.3 The bacterium *Pseudoalteromonas marina*

Marine, aerobic, heterotrophic bacteria of the genus *Pseudoalteromonas* (Gauthier et al. 1995) have received significant interest during the last years because of their easy cultivation and their widespread occurrence in the marine environment. Furthermore some species of the genus *Pseudoalteromonas* showed antibacterial activity and VLP production (Soiza et al. 2008, Holmström et al. 1999, Gram et al. 2009, Longeon et al. 2005, Bowman 2007, Nevot et al. 2006). *Pseudoalteromonas* is with more than 30 species one of the largest genera within the *γ- Proteobacteria* (Ivanova et al. 2004). *Pseudoalteromonas marina* was first described by Nam et al. (2007). The cells are gram-negative, rod-shaped growing on marine agar (MA) (measuring 0.5- 0.7 x 2.1- 3.0 µm) and motile. Cells do not form endospores and

the colonies are colored pale yellow, 0.2- 0.5 mm in diameter, smooth and circular to slightly irregular in shape after 3 days of culture. *Pseudoalteromonas marina* is able to grow at 4°C- 37°C and at pH= 5.3- 8.8, but not at pH- values lower than 4.1 or higher than 9.3. Growth occurs in presence of 3- 12% NaCl, but not without NaCl or concentrations higher than 15%.

Table 1: Classification of *Pseudoalteromonas marina*

Classification of *Pseudoalteromonas marina*	
Domain	Bacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Alteromonadales
Family	Pseudoalteromonadaceae
Genus	Pseudoalteromonas
Species	*Pseudoalteromonas marina*

1.4 Antibacterial activity of marine bacteria

Marine bacteria are substantial for the nutrient turnover in the oceans. Growth and metabolism of those microorganisms have been studied to investigate their ecology and role in biochemical processes (Gram et al. 2009).

Some marine bacteria are inhibitory to other bacteria, thus playing an important role in marine ecology. Additionally, as producers of antibacterial compounds they are thought to be of high medical relevance, especially considering the rapid increase and spread of antibiotic-resistant bacteria. Because of this rapid increase over the past decade, there is an urgent need to discover novel antimicrobial compounds. The aquatic environment and especially marine systems are major source-candidates for such compounds. First observations of antibacterial activity of marine bacteria were made more than 40 years ago (Burkholder et al. 1966), based on the production of antibiotic substances of marine bacteria. Isolates were observed to inhibit gram- positive bacteria, especially *Staphylococcus aureus*. Over the past 30- 40 years, there has been an ongoing quest to discover chemotherapeutic

agents from the sea (Anand et al. 2006). At first sight these growing interest can be explained by the enormous biodiversity of marine microorganisms, which is a promising resource for the discovery of new medicines. Screening of marine bacteria isolated from the surface of marine algae and invertebrates has shown that a high percentage produce antimicrobial metabolites. The importance of searching for novel antibiotics increases because of the growing occurrence of multi- drug resistant human pathogens (Ahmed et al. 2008). Long and Azam (2001) discovered that members of the orders *Vibrionales* and *Alteromonadales* were the most eminent producers of antibacterial substances.

Several species within the genus *Pseudoalteromonas* often associated with surfaces produce antibacterial compounds (Bowman et al. 2007), so it can be expected that attached bacteria have a higher production of antibacterial substances than their planktonic correspondent (Gram et al. 2009). This finding may suggest that the antibacterial activity can be switched off or expressed depending on the immediate environment of the cell. Moreover, *Pseudoalteromonas* is mostly found associated with eukaryotic hosts in a world- wide variety of habitats. This suggests that the adaptive and survival strategies are diverse, efficient and consequently important for basic and applied research. It is also well established that antibacterial compounds of *Pseudoalteromonas*- like species can be divided into two classes: First, cell bound polyanionic macromolecules, which are partly diffusible in culture media. A study by McCarthy et al. (1994) demonstrated that these high molecular compounds are associated with proteins. The second group of antibiotics involves small brominated compounds which are cell-bound and not diffusible into the media (Holmström et al. 1999, Hayashida-Soiza et al. 2008). Hence, the antibacterial activity can be justified per extra cellular substances. Finally, Wilson et al. (2009) found out that the antibacterial factors produced by *Pseudoalteromonas* are proteins of high molecular weight.

Antibacterial activity among marine bacteria is a well- known phenomenon and has been demonstrated in a number of studies (Gram et al. 2009, Wilson et al. 2009, Kennedy et al. 2008 etc.).

2 Aims of the Diploma Thesis

In general the results of this study should offer valuable clues about the bacterial strain *Pseudoalteromonas marina* and its growth behavior, its morphotype variation during bacterial growth, and its particle- production and -function.

The first aim of this work was to determine the source of the particle genome. Sequence analysis of the VLP-DNA should make clear if the VLPs contain viral or bacterial DNA or both.

The second aim of this study was to test if the species *Pseudoalteromonas marina* has an antibacterial effect on pathogenic bacteria and if so, if this antibacterial activity is induced by the bacteria itself or mediated by their VLPs.

3 Material and Methods

Materials used for more than one experiment are listed in 3.3 and the suppliers of the used chemicals can be found at the end of this chapter in 3.4.

3.1 Laboratory techniques

3.1.1 Cultivation of *Pseudoalteromonas marina*

In this study the aerobic, gram- negative marine bacterium *Pseudoalteromonas marina* was used as the source of virus like particles (VLPs). This bacterium has been cultivated in a *514.Bacto Marine Broth (DIFCO 2216).*

The bacterium was stored as glycerol culture at -80°C and stroked out onto an agar plate, which contained the *514.Bacto Marine Broth (DIFCO 2216)* medium. The bacteria were growing at 26°C in the incubator. After 24 h of growing the bacteria were transferred to a, which was incubated at the same temperature for 24 h. 500 µl of the preculture were then used to inoculate 250 ml of fluid medium and incubated at 26°C and shaking at 200 rpm. The growth behavior and growth speed was observed by O.D. - measurements with a Biophotometer plus.

3.1.2 Separation of Virus- like- particles by filtration and ultracentrifugation

To separate the VLPs from the bacterial cells, the culture had, in the first step, to be centrifuged at 7.500x g and 4°C for 40 min. Afterwards the supernatant was successively filtered through a 0.45 µm, a 0.22 µm and finally a 0.22 µm sterile membrane filter. Then the filtrate was centrifuged at 42.000 rpm or 25.000 rpm for 4 h using a *Beckman XL-70*Ultracentrifuge with a *55.2Ti* rotor to pellet the VLPs. The supernatant was discarded and the VLP- pellets resuspended in 300µl of Cell- Lyses- Solution or 100 µl of TBT- buffer (depending on the DNA- extraction method). The obtained suspension was dissolved over night at 4°C.

Materials
Whatman filters
TBT- buffer

3.1.3 DNA – Extraction

In this study three DNA-fractions were isolated: bacterial genomic DNA, DNA from VLPs and plasmid DNA.

3.1.3.1 Isolation of bacterial DNA

To prepare genomic bacterial DNA as template for PCR the *Capture Column Kit (QIAGEN)* was used. The DNA was isolated following the protocol for *DNA- Purification from Gram-Negative Bacteria*. As starting material 1.5 ml of overnight bacterial cultures were used.

Protocol

- Pelletation of bacteria by centrifugation at 16.000 x g for 1 min
- Supernatant was discarded except of 200 µl residual fluid in which the pellet was resuspended
- For each sample 1 Capture Column placed in a blue Waste Collection Tube, 1 additional blue Waste Collection Tube and 1 clear DNA Collection Tube were labeled

- Addition of 200 µl sample to the Capture Column
- Incubation at room temperature for 5 min
- Addition of 400 µl DNA Purification Solution 1
- Incubation at room temperature for 1 min
- Centrifugation for 10 s at 10.000x g
- The flow- through was discarded and the Capture Column transferred to the second blue Waste Collection Tube
- Addition of 400 µl DNA Purification Solution 1
- Incubation at room temperature for 1 min
- Centrifugation for 10 s at 10.000 x g - the flow- through was discarded
- 200 µl DNA Elution Solution 2 were added
- The samples were centrifuged 10 s at 10.000 x g and the Capture Columns were transferred to clear DNA Collection Tubes (the wastes were discarded)
- 100 µl DNA Elution Solution 2 were added and the samples incubated at 99°C for 10 min
- Centrifugation for 20 s at 10.000x g yielded 100 µl of purified DNA solution

Materials
Capture Column Kit (Qiagen)

3.1.3.2 Isolation of VLP- DNA

In case of VLP- DNA isolation several different protocols were tried in order to find the best working method.

Protocol I

In this protocol GES- reagent was used which lyses the membranes and inhibits nucleases. The GES- reagent has to be used freshly prepared: 60g Guanidium thiocynate were dissolved in 20 ml of A.d. and 20 ml of 0.5 M EDTA at 65°C. After cooling down 5 ml 10% Sarkosyl were added and the volume filled up to 100 ml with A.d.

- Addition of 100 µl of VLP- resuspension (in TBT- buffer) to 500 µl of GES- reagent
- Incubation for 5 min at room temperature and another 5 min on ice
- Addition of 250 µl ice- cold ammonium acetate (7.5 M)
- The sample was mixed and incubated for 10 min on ice
- 460 µl of ice- cold 2- propanol were given to the sample, mixed and incubated further 10 min on ice
- Centrifugation for 5 min at 16 000 x g and the supernatant was discarded
- 2 Washing steps: once with 200 µl of 70% ice- cold EtOH and once with 100 µl of ice- cold EtOH abs.
- Drying of the pellet for about 15 min at room temperature
- Resuspension in 10 µl of 1x TE buffer over night

Materials
Ammonium- acetate (7.5 M)
70% EtOH (-20°C)
EtOH abs. (-20°C)
GES- reagent: 60% (w/v) Guanidium thiocyanat; 0.5% (w/v) Sarkosyl; 100mM EDTA

Protocol II

In this protocol the *Gentra Puregene-Kit from Qiagen* was used for VLP-DNA extraction. The instructions for "*DNA Purification from Gram Positive Bacteria"* were followed. The original protocol was slightly modified.

- The VLP- pellet was resuspended in 300 µl Cell- Lyses Solution and the suspension transferred to 1.5 ml tubes
- Incubation for 5 min at 80°C to lyse the VLPs
- Addition of 100 µl Precipitation Solution - vortex 20 s at high speed
- Centrifugation for 3 min at 16.000 x g
- Incubation for 5 min on ice (-20°C)

- Centrifugation for 3 min at 16.000 x g
- 300 µl isopropanol were provided in a clean 1.5 ml microcentrifuge tube and the supernatant from the last centrifugation was added - mixing by inverting for 50 times
- Centrifugation for 1 min at 16.000x g
- Supernatant was discarded
- Addition of 300 µl of 70% ethanol (invert several times)
- Supernatant was discarded and the pellet dried
- The DNA- pellet was dissolved in 60 µl 1x TE buffer by incubation at room temperature overnight
- <u>At this point a protein-precipitation step was included:</u>
- 230 µl A.d. (to increase the volume) and 200 µl PCI were added to 40 µl of VLP-DNA
- The mixture was mixed by inverting several times
- Centrifugation for 5 min at 16.000x g
- The supernatant was transferred to a fresh micro centrifuge tube
- Addition of 200 µl CI (24:1)
- The sample was mixed and centrifuged for 5 min at 16.000x g
- 270 µl of the supernatant were transferred to a fresh micro centrifuge tube
- Addition of 30 µl 3M NaAc and 600 µl EtOH abs.
- The sample was mixed and incubated for 1 h at -20°C
- Centrifugation for 5 min at 16.000 x g
- The supernatant was discarded and 200 µl 70% EtOH were added
- Centrifugation for 5 min
- The supernatant was discarded and the pellet air dried
- The VLP- DNA was dissolved in 10 µl A.d. at 4°C over night

Materials
70% EtOH
Gentra Puregene-Kit (Qiagen)
TE- buffer (1x)
CI (24:1)
EtOH abs.
Natrium- acetate (3M)
PCI

Protocol III

Another method for VLP- DNA extraction was similar to Procedure II with the difference that the VLP- pellet was resuspended in 400 µl of Cell- suspension buffer and after incubating over night at 4°C the resuspended VLPs were centrifuged for 30 min at high speed. Then the supernatant was discarded and the pellet resuspended in 300 µl of Cell-Lysis Solution. The following steps were the same as in Procedure II, but without protein precipitation and the DNA- pellet was dissolved in 10 µl TE buffer.

3.1.3.3 QIAprep Spin Miniprep Kit (QIAgen)

Plasmids are double- stranded, circular, extrachromosomal DNA molecules. Within cloning procedures they are commonly used as vectors to introduce foreign DNA into bacterial cells by transformation. To recover (recombinant) plasmid- DNA many methods and kits are available, eg. the *QIAprep Spin Miniprep* Kit from Qiagen, by which small amounts of highly purified DNA are isolated.

In this work the protocol *Plasmid DNA Purification Using the QIAprep Spin Miniprep Kit and a Microcentrifuge* was applied.

Protocol

- 1.5 ml appropriate medium was inoculated with a single colony and incubated overnight at 37°C and 250 rpm

- The samples were transferred to microcentrifuge tubes and centrifuged for 1 min at 16.000 x g
- The supernatants were discarded and the pelleted bacterial cells of each sample resuspended in 250 µl Buffer P1
- 250 µl Buffer P2 were added to each sample and mixed thoroughly by inverting the tubes 4–6 times
- Addition of 350 µl Buffer N3, mixing immediately and thoroughly by inverting the tubes 4–6 times
- Centrifugation for 10 min at 16.000 x g
- The supernatants were applied to the QIAprep spin columns by decanting
- Centrifugation for 30–60 s; flow-through was discarded
- Washing steps:

  Once: addition of 500 µl ml Buffer PB to the QIAprep spin columns; centrifugation for 30–60 s; the flow-through was discarded

  Twice: addition of 750 µl ml Buffer PE to the QIAprep spin columns; centrifugation for 30–60 s; the flow-through was discarded
- Centrifugation for 1 min to remove residual wash buffers
- The QIAprep columns were placed into clean 1.5 ml microcentrifuge tubes
- Addition of 50 µl A.d. to the center of each QIAprep to elute DNA

Material
QIAprep Spin Miniprep Kit (Qiagen)

3.1.4 Polymerase Chain Reaction

The polymerase chain reaction (PCR) allows producing millions of copies of a specific DNA sequence in only about two hours provided that its flanking regions are known, so that primers can be designed. The PCR reaction is performed in 20 to 40 cycles. Each cycle starts with a denaturing step in which the samples are heated to 94- 96°C. Then, for primer annealing (hybridization of the primers to regions flanking the desired sequence), the

temperature is lowered to the annealing temperature, usually to 50- 65°C. The last step within one cycle is the DNA polymerization with a heat stable polymerase at 68° - 74°.
In this study PCR was used for screening bacterial genomic 16S rDNA.

3.1.4.1 Primer

Primers are single- stranded oligonucleotides and generally 15-30 bases long. Within the PCR they are the starting molecules for the polymerase. Primers should be composed of about 50% GC and should have a T_m- value of 55- 70°C. This T_m- value can be roughly calculated by using the following formula: T_m= [4x (G+C)] + [2x (A+T)]. The annealing temperature is 5°C below the calculated T_m- value and for each mismatch the annealing temperature should be decreased by 1°.
In this work genomic DNA of *Pseudoalteromonas marina* was used as template DNA for amplification of a part of the 16S rDNA via PCR. Therefore the degenerated primers 16F27 and 16R1492 were used. The primer 11F is adduced to show the mismatches in 16F27 as to the 16S rDNA of Pseudoalteromonas sp. 1394 (Table 2).

Table 2: degenerated Primers; 16F27 and 16R1492 were used for amplification of a part of the 16S rDNA via PCR; primer 11F is adduced to show the mismatches in 16F27 as to the 16S rDNA of Pseudoalteromonas sp. 1394.

Primer	Sequence	References
16F27	5´- AGA GTT TGA TC(AC) TGG CTC AG -3`	**Bennasar et al. (1998)**
16R1492	5`- TAC GG(CT) TAC CTT GTT ACG ACT T- 3`	**Lane D. (1991)**
***11F (Pseudoalteromonas sp.* 1394)**	5´- GTT GTA TC(AC) TGG CTC AG- 3´	**Green et al. (2004)**

3.1.4.2 PCR- based detection of 16S rDNA

After the bacterial DNA purification the 16S rDNA of *Pseudoalteromonas marina* was amplified by PCR to verify the species. *E.coli* served as a positive, PCR- water as a negative control.
One PCR set- up comprised 12.5 µl *GoTaqGreen*- Master- Mix, 7.5 µl Primer Mix and 10 µl DNA.

The T 3000 Thermocycler was programmed as follows:

- 5 min denature DNA (96°C)
- 30 cycles
    - 30 s denature DNA
    - 30 s primer annealing (50°C)
    - 1h 20 min polymerization (72°C)

  } 1 cycle
- 10 min polymerization (72°C)

Material
GoTaqGreen- Master- Mix (Promega)
Primer Mix (16F27, 16R1493)

3.1.5 Amplification of high molecular weight VLP- DNA

In order to amplify small amounts of high molecular weight VLP-DNA the *Repli- g® Mini Kit* (Qiagen) according to the instructions of the protocol *Amplification of Purified Genomic DNA* was used. The principle of this method is to amplify large DNA molecules from small amounts of template DNA and ensures minimal sequence bias.

Protocol

- To be done before starting with the protocol: Buffer DLB was prepared by adding 500 µl nuclease-free water to the tube; all buffers and reagents were vortexed before use; the heating block was set to 30ºC; 41 µl of Buffer D1 (denaturation buffer: 9 µl Reconstituted Buffer DLB and 32 µl Nuclease-free water) and 80 µl of BufferN1 (neutralization buffer: 12 µl Stop solution and 68 µl Nuclease-free water) were prepared
- 5 µl of template DNA were placed into a microcentrifuge tube
- Addition of 5 µl Buffer D1 to the DNA and incubation for 3 min at room temperature
- 10 µl Buffer N1 were added to the samples
- Samples were vortexed and centrifuged

- Preparation of the Master mix (on ice): 29µl REPLI-g Mini Reaction Buffer and 1 µl REPLI-g Mini DNA Polymerase
- Addition of 20 µl of denatured DNA to 30 µl of the master mix
- Incubation for16 h at 30°C (lid temperature: 70°C)
- After incubation: heating block was heated up to 65°C for 3 min (inactivation of the polymerase)
- Amplified DNA can be stored at 4°C for short-term

Material
Repli- g®Mini Kit **(Qiagen)**

3.1.6 Agarose gel electrophoresis

The agarose gel electrophoresis is the easiest and commonest method to separate, identify and analyze DNA. Basically the negative loaded DNA molecules migrate to the positive pole, whereat the velocity of migration depends on the size of the DNA fragments. High molecular weight DNA fragments migrate slower than short DNA fragments. To detect the DNA under UV- light MidoriGreen was included within the gel and/or the gel was incubated for about 20 min in ethidium bromide solution. In both cases the DNA became visible under UV light.

Two different gel concentrations depending on the molecular weight of the DNA were used in this study: 1% and 0.5% agarose gels.

- The solid agarose was melted by boiling and cooled down to about 50°
- Addition of MidoriGreen (3 µl to 100 ml gel)
- Casting the gel into the gel casting platform with the inserted comb
- After polymerization the samples were loaded and the electrophoresis started
- The gel was incubated in ethidium bromide solution for about 20 min

Material
Agarose
Ethidium bromide solution
MidoriGreen
TAE- buffer (1x)

3.1.7 Fluorescence microscopy

The technique of fluorescence microscopy has become an essential tool in biology and the biomedical science. Fluorescence is a result of physical phenomena by absorption of light by organic or inorganic specimens. Usually a component of interest in the specimen can be labeled specifically with a fluorescent molecule called a "fluorophore". By illuminating the specimen with light of a specific wavelength, which is absorbed by the fluorophores, causes radiation of light of longer wavelengths. To guarantee the separation of the illumination light and the emitted fluorescence a spectral emission filter is used. The basic components of a fluorescence microscope are the light source, the excitation filter, the dichroic mirror and the emission filter.

The Fluorescence microscopy has been employed, so that contamination in the bacteria cultures could be precluded and for observing the morphotype or potential aggregate formation of the bacteria during their growth. In this study staining with Acridineorange was used to detect the bacteria and with SYBR Gold to detect the much smaller VLPs.

Staining with Acridineorange

- 0.2µm *Whatman* filters were used
- Addition of 990 µl A.d. to 10µl of the bacterial fluid culture (mixed vigorously)
- 500 µl of the mixture were applied to the filter, then one drop of Acridineorange and afterwards the residual 500 µl of the sample were added
- After incubation of 1 min the sample was filtered through the filter system
- Before fluorescence microscopy the filter has to be dried

Acridineorange intercalates into nucleic acids and colors DNA green to yellow-green (excitation maximum at 502 nm and an emission maximum at 525 nm) and RNA almost red (the excitation maximum shifts to 460 nm and the emission maximum shifts to 650 nm).

Staining with SYBR® Gold

- 0.05 µm *Whatman* filters were used
- Addition of 990 µl A.d. to 10µl of the resuspended VLPs (mixed vigorously)
- The mixture was applied to the filter and filtered through
- Placing the filter on 60 µl of Sybr®Gold
- Incubation for 30 min in the dark
- Placing the filter back on the filter system
- 2 Washing steps: filter 1 ml A.d. each
- Before fluorescence microscopy the filter has to be dried

SYBR® Gold nucleic acid gel stain is the most sensitive fluorescent stain available for detecting double- or single-stranded DNA or RNA. SYBR® Gold stain is a proprietary unsymmetrical cyanine dye that exhibits >1000-fold fluorescence enhancement upon binding to nucleic acids and has a high quantum yield (~0.6) upon binding to double- or single-stranded DNA or to RNA.

Excitation maxima for dye–nucleic acid complexes are at about 495 nm in the visible and about 300 nm, in the ultraviolet. The emission maximum is about 537 nm. Upon excitation with an appropriate light source, a green light is emitted that can be imaged and quantified.

Materials
SYBR®Gold (10.000 x conc. In DSMO; add 10 µl aliquot to 390 µl A.d.)
Acridineorange (0.3 g Acridine orange, 100 ml A.d. and 3 ml Formaldehyd)
Whatman filter 0.05 µm
Whatman filter 0.2µm

3.1.8 Electron microscopy

Aliquots of the bacterial fluid culture and the resuspended VLPs were prepared for the electron microscopy to analyze the bacteria and their VLPs in more detail. The samples were fixed and followed up to the department of Ultrastructure Research, Schwarzspanierstr.17, 1090 Vienna for further preparation (Regina Wegscheider) and examining (Univ.Prof. Adolf Ellinger).

Protocol

- Centrifugation of 1 ml of bacterial culture or resuspended VLPs for 10 min at 16,000 x g (in case of resuspended VLPs 2 µl Roti®- Methylenblue- stain were additionally added to stain the pellet)
- The supernatant was discarded and the pellet resuspended in 1 ml PBS (pH= 7.4)
- The suspension was centrifuged for 10 min
- The supernatant was discarded and the pellet resuspended in 1 ml of 2.5% Glutaraldehyde in PBS to fix the pelleted bacteria (or VLPs)
- The sample was incubated at 4°C for 2 h
- Washing steps: twice with 1ml PBS (centrifugation 10 min)
- Addition of 1ml of PBS to the final pellet

Materials
2.5% Glutaraldehyde in PBS
PBS
Roti®- Methylenblue- stain

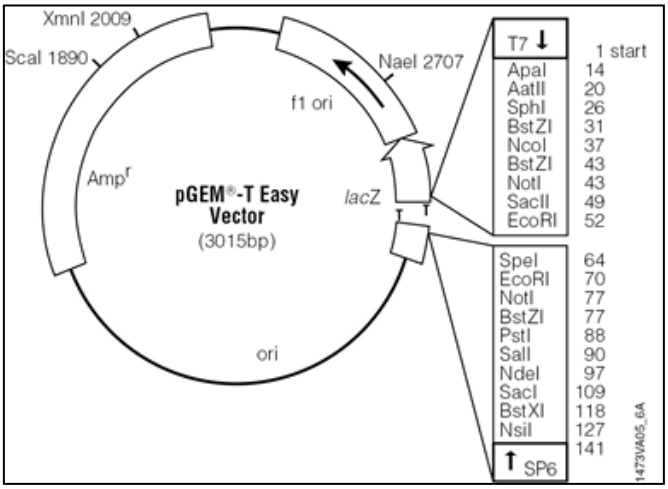
3.1.9 Cloning procedures

3.1.9.1 Cloning of PCR – products (pGEM®-T-easy-vector)

The PCR products were ligated into plasmids and the recombinant plasmids were transformed into an appropriate bacterial strain.

In this study the *pGEM®-T Easy vector* system from Promega was used. The vector counts 3015 bp in length and is cut at position 60 with EcoRV. After adding a single thymidine-overhang at its 3'-terminus a sticky end ligation with the PCR products that carry a single adenosine-overhang at their 5`-terminus, produced by the Taq polymerase during PCR, is possible. The vector contains an ampicillin resistance gene, the lac control elements as well as the β-galactosidase encoding region with a multiple cloning site including the EcoRV restriction site. To select the colonies containing a recombinant plasmid, ampicillin and blue/ white selection by IPTG and X- Gal were used. Bacteria which carry the vector containing the ampicillin resistance gene are able to grow on LB_{amp}- plates. Those which have taken up a recombinant plasmid appear white, because the insert disrupts the β-galactosidase encoding region. The uptake of an empty vector results in a blue colored colony (β-galactosidase will be expressed and turns the colorless X-Gal into an indoxyl derivative **which anodises to 5,5'-dibromo-4,4'-dichloro-indigo).**

Figure 2: pGEM®-T- Easy vector (www.promega.com)



3.1.9.1.1 Purification of DNA fragments with GFX PCR DNA and Gel Band Purification – Kit (GE Healthcare)

The PCR products were purified by agarose gel electrophoresis followed by elution of the DNA from the gel by the *GFX PCR DNA and Gel Band Purification – Kit (GE Healthcare).*

Protocol

- The slice of agarose containing the DNA of interest was cut out and transferred to a pre-weighed 1.5 ml microcentrifuge tube (weigh tube containing the agarose slice and subtract the weight of the empty tube)
- Addition of 300 µl of Capture Buffer Type 3 to the 300 mg weighting gel slice (vortex vigorously)
- Incubation for 15 min at 60°C on the thermoblock until the agarose was completely dissolved
- The GFX MicroSpin Column was placed in the Collection tube
- 500 µl of the Capture buffer type 3- sample mix were dropped off and transferred to the GFX MicroSpin Column of a Collection tube
- Incubation for 1 min at room temperature
- Centrifugation for 30 s at 16.000 x g
- The flow- through was discarded and the residual sample was transferred to the GFX MicroSpin Column (placed in the Collection tube)
- Incubation for 1 min at room temperature (centrifugation for 30 s at 16.000 x g; flow-through was discarded)
- Addition of 500 µl Wash Buffer type 1 to the GFX MicroSpin Column
- The assembled column and Collection tube were centrifuged 30s at 16.000 x g
- The Collection tube was discarded and the GFX MicroSpin Column was transferred to a fresh DNase- free 1.5 ml microcentrifuge tube
- Addition of 30 µl of A.d. to the center of the membrane in the assembled GFX MicroSpin column (placed in the sample Collection tube)
- Incubation for 1 min at room temperature
- Collect the DNA-solution by centrifugation for 1 min at 16.000 x g

Materials
GFX PCR DNA and Gel Band Purification – Kit (GE Healthcare)

3.1.9.1.2 Ligation into pGEM®-T- Easy vector

The purified PCR products were ligated into the *pGEM®-T Easy* vector.

Protocol

For 10 µl ligation reaction:

- 3 µl DNA
- 5 µl 2X Rapid Ligation Buffer
- 1 µl pGEM®-T Easy vector
- 1 µl T4 DNA Ligase

The ligation reaction was incubated for 2 hours at room temperature.

Materials
pGEM®-T- Easy vector Kit (Promega): **2xLigation Buffer:** 60mM Tris- HCl (pH 7.8), 20mM $MgCl_2$, 20mM DTT, 2mM ATP, 10% polyethylene glycol **pGEM®-T- Easy vector (50 ng/µl)** **T4 DNA Ligase (3 u/µl)**

3.1.9.1.3 Transformation in E.coli strain JM109 (competent cells)

Transformation is the uptake of free, non- viral DNA by competent cells. Cells can be made permeable to DNA by electroporation or chemically. In this work the *E. coli strain JM109* was made competent chemically and stored at -80°C until needed.

The *pGEM®-T Easy* vector containing the PCR product was used to transform the target DNA into competent cells of the *E.coli* strain JM109.

Protocol

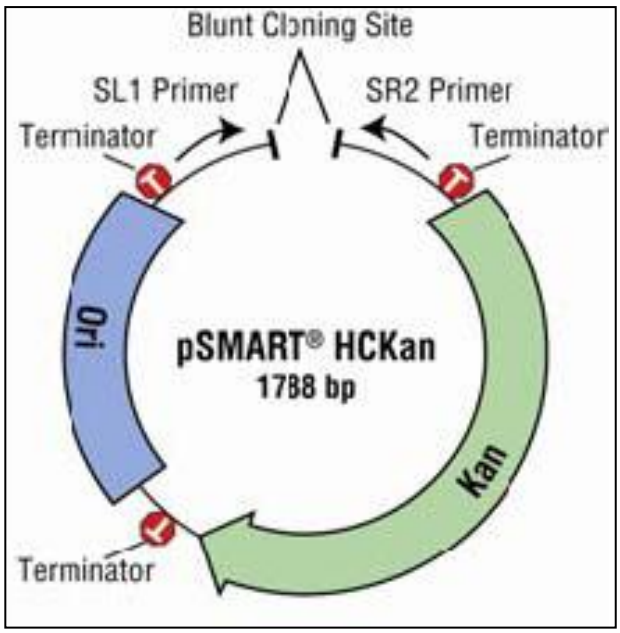
- Thawing 100 µl competent cells on ice
- Addition of 2 µl ligation reaction
- Incubation on ice for 20 minutes
- Heat shock for 45 seconds at 42°C using a GFL Water Bath Type 1012
- Incubation on ice for 2 minutes
- Addition of 950 µl SOC medium
- Incubation for 1,5 h at 37°C and 150 rpm
- The appropriate number of LB plates containing ampicillin were plated with 40 µl IPTG (0,1M; activates lac-operon) and 40 µl X-gal (2%; substrate for β-galactosidase) each for blue-white selection
- Plating out 100-330 µl of the transformation mixture on each plate
- Incubation at 37 °C overnight

Materials
Ligation reaction
Competent cells
SOC medium: (= SOB medium + 20 mM glucose): for 1000ml: 20g peptone, 5g yeast extract, 5g NaCl dissolved in A. dest.; 10 ml KCl (250mM) added; pH value adjusted to 7,0; autoclaving; when cooled down to < 60 °C 5 ml $MgCl_2$ (2M) and glucose (final concentration: 20mM) added;
X- Gal (2%): (w/v; 5-bromo-4-chloro-3-indolyl- β-D-galactosidase): for 5 ml: 100 mg dissolved in 5 ml N,N'-dimethylformamide;
IPTG (0.1%): isopropyl β-D-1-thiogalactopyranosidase: for 50 ml: 1,2 g; sterile filtration;

3.1.9.2 Cloning of blunt-ended fragments using the pSMART® Vector with the *CLONE SMART- Blunt Cloning Kit*

For construction of the shotgun library (see chapter 3.1.12) the *CLONESMART® HC_{Kan} Chemically Competent Blunt Cloning Kit* (*Lucigen® Corporation)* was used. This kit

Figure 3: pSMART® HCKan (www.lucigen.com)



contains the linearized and blunt ended vector pSMART®-HC_{Kan} and the chemically competent cells *E. cloni* 10G. The kit was designed to eliminate cloning bias and maximize cloning efficiency. The pSMART®- HC_{Kan}-vector is qualified to produce 99.5% recombinant clones so that no screening is needed.

3.1.9.2.1 Removing of RNA residues by RNAse A digestion

RNA digestion was carried out for 1 h at 37 ° in 40 µl total volume containing:

- 25 µl of the isolated VLP- DNA
- 4 µl 10x restriction- enzyme buffer
- 4 µl RNAse A
- 7 µl A.d.

Materials
Restriction- enzyme buffer (10x)
RNAse A

3.1.9.2.2 Purification of DNA fragments with *GFX PCR DNA and Gel Band Purification – Kit* (GE Healthcare)

The DNA out of the RNAse reaction (40µl) was purified by using the *GFX PCR DNA and Gel Band Purification – Kit (GE Healthcare)*. The procedure was carried out by following the instructions of the *Protocol for purification of DNA from solution or an enzymatic reaction.*

Protocol

- One GFX column was placed in a collection tube for each purification
- Addition of 500 µl of capture buffer type 3 to the GFX column
- 40 µl DNA solution (up to 100 µl are possible) were transferred to the GFX column
- The sample was mixed thoroughly by pippetting up and down 4–6 times
- Centrifugation of the columns at 16.000 x g for 30 s
- The flow- through was discarded
- Addition of 500 µl wash buffer type 1 to the column
- Centrifugation at full speed for 30 s
- The collection tube was discarded and the GFX column transferred to a fresh 1.5 ml microcentrifuge tube
- 40 µl A.d. were applied directly to the top of the glass fiber matrix in the GFX Column
- The sample was incubated for 1 min at room temperature
- Centrifugation for 1 min at 16.000 x g
- The flow- through contained the purified DNA

Materials
***GFX PCR DNA and Gel Band Purification – Kit* (GE Healthcare)**

3.1.9.2.3 VLP- DNA End- Repair and DNA-precipitation

End- repairing was carried out by the *DNA Terminator- End Repair Kit (Lucigen Cooperation),* which is used to transform 3' and 5' overhangs into blunt ends for ligation into blunt cloning sites. This kit has been developed to maximize the efficiency of shotgun library construction, in which insert DNA is fragmented by shearing or restriction digestion. To reduce the volume of the DNA solution the DNA was precipitated and solubilized in 10 µl A.d.

Protocol

- Following components were mixed to get 50 µl final volume:

  38 µl purified, fragmented DNA

  10 µl 5x DNATerminator® End Repair Buffer

  2 µl DNATerminator® End Repair Enzymes

- Incubation of 30 min at room temperature
- Reaction was stopped by incubating at 70ºC for 15 min (thermoblock)
- To precipitate the DNA following components were added:

  40 µl A.d.

  10 µl 3M NaAc (10%)

  200 µl EthOH abs. (2xV)

- The sample was mixed and incubated 15 min at -20°C
- Centrifugation step of 5 min at 16.000 x g
- The supernatant was discarded
- Two washing steps each with 200 µl 70% EthOH (ice- cold)
- Dry pellet at room temperature (about 15 min)
- The DNA was solubilized in 10µl A.d. by over night incubation at 4°C

Materials
DNA Terminator- End Repair Kit (Lucigen Corporation)
EthOH 70%
EthOH abs.
NaAc (3M)

3.1.9.2.4 Size fractionation by agarose gel electrophoresis and DNA recovery with GFX PCR DNA and Gel Band Purification – Kit (GE Healthcare)

For construction of the shotgun library DNA fragments of sizes between 1000 and 5000 bp should be cloned. Therefore the shared and end-repaired DNA was size-fractionated by agarose gel electrophoresis and the DNA fraction of the desired size eluted.

Protocol

- The slice of agarose containing the desired DNA fraction was cut out and transferred to a pre-weighed 1.5 ml microcentrifuge tube (weigh tube containing the agarose slice and subtract the weight of the empty tube)
- Addition of 450 µl of Capture Buffer Type 3 to 450 mg gel slice (vortex vigorously)
- Incubation for 15 min at 60°C on the thermoblock until the agarose was completely dissolved
- One GFX MicroSpin Column was placed in one Collection tube
- 500 µl of the Capture buffer type 3- sample mix were dropped off and transferred to the GFX MicroSpin Column of a Collection tube
- Incubation for 1 min at room temperature
- Centrifugation for 30 s at 16.000 x g
- The flow- through was discarded and the residual sample was transferred to the GFX MicroSpin Column of the Collection tube
- Incubation for 1 min at room temperature
- Centrifugation for 30 s at 16.000 x g
- The flow- through was discarded
- Addition of 500 µl Wash Buffer type 1 to the GFX MicroSpin Column

- The assembled column and Collection tube were centrifuged 30s at 16.000 x g
- The Collection tube was discarded and the GFX MicroSpin Column was transferred to a fresh DNase- free 1.5 ml microcentrifuge tube
- Addition of 30 µl of A.d. to the center of the membrane of the GFX MicroSpin column and incubation for 1 min at room temperature
- Centrifugation for 1 min at 16.000 x g - thus collecting the purified DNA solution

Materials
GFX PCR DNA and Gel Band Purification – Kit (GE Healthcare)

3.1.9.2.5 Ligation to the pSMART® Vector with the *CLONE SMART- Blunt Cloning Kit*

In the CloneSmart ligation reaction, the pre- processed pSMART®-HC_{Kan}- vector is ligated with a blunt-end, phosphorylated insert in a total volume of 10 µl.

Protocol

- The CloneSmart Vector Premix was briefly centrifuged before usage and mixed by gently pippeting up and down
- Following components were combined in a 1.5-ml tube, adding the enzyme last:

  6.5 µl Insert DNA (100-500 ng, blunt-ended, 5'-phosphorylated)
  2.5 µl 4X CloneSmart Vector Premix (pSMART vector, ATP, buffer)
  1.0 µl CloneSmart DNA Ligase (2 U/µl)
  →10.0 µl total reaction volume
- The reaction mixture was mixed by pippeting up and down and incubated first 2 h at room temperature and then 15 min at 70°C on the thermoblock to stop the reaction

Materials
CLONE SMART- Blunt Cloning Kit (Lucigen Corporation)

3.1.9.2.6 Transformation

Transformation means the uptake of free DNA (recombinant plasmid) by competent bacterial cells. The used cloning kit contains chemically competent *E.coli* cells (*E.cloni*), which are kanamycin-sensitive and can grow on kanamycin containing plates only after the uptake of a plasmid with a kanamycin resistant gene.

Protocol

- 40 µl of the competent *E. cloni* cells were thawed on ice
- Addition of 1 µl of the heat-denatured CloneSmart Ligation to the cells (mix carefully)
- The cells were put on ice for 30 min and then heat shocked by placing them in a water bath (42°C) for exactly 45 s
- The cells were returned on ice for 2 min
- Addition of 960 µl of recovery medium
- Incubation of the culture tube for 1 h at 250 rpm and 37°C
- 100 µl of the transformed cells were then platted on altogether 7 YT- LB_{Kan}- agar plates
- Incubation at 37°C over night

Materials
LB- Kanamycin- plates
Recovery- medium (room temperature)
Lucigen's E. cloni® 10G ELITE
Ligation reaction

3.1.10 Plasmid preparation and restriction digest

Plasmid preparation was done as described under chapter 3.1.3.3. The insert sites of the pGEM®-T Easy vector and the pSMART-HCKan-vector are both flanked by two EcoRI restriction sites digestion with EcoRI resulted in the separation of vector and insert DNA. The agarose gel electrophoresis offered information about the size of the insert.

Protocol

For 10 µl restriction reaction:

- 1 µl DNA (prepared using the QIAprep Spin Miniprep Kit from)
- 1 µl EcoRI (10 u/; Roche)
- 1 µl 10x EcoRI Buffer
- 7 µl A. dest.

The digest lasts 2 h at 37°C. Afterwards an agarose gel was charged with the digest products.

Materials
EcoRI (10 u/ µl)
10 x EcoRI Buffer
A.d.

3.1.11 Sequencing

To determine the concentration of the recombinant plasmid DNA the *Biophotometer plus* was used (described in 3.1.3.3 *QIAprep Spin Miniprep Kit*). The clones were shipped to "4base-lab" (Germany) for sequencing.

Primers used for sequencing:

Table 3

Primer	Sequence
M13-40	5´-GGT AAC GCC AGG GTT TTC C – 3’
M13-rev	5´-CAG GAA GCA GCT ATG AC – 3’
SL1	5´-CAG TCC AGT TAC GCT GGA GTC–3’
SR2	5´-GGT CAG GTA TGA TTT AAA TGG TCA GT–3’

M13-40 and M13-rev were used for sequencing insert DNA of the pGEM®-T- Easy vector, SL1 and SR2 for insert DNA of the pSMART® HC_{Kan}- vector.

3.1.12 Library preparation for long time storage

A shotgun library has been constructed to provide a reagent library that can be used many times. Shotgun libraries are used in case of analyzing longer sequences, which are subdivided into smaller fragments, and subsequently re-assembled to give the overall sequence. Multiple overlapping reads for the target DNA are obtained by performing several rounds of this fragmentation and sequencing. In the applied protocol, bacteria containing the recombinant clones are grown on agar plates, washed off the plates and finally stored in glycerol. The achieved shotgun library can be stored at -80°C for almost one year.

Therefore 5 plates out of the transformation step were prepared, containing the *pSMARTt® HC* $_{Kan}$ *-Vector* with VLP- DNA inserts.

Protocol

- Each LB_{Kan} plate was flooded with 2 ml of LB medium
- The colonies were rubbed off the agar with a sterile rubber policeman
- The suspension of all plates was pooled into a sterile 50 ml tube resulting in a total volume of 5.742 ml
- The suspension was mixed properly with 1.013 ml sterile glycerol so that a final glycerol concentration of 15% was reached
- 500 µl of the suspension were dispensed into 1- ml tubes and stored at -80 °C

Material
LB-medium
Sterile rubber policeman
Glycerol- solution

3.1.12 Antibacterial activity of *Pseudoalteromonas marina* and derived VLPs

3.1.12.1 Pathogenic bacteria

For testing the antibacterial activity of the bacterium *Pseudoalteromonas marina* and the virus like particles produced of it, the following pathogenic bacteria were provided as opponents:

- *Bacillus subtilis*
- *Bacillus cereus*
- *Staphylococcus aureus*
- *Lysteria monocytogenes*
- *Vibrio cholerae*
- *Vibrio alginolyticus*

3.1.12.2 Disc- diffusion assay

The disc- diffusion assay is a method for testing the antibacterial activity of bacteria. The pathogenic bacteria are cultivated on agar plates. To test the antibacterial activity of *Pseudoalteromonas marina* and its VLPs sterile filter paper discs were saturated with the different fractions and placed onto the plates with the pathogenic bacteria. Antibacterial activity should give a region of drastically reduced bacteria concentration around the filter.

Protocol

- *Pseudoalteromonas marina* was plated on the appropriate agar plate (Bacto Marine Broth) and incubated 24 h at 26°C
- 5 ml medium (Bacto Marine Broth) were inoculated with a single colony and incubated 24 h at 26°C with shaking to prepare a preculture
- 250 ml medium (Bacto Marine Broth) were inoculated with 500 µl of the preculture and incubated at 26°C with shaking at 200 rpm for 72 h, 96 h, 120 h and 144 h

- The pathogenic bacteria were plated on LB- agar plates and incubated at 26°C overnight
- 10 ml LB medium were inoculated with the a single colony of a pathogenic bacterium and incubated at 26°C overnight to prepare a preculture
- 100 µl of this preculture were plated on the appropriate agar plates (Bacto Marine Broth) and the sterile filter paper discs placed onto it, were saturated with
    1. Tris/ HCl
    2. 514+4% -medium
    3. TBT- buffer
    4. Pellet- suspension (bacterial suspension)
    5. Supernatant of the bacterial culture after centrifugation
    6. Isolated VLPs

Material
Antimicrobial susceptibility test discs (sterile filter paper discs)
Bacto Marine Broth
Retilabo®- syringe filters 0.22µm sterile
TBT- buffer
Tris/ HCl (pH= 7.5)

3.2 In- silico- analysis

In-silico-analysis was done using the the following programs:

- BLAST- search for screening databases and proteins (http://blast.ncbi.nlm.nih.gov/Blast.cgi)
- CLC Sequence Viewer 6 to create reverse complement sequences and alignments

3.3 Solutions

- **Ammonium- acetate** (7.5 M): dissolve 57.81 g in 70 ml A.d., filling up to 100 ml, filter sterile and store at 4°C
- **Bacto Marine Broth 514** (DIFCO 2216): (1 litre) 40.1 g Marine Bouillon, 40 g NaCl; pH- value adjusted to 7.4- 7.8 with NaOH; autoclave
- **Bacto Marine Broth- plates 514**: (1 litre) before autoclaving the 514. Bacto Marine Broth - medium 15 g agar are added
- **DAVIS salt solution**: (1 litre) 2 g KH_2PO_4, 7 g K_2HPO_4, 1 g $(NH_4)_2SO_4$, 0.5 g sodium citrate
- **Ethidium bromide solution**: Stock solution: 10mg/ml A.dest.; working concentration: 0.5µg/ ml
- **Glutaraldehyde:** 2.5% in PBS
- **GES- reagent**: Guanidium thiocynate (Sigma), 0.5 M EDTA, A.d., 10% Sarkosyl
- **Glycerol Solution**: (100ml) 10 ml $MgSO_4$(1 M), 2.5 ml Tris- HCl pH=8 (1 M), 22.5 ml A.d.; autoclave; 65 ml Glycerol are added
- **LB- medium containing ampicillin**: 2 ml of ampicillin (25 mg/ml) to 1 litre of hand- hot LB- medium
- **LB- medium containing kanamycin** (with YT- medium): 3 ml of kanamycin (30 µg/ml) to 1 litre of hand- hot LB- medium;
- **LB- medium**: (1 litre) 10 g Trypton, 5 g Yeast extract, 10 g NaCl, pH- value adjusted to 7.2- 7.6 with NaOH; autoclave
- **LB- plates**: before autoclaving the LB- medium 15 g agar are added; for ampicillin or kanamycin containing plates the ampicillin/ kanamycin is added after autoclavation
- **PBS:** (1 litre, 1x) 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4; autoclave; pH= 7.4
- **Recovery- medium**
- **TAE- buffer (** 2 litres, 10x) 96.8 g Tris (40 mM), 22.84 ml acetic acid, 40 ml EDTA (500 mM)

- **TBT- buffer (**1 litre, 10x) 12.11 g Tris (100 mM), 0.45 $MgCl_2$ (10 mM), 5.84 NaCl; pH- value adjusted to 7.6 with HCl; autoclave
- **TE- buffer (**1 ml, 10x**)**: 20 µl EDTA (500 mM; pH= 8), 100 µl Tris- HCl (1 M; pH= 7.5)

3.4 Suppliers of the used chemicals and materials

Reagent/ chemicals	Suppliers
A	
Agar- Agar	Merck GmbH
Agarose	Promega GmbH
Acridinorange	Merck GmbH
Ammonium acetate	Merck GmbH
Ammonium sulfate	Merck GmbH
Ampicillin(50 ng/ml)	Carl Roth GmbH & Co. KG
Arginine	Merck GmbH
C	
Chloroform	
Citifluor	Christine Gröpl Elektronenmikroskopie
CloneSmart®Blunt Cloning Kit	Lucigen Corporation
D	
DNA Terminator End Repair Kit	Lucigen Corporation
D- Glucose	Fluca Chemie AG
Di- Potassium hydrogen phosphate	Merck GmbH
E	
EDTA	Sigma-Aldrich Handels GmbH
Ethanol	Carl Roth GmbH & Co. KG
Ethidium bromide (10mg/ml)	Carl Roth GmbH & Co. KG
EcoRI(10u/µl)	Roche Diagnostics GmbH
EcoRI- buffer	Fermentas
F	
Formaldehyd	Carl Roth GmbH &Co. KG
G	
GFX PCR DNA and Gel Band Purification – Kit	GE Healthcare

Glycerol	Carl Roth GmbH & Co. KG
GoTaq®Green- Master- Mix	Promega GmbH
Gene- Ruler ™ High range DNA ladder	Fermentas
Guanidium thiocynate	Sigma-Aldrich Handels GmbH
H	
Histidine	Sigma-Aldrich Handels GmbH
I	
IPTG	Carl Roth GmbH + Co. KG
(isopropyl β-D-1-thiogalactopyranosidase)	
Isopropanol	Fluka Chemie AG
K	
Kanamycin (30 µg/ml)	Carl Roth GmbH & Co. KG
L	
Leucine	Fluka Chemie AG
Loading dye	Fermentas
Ladder marker (1kb)	Fermentas
M	
Marine Bouillon	Carl Roth GmbH & Co. KG
Magnesium dichloride	Carl Roth GmbH & Co. KG
Magnesium sulfate	Carl Roth GmbH & Co. KG
MidoriGreen	Nippon Genetics Europe GmbH
N	
Natriumchloride	Carl Roth GmbH & Co. KG
P	
Paraffin oil	Kwizda Pharma GmbH
Primer 16F27, 16R1493	VBC- Biotech, Austria
pGEM®-T- easy vector Kit	PromegaGmbH
Phenol	Carl Roth GmbH & Co. KG
pSMARTHC Kan Vector	LucigenCorporation
Proline	Sigma-Aldrich Handels GmbH
Potassium dihydrogen phosphate	Merck GmbH
Q	
QIAprep®Miniprep	QIAgen GmbH

R	
Repli- g® Mini Kit	QIAgen GmbH
RNAse A	Roche
Roti®- Methylenblue- stain	Carl Roth GmbH & Co. KG
Recovery medium	LucigenCorporation
S	
Sarkosyl (10%)	Sigma-Aldrich Handels GmbH
Sodium acetate	MERCK GmbH
SYBR® Gold	Invitrogen GmbH
T	
Thiamine- hydrochloride	Carl Roth GmbH & Co. KG
Threonin	Fluka Chemie AG
Trypton/ Pepton	Carl Roth GmbH & Co. KG
Tris	Carl Roth GmbH & Co. KG
Tri- Sodium Citrate 2- hydrate	Carl Roth GmbH & Co. KG
X	
X- Gal	Carl Roth GmbH & Co. KG
Y	
Yeast Bact. Kit	QIAgen GmbH
Yeast extract	Carl Roth GmbH & Co. KG

3.5 Suppliers of the used instrumentation

Instrumentation	Suppliers
Antimicrobial susceptibility test discs	OXOID
CertoClav	KELOMAT Sterilizer- Division
Biophotometer plus	Eppendorf AG
Centrifuge 3K30	Sigma Laboratory Centrifuges
Microcentrifuge 5415 D (max. 16.000 x g)	Eppendorf AG
Dry Heat Sterilizer	Binder
Incubator innova®40	New Brunswick
Vertical Laminar Airflow Cabinet Faster;	Szabo- Scandic

Biohazard BH- EN 2004	
***Quickseal*- Centrifuge tubes**	Beckman Instruments, Inc.
Retilabo®- syringe filters, PVDF • **0.44 µm** • **0.22 µm** • **0.22 µm sterile**	Carl Roth GmbH & Co. KG
Sterile rupper policeman	Semadeni (Europe AG)
ThermoStat plus	Eppendorf AG
T3000 Thermocycler Combi Bloc	Whatman biometra®
XL-70 Ultracentrifuge with 55.2Ti rotor	Beckman Instruments, Inc.
Waterbath Type 1012	Johann AIGNER; Ges.mbH & Co. KG
Whatman filters • **0.2 µm** • **0.05 µm (Anodisc 25)**	Whatman Inc.GmbH

3.6 Used bacterial strains

Baterial strain	***Stock No./ Origin***
Bacillus cereus	*Mag. Alexander Knoll, VetMed Vienna*
E. coli AB1157	*DSM4509*
Bacillus subtilis	*DSM10*
Listeria monocytogenes	*Mag. Alexander Knoll, VetMed Vienna*
Pseudoalteromonas marina	*DSM17587*
Staphylococcus aureus	*DSM20231*
Vibrio alginolyticus	*ATCC17749*
Vibrio cholerae	*Neusiedlersee; isolated by Dr. Kirschner, MedUni Vienna*

4 Results

4.1 Cultivation of *Pseudoalteromonas marina*

Pseudoalteromonas marina showed some morphotype variations during their growth. Under observation by fluorescence microscopy long rod- shaped structures were visible in the lag- and exponential phase. During the exponential phase the morphotype changed into smaller rod- shaped structures and finally in the stationary phase they seemed to be nearly globosely. The morphotype variations during growth of *Pseudoalteromonas marina* are demonstrated in Figure 4.

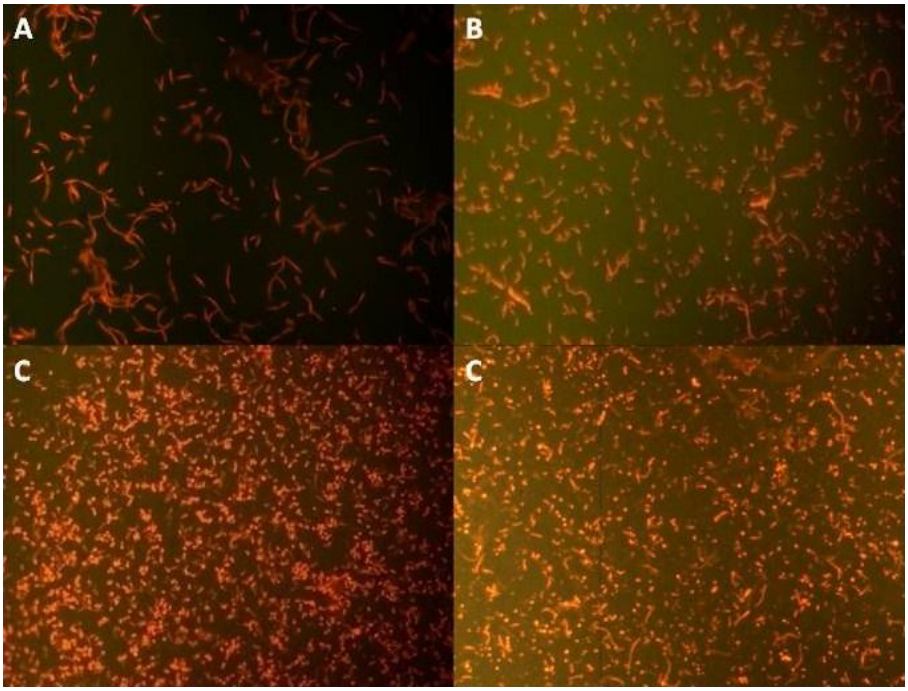


Figure 4:

Pseudoalteromonas marina culture after different incubation times: 25 h **(A)**, 48 h **(B)**, 69 h **(C)** and 143 h **(D)**;

The varying density was determined by measuring the optical density (OD) of the bacterial culture in roughly regular intervals during bacterial growth. The density differences were similar in both culture media with variations in the stationary phase (Figure 5).

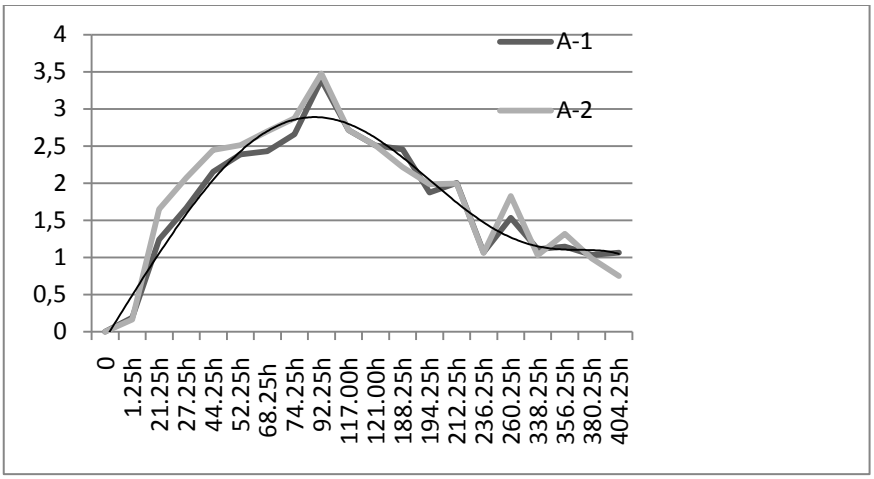


Figure 5:

OD- curve of *Pseudoalteromonas marina* of two different fluidal cultures (**A-1, A-2**);
x- axis: time in h
y- axis: Optical density;

Cross- sections of bacterial cells and VLPs of *Pseudoalteromonas marina* were also observed by electron microscopy to make the structure of the bacteria and VLPs visible and to get a basic orientation of the VLP´s size and production mechanism concerning to the bacterial age. Several periods of growth were monitored to detect more detailed information about the morphotype variations, structure of bacteria and VLPs in general. The results are shown in Figure 6. After 121 h of growth *Pseudoalteromonas marina (P.m.)* showed a kind of reticulate structure and the inner membrane seems to disassociate from the outer membrane. In some cases an electron dense structure (nearly black under electron microscopy) is visible, which is supposed to be a precursor of a VLP.

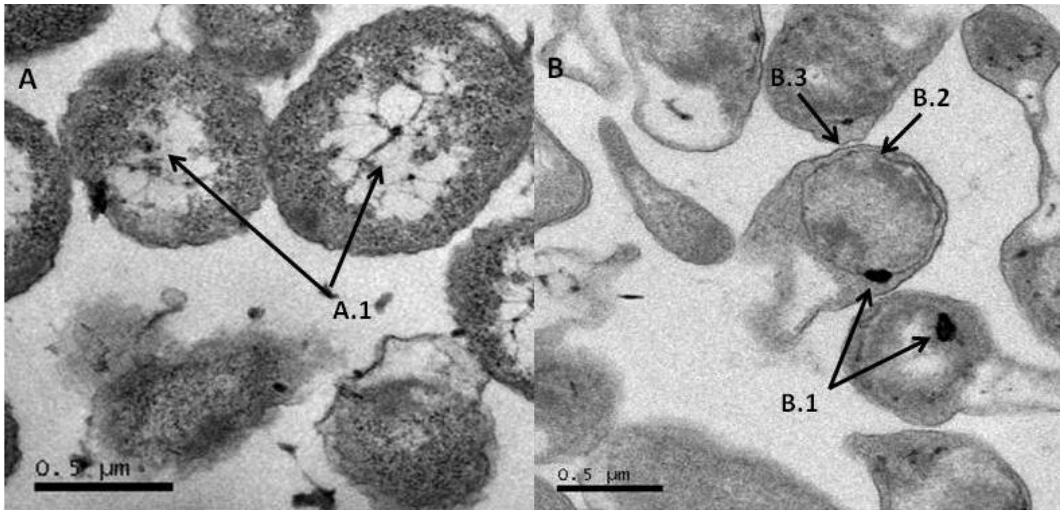


Figure 6:
Pseudoalteromonas marina (P.m.) after 121 h of growth **(A)** and after 167 h of growth **(B)**; P.m. showed a kind of reticulate structure and the inner membrane of the bacterium seems to disassociate from the outer membrane; the supposed precursors of VLPs appear nearly black under electron microscopy (electron dense structure);
A.1) reticulate structure of P.m. **A.2); B.1)** electron dense structure
B.2) inner membrane of P.m.
B.3) outer membrane of P.m.

4.2 Identification of *Pseudoalteromonas marina*

The 16S rDNA sequence is one of the most common genetic marker for identifying and classifying bacteria. In this study the 16S rDNA was used to verify that the strain under investigation is in fact *Pseudoalteromonas marina*.

The DNA of the questionable strain was extracted and served as template for PCR which was carried out using the primer pair 16F27 and 16R1492 (see Material and Methods; Table 2). The result is shown in Figure 7.

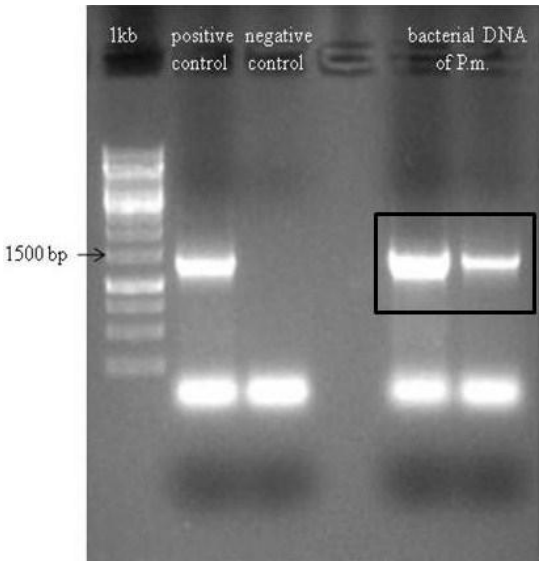


Figure 7:

Bacterial DNA of *Pseudoalteromonas marina* (P.m.) after gel electrophoresis; 1kb-ladder marker shows the size of the DNA fragment, the positive and negative control show that the PCR was working; the PCR resulted in 1.5 kb long 16S rDNAs (labeled);

After electrophoreses the 16S rDNA fragments were eluted and cloned. The recombinant plasmids were isolated, digested with the restriction enzym EcoRI and electrophorized (Figure 8). Based on the restriction digest the insert DNA has a length of about 1500 bp.

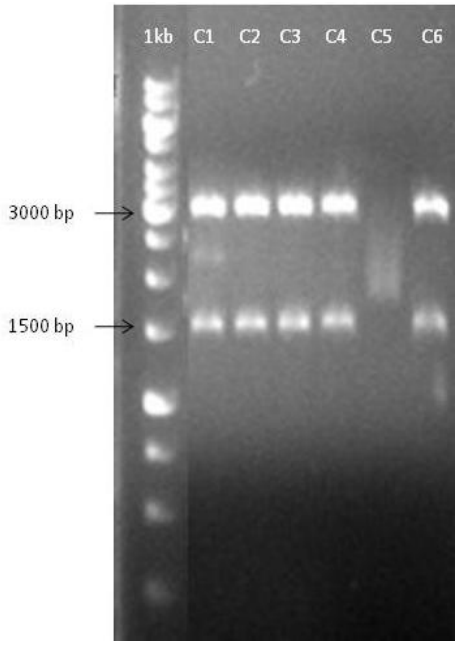


Figure 8:

Gel electrophoresis of the EcoRI digested clones (C1- C6) of the 16S rDNA of *Pseudoalteromonas marina* before sequencing to check the size of the 16S rDNA insert (~1.5 kb); from left to right all samples except C5 showed the expected insert size of about 1500 bp.; for length control of the obtained fragments the 1 kb ladder- marker (1 kb) was applied to the gel.

Sequencing of the clone was done by the "4base lab". Blast search showed that the investigated bacterial strain was in fact a Pseudoalteromonas strain: the blast-alignment is shown in Figure 9.

Figure 9:
The Blast- search showed that the cloned sequence has a 99% identity with the 16S rDNA of *Pseudoalteromonas sp.* BSw20582 (E- value: 0.0).

```
Query  5     GGTTACCTTGTTACGACTTCACCCCAGTCATGAATCACTCCGTGGTAAACGTCCTCCCGA  64
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```

4.3 Virus- like- particles produced by *Pseudoalteromonas marina*

To find out the point of time when the bacterium *Pseudoalteromonas marina* starts the production of VLPs cultures of different growth stages were investigated by fluorescence microscopy. The growing conditions were maintained at 26°C and 200 rpm. After filtration and ultracentrifugation the "VLP-pellet" was resuspended, stained and monitored by fluorescence microscopy (Figure 10). It turned out that the VLP production increased during

120 h of growth - after this time the highest concentration of VLPs was visible. After 144 h of bacterial growth the VLP production seems to be reduced, but we do not have direct counts to confirm these observations. These results indicate that the VLP production takes place quite all the time during bacterial growth.

Fluorescence microscopy gave an overview in which stages of bacterial growth VLPs occur. Electron microscopy was used to get an idea of their structure or even to observe their origin within the bacterium and their secretion into the environment. The results of the electron microscopy are shown in Figure 11. In some cases an electron dense structure (nearly black under electron microscopy) is visible, which is supposed to be a precurser of a VLP (Figure 12).

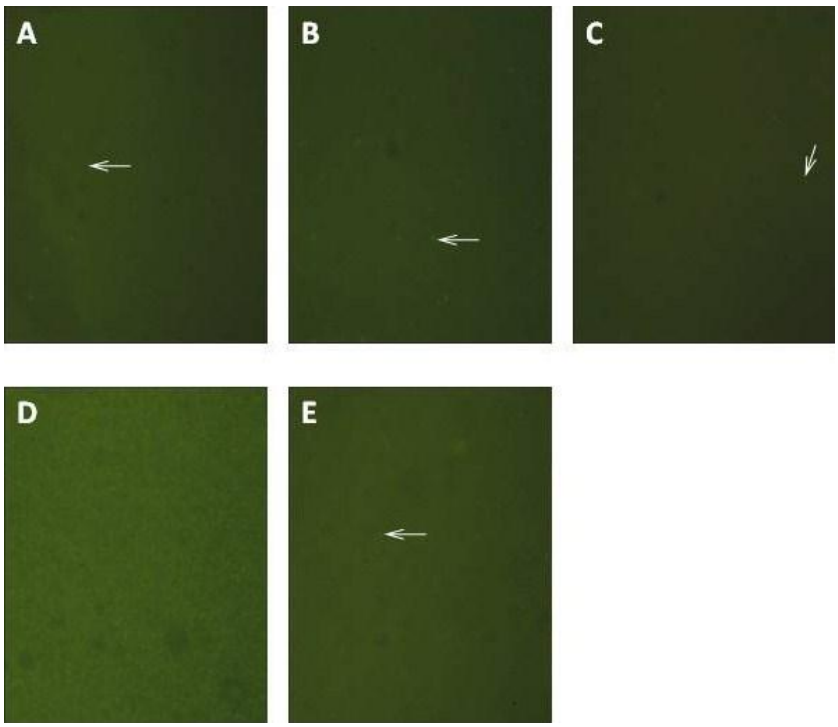


Figure 10:

isolated VLPs after different growth times **A)** 48 h **B)** 78 h **C)** 96 h **D)** 120 h **E)** 144 h
In all samples VLPs are visible as small punctual structures; the difference between the samples is the varying density of the VLPs

Figure 11: *Pseudoalteromonas marina* after 72 h of growth; electron dense structure (under electron microscopy nearly black) is visible, which is supposed to be a precursor VLP; Figures **A)** and **B)** show bacteria which seem to release VLPs in the surrounding area; in Figure **C)** the produced VLP is almost released completely from the bacterium;
A.1) VLP associated with P.m. **B.1)** multiple VLPs associated with P.m. **C.1)** VLP almost released from P.m.

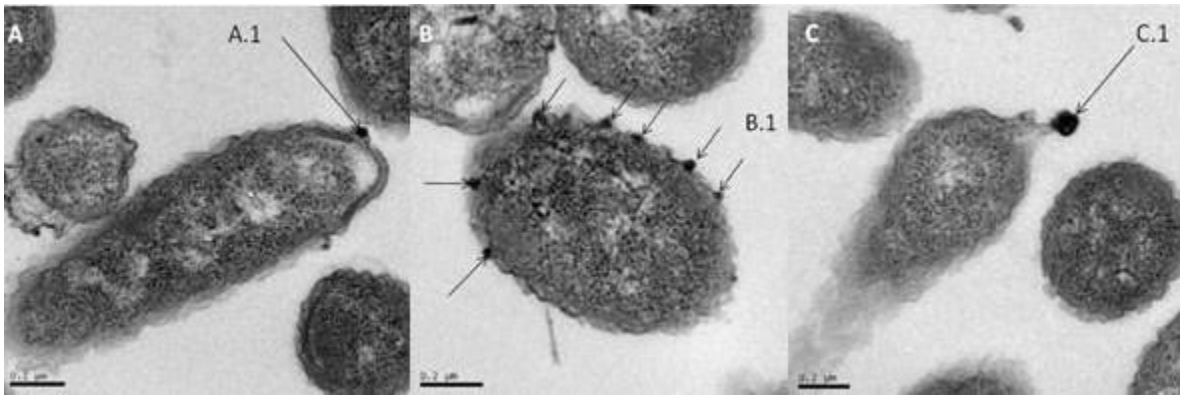
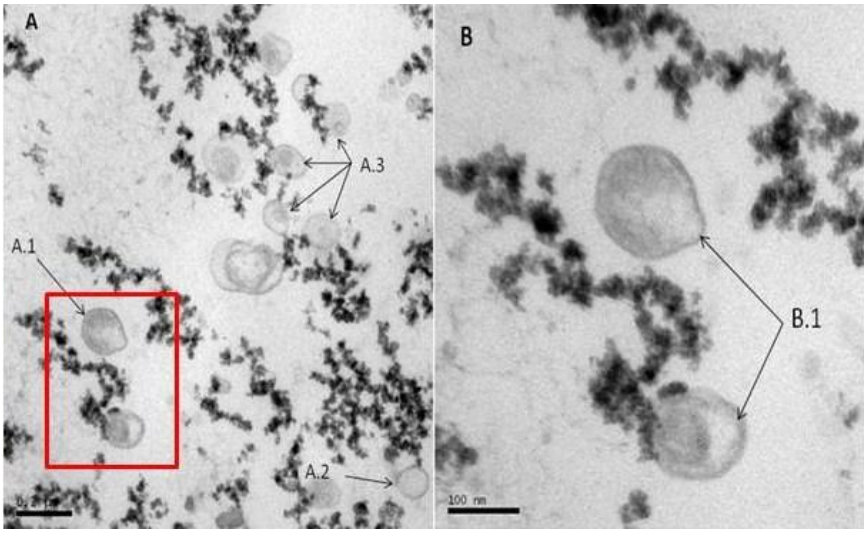


Figure 12: Electron microscopy of isolated VLPs after 72 h of bacterial growth; VLPs differ in brightness, shape and dimension; variety in dimension: 0.05 µm- 0.2 µm;
A.1) VLP with a diameter of about 0.2 µm, colored grey under electron microscope (high electron dense structure), well defined outer membrane visible;
A.2) VLP with a diameter of about 0.1 µm, colored nearly white under electron microscopy (low electron dense structure), well defined outer membrane visible;
A.3) VLPs with a diameter of about 0.1 µm, two different electron dense structures visible, bright electron dense structure in the periphery of the VLP with darker spots within the VLP, well defined outer membrane visible;
B.1) Enlargement of the red marked section in A); VLPs seem to have an inner membrane also;



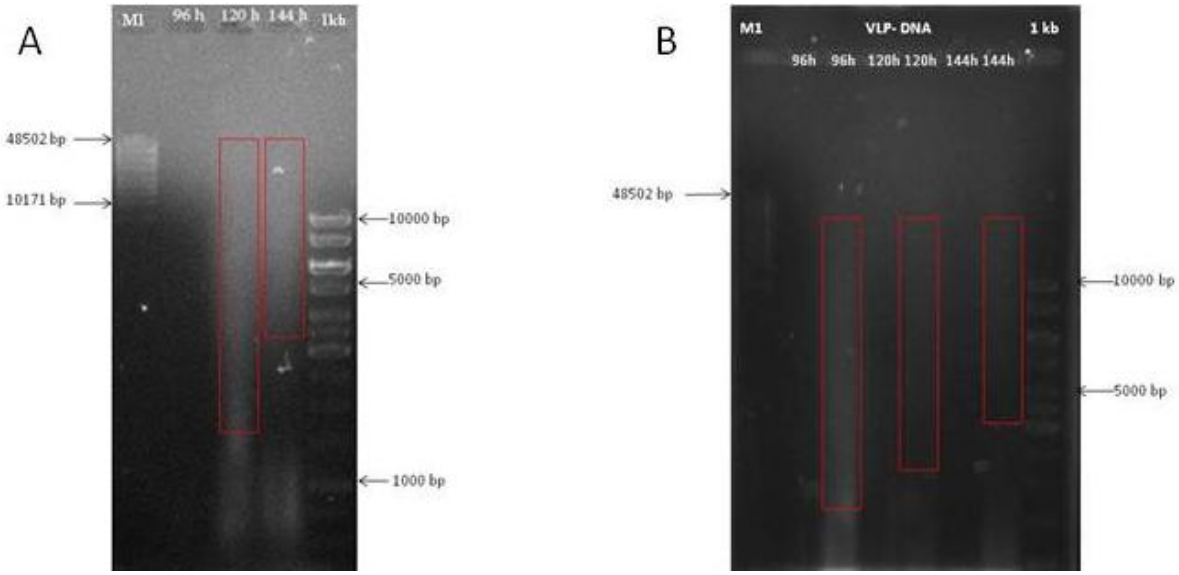
4.3.1 DNA- Extraction of Virus- like- particles

Assuming, that the VLPs produced by *Pseudoalteromonas marina* carry DNA, the VLPs were isolated and different DNA- extraction methods were applied. If no other declarations are given the VLPs of 120 h old fluidal cultures of *Pseudoalteromonas marina* were used.

Protocol I

VLP-DNA extracted by using the protocol including GES- reagent (see Material & Methods; 3.1.3.2) was electrophorised (Figure 13, A and B). DNA within the VLPs could be detected after a culture growth of 96 h, 120 h and 144 h. No DNA could be obtained using VLPs of earlier or later stages.

Figure 13:
Extracted VLP- DNA using GES- reagent (VLPs isolated out of two different bacterial fluid cultures)
A) VLP- DNA (extracted out of isolated VLPs after 96 h, 120 h and 144 h of bacterial growth)
B) VLP- DNA (extracted out of isolated VLPs after 96 h, 120 h and 144 h of bacterial growth)
The red boxes mark the fragments of the VLP- DNA (the fragmented DNA can be caused by the treatment of the DNA during the experiment). For length control of the obtained fragments two molecular weight markers were applied to the gel, the 1 kb Ladder- marker (1 kb) and the High Range Marker (M1).



Protocol II

In this protocol the *Gentra Puregene-Kit from Qiagen* was used for VLP-DNA extraction (see Material & Methods; 3.1.3.2). VLPs obtained after 120 h of bacterial growth were used. The gel electrophoresis showed only a very small amount of VLP- DNA (Figure 14) so the VLP- DNA was amplified by a new technique, which is described in *Repli- g® Mini Kit (QIAgen)* (see Material and Methods; 3.1.5). The amplification result is shown in Figure 15. Sometimes high amounts of proteins sticking in the slots were visible. Thus a protein precipitation step was applied. The results are shown in Figures 16 and 17. The VLP- DNA after protein precipitation shows not the expected high molecular weight DNA, but in most cases high amounts of RNA. The expected length of VLP- DNA is between 30 kb and 40 kb.

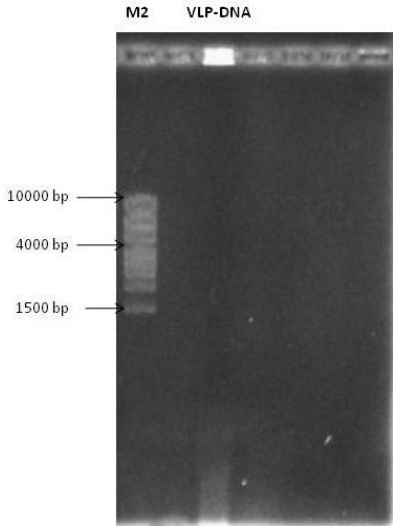


Figure 14: VLP- DNA isolated after 120 h of bacterial growth; small amount of VLP- DNA after gel electrophoresis detected; arrow marks the VLP- DNA; For length control of the obtained fragment the High Range Marker (M1) was applied to the gel.

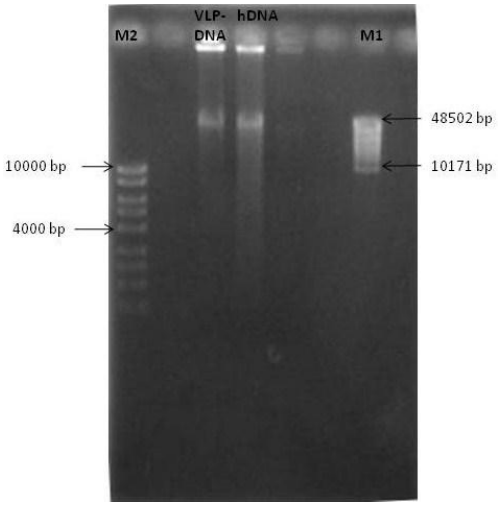
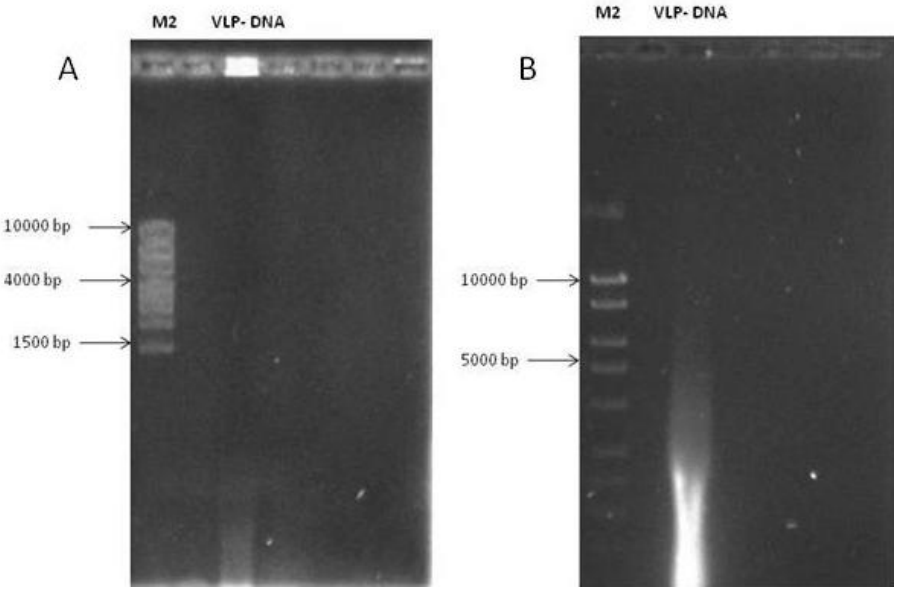


Figure 15: amplified VLP- DNA shows a length of about 48000 bp (red box); human DNA was used as a positive control; For length control of the obtained fragments two molecular weight markers were applied to the gel, the Mass Ruler (M2) and the High Range Marker (M1).

Figure 16: VLP- DNA **A)** before protein precipitation and **B)** after protein precipitation; **A)** shows high amounts of proteins sticking in the slot; in B) high amounts of RNA are visible but no high molecular DNA could be detected; For length control of the obtained fragments the Mass Ruler (M2) was applied to the gel.



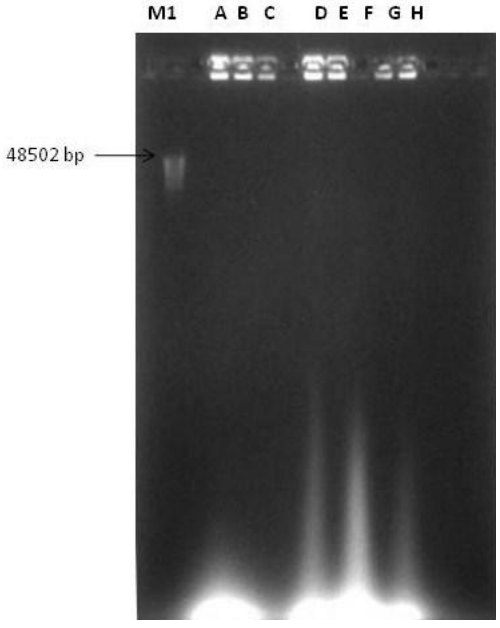


Figure 17: Gel electrophoresis after VLP- DNA extraction: All samples show high amounts of RNA; With sample D) a protein precipitation step was conducted, but still there are proteins in the slot. In case of sample F) no proteins are visible although no protein precipitation was accomplished. There is also no high molecular weight VLP- DNA visible. **A)** no protein precipitation; **B)** no protein precipitation; **C)** no protein precipitation; **D)** protein precipitation; **E)** no protein precipitation; **F)** no protein precipitation; **G)** no protein precipitation; **H)** no protein precipitation;
For length control of the obtained fragment the High Range Marker (M1) was applied to the gel.

Protocol III

The third sampled method for VLP- DNA extraction was similar to Procedure II (see Material & Methods; 3.1.3.2). Within this method no protein precipitation step was necessary. The obtained VLP- DNA is high molecular, but fragmented probably due to handling during DNA extraction (Figure 18). This sample again showed high amounts of RNA which could be eliminated by an RNAse digest (Figure 19). Afterwards the VLP-DNA was purified, concentrated and end- repaired.

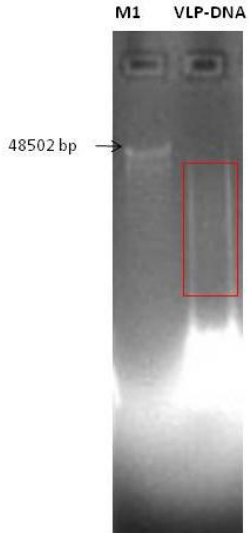


Figure 18: VLP- DNA before RNAse digest; VLP- DNA after gel electrophoresis visible (red box) among high amounts of RNA; For length control of the obtained fragment the High Range Marker (M1) was applied to the gel.

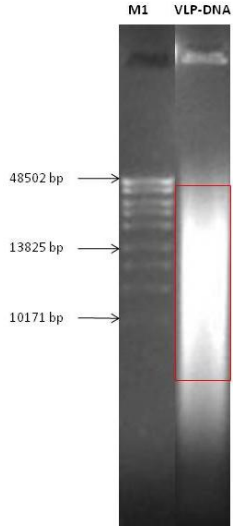


Figure 19: VLP- DNA after RNAse digest; fragmented VLP- DNA after gel electrophoresis visible (red box); the fragments show a length of about 10000 bp up to 40000 bp. For length control of the obtained fragment the High Range Marker (M1) was applied to the gel.

4.3.2 VLP- DNA after End- repair

The end repaired DNA was size fractionated and the fragments between 1000 bp and 5000 bp were eluted by the *GFX PCR DNA and Gel Band Purification – Kit (GE Healthcare)* (see Material & Methods; 3.1.9.1.1; Figur 20).

The eluted VLP- DNA was ligated in a compatible vector (pSMART®-HC_{Kan}).

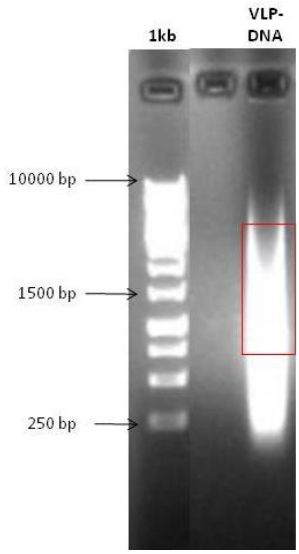


Figure 20: VLP- DNA after end- repair step; the labeled region was eluted; for length control of the obtained fragments the 1 kb Ladder- marker (1 kb) was applied to the gel.

4.3.3 Transformation and Restriction digest

The transformation resulted in more than 100 colonies each plate. 40 colonies were selected for further analysis. After plasmid- DNA purification the DNA concentration was establish by using a *Biophotometer plus.* Then a restriction digest was accomplished to establish the insert length. The maintained sequence length from the "4base lab" could then be compared with the length visible after gel electrophoresis. The restriction digest gave the results shown in Figure 21.

Figure 21: EcoRI- restriction digest after plasmid DNA purification to estimate the length of the insert DNA; clones 1- 37 are visible; for length control of the obtained fragments the 1 kb Ladder- marker (1 kb) was applied to the gel.

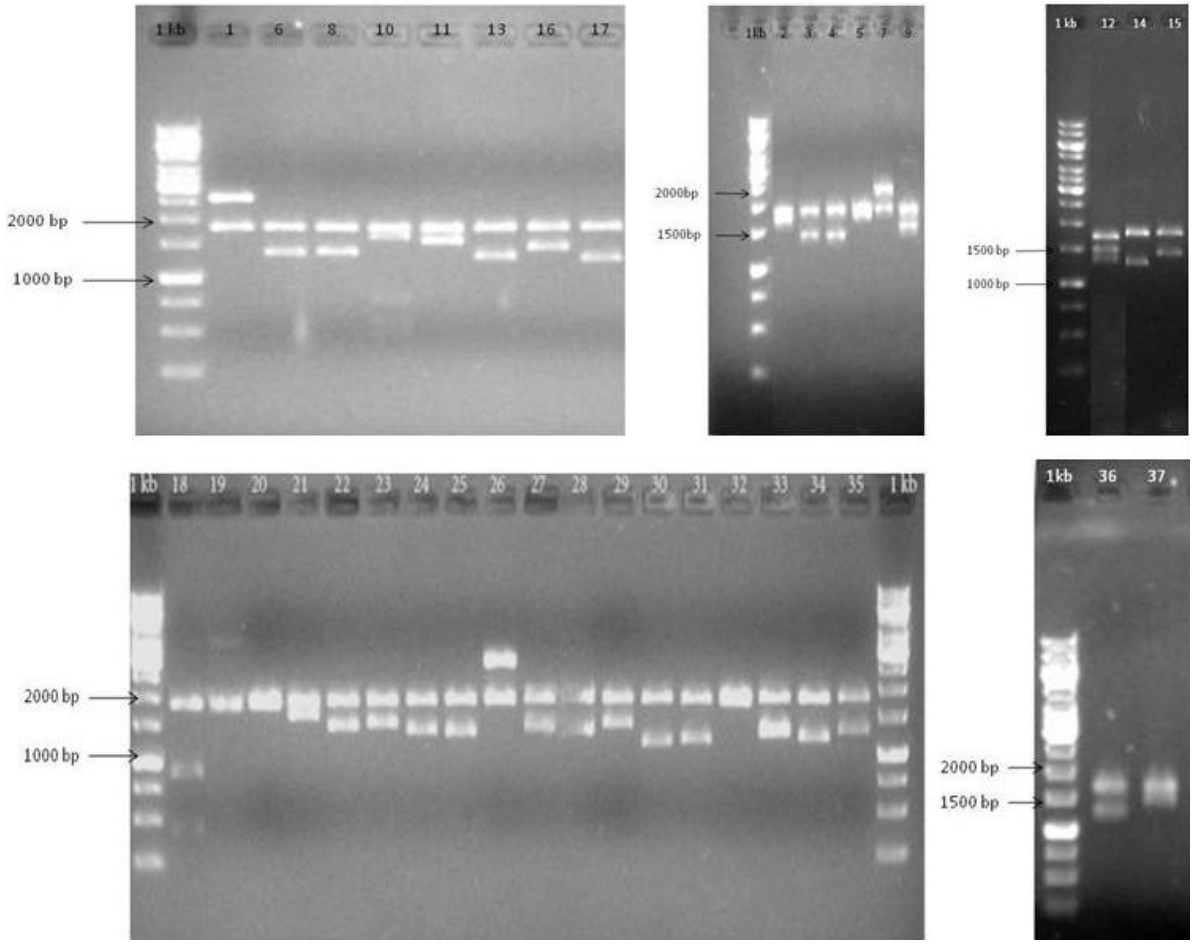


Table 4

Clone	Sequence length per restriction digest (in bp)	sequenced by "4base lab" (in bp)	Overlapping region
1	~ 2500	1720	No
2	~ 1600	1628	Yes
3	~ 1500	1762	No
4	~ 1400	460	No
5	~ 1800	1157	No
6	~ 1400	1323	Yes
7	~ 2400	1199	No
8	~ 1400	1329	Yes
9	~ 1400	1797	No
10	~ 1700	1647	No
11	~ 1600	1549	Yes
12	~ 1500	713	No
13	~ 1300	1278	Yes
14	~ 1400	1494	No
15	~ 1300	1240	Yes
16	~ 1500	1454	Yes
17	~ 1400	656	No
18	~ 1000	1308	Yes
19	~ 1900	1750	No
20	~ 1900	1336	No
21	~ 1700	1536	Yes
22	~ 1500	1364	Yes
23	~ 1500	1402	Yes
24	~ 1400	1301	Yes
25	~ 1300	1268	Yes
26	~ 3000	1727	No
27	~ 1500	1348	Yes
28	~ 1400	1270	Yes
29	~ 1500	1358	Yes
30	~ 1200	1124	Yes

31	~ 1200	1177	Yes
32	~ 1900	1770	No
33	~ 1500	1740	No
34	~ 1300	1171	Yes
35	~ 1400	1262	Yes
36	~ 1300	1342	Yes
37	~ 1500	1527	Yes

4.3.4 Sequence analysis

Each clone was sequenced from both directions (4baselab) leading to two sequences. From one of them the complementary sequence was created. Then both sequences were brought together and aligned to find out, if the clone was sequenced completely - in this case an overlapping region was expected. This overlapping region as well as flanking vector sequences were eliminated and the sequence analyzed by blastx. By this also the Conserved Domain Database was searched. Green labeled sequence sections stand for the "left" DNA-sequence homologous to the protein-coding region, blue labeld sequence sections for the "right" DNA-sequence with the homology to the protein-coding region.

Clone 1)

Sequenced: 1720 bp

Sequence length according to restriction digest: 2.5 kb

Missing: 780 bp

GGTCTTATTTGAGCGAAAAGCGGAGGGTCGGGTAAACTCGTTTTTGGACAGAAATGAGTTAGAGCCAACGGACGGCTCTGTGCCATTTTCGGT
CTTTCGACTGCAATATTACATCTTAAAACTAATTGCGACTACAGATGTTCACCATCTCATTTTTGATTAAACTTATCGTTGAGTATTATTTTC
CCTGCTGAGAATACCAATAGTTTTTATACGCCAAACTTCCCCACAATACATGGGCCAATGCAATTTCTGAAGTATCATGATTAACATCTATTG
GTGATAGTGTGTTGAGTTCATAATTATATTGAAAGTTCAAAGGTGCTTTACTTGGCTGAAGGATGGTTACTTCATCATTAACCATATAAGCAA
AATTCTCTGCATACTGCATCATAGCTCTATTAGAAGCCGTTGGAACTGTTAGGTCTTGACCTAACATTGGTGAAATATTCTCTATACCCATTA
ATGACAAAAGAGTTGGTGCTAAATCGATTTGACTGACTATTCGTTGATCTAAAGTGGGTTCTTTACCTGAGTTTAGGATCACACCCGGAATAT
GGAAATTGTTAATAGGAACAAGATCTTTACCCATTGCTCTTGCATCATGGTCGGCAACAACGAGGAATATTGTGTTTTCCCAATAGGCTTGCA
TCTTTGCTTTTGCAATAAATTGTCCTAAGGCATAATCTGCATATTGTATGGCTTTATGGCGCAGTAATTCTTTGTCATCATACTTCGTCAATT
GTTTATCGCTATACTGGATAGAGGTAACAATACCGTCAGGAATTTCAAATGGATCATGGTTACTGACGTAAATATGAGCTAAAAAATGGTTTT
CTTCGTTATGAAGCTTAGTACATCAATCAGCTGGANNNNNNNNTCAGTAACTGCCATTATTTTTTAACAGGAAATAGCAATAGAGCAAACACT
ATCAATATTGTTATCAATGACCATCACCAGCATTTAGACACTTCATCAGGATAAGCTAGATATCAATAAATAGCTGTTTGGTCTAAAGCGTAT
TCATTGATAAATGCGGGCGTTGCTAATTCAAAAAACAATATGCCCATAAAGCAGATCAAAAACCAAATTCGCAAAACCAATGACCAATATTCT
TGAAATCTAGTTTTTGACACCCAAGGGTGCAATAACGCCGGGATGGCGCATAAGTAACCTACGCTACTTAAGTCTATACGAAAGCCATTTGTG
ATTAGGCTCCATATACTATTTAAGTCATTAAATCTATCTAATTGCCAGATAGCCAACCCTGTTCGTACTAAGGTTAGGAATAGCATGACAGAA

ACAGAGAATAGTAGGAATGGTCTGATAAAGCTGTAGATAGAAGTAAACATCGCAATTCTTTTTAGTGAATAACACGATGTTTACTGTATTAGG
TTAAACTTAAGAATTGCTTAATTAAAACTGATAATTAGACATTAATGTTGTTTTAAAGCAGGTCGTTGAGGTAGAAGTATGATCAAAACTACT
ATTACAAACAGTAGTGTCGCGGTAGCTGAATATCTACTCAATTCCAAACCACCGTGCGCAACAGGCTTGTCTAAAAAGTCACCAACAACCGCG
CCTAAGGGACGAGTCAAAACAAATGCAAGCCAAAATAACATTGTTCTAGATACTTTAGTGAACATATATAGGGTTAATACAATTCCTAAGCCT
GTACTGAAAATATATGCAGCTCCAATATAACCCAATCCAGCAGTATCAGCAGTC

Over the complete clone the blastx search addicted a homology of the DNA- sequence to the protein *Phosphoglycerol transferase [Pseudoalteromonas tunicata D2]* with an E- value of 4e-52. This protein belongs to the Multi- domain *MdoB*, based on the Conserved Domain Database. It is responsible for the cell envelope biogenesis and anchors in the outer membrane. The protein is also a member of the Superfamily *Sulfatase* (cl10460).

Clone 2)

Sequenced: 1628 bp

CTATGAATCAGCTTGATTCGTCCATGTTATTACATGGGCTTTTTTGTAACTAATTAGCGGTTAGCATCAATTAGCACTGTGCGTAATAGCATG
CAAACGGCGCTAATAGTAATTCATCGTTCGTTACATGGCCTTTATGGTGGCTAAGCTCACTAAACTCTGTTAATACATCAGTTGGCATTGATA
GCGATGCTTCAGTGCCACTTAAATTAAAAACACACAACATTTTTTCATTTTCGAATGTTCTGTAAAAAGCAAGTACAGGCTCATGTGTGTTAA
TAAATTCAATATCGCCTTCAAGTAGCACGGTTTTTGTTTTTCGCCACGCCATAAATTCACGATAAGCATTTAAGGTAGAATCATGATCATGTG
TTTGCTGTTTTACTGATTGCTGTTTATGCTGCTCGTCTACAGGCAGCCATGGCTTAGTGTCACTAAAGCCCGCATGGTGTAAATCGGCATTAT
CCCATGGCATCGGCGTTCTACAGCCATCACGGCCTTTAAAGTTTGGCCAAAAGGTTATTCCATACGGGTCTTGCAAATCCTCAAAGGCTACGT
TTGCTTCCCCTAGGCCTAATTCTTCACCTTGGTACATACATACGCTACCGCGCAAGGATGCGAGTAGGGCGGTGAGCATTTTACATTGCTGTG
AATTAACTTCGCCATTTTGACTCCAACGGCTTGCTACACGTTCAACGTCGTGATTGCTAAACGCCCAACAAGGCCAGCCTTCTGTCATTCCTT
TTTCAAAGGTTTGCACGGTTGTTCTTATATACTCACTTGAATAATCGTTAGTCAAAAGCTCAAAGCTATAACCCATATGCAGCTTATCACCGC
CTTGTGTGTACTCTGCCATAGTGGCTAAAGAGTCCTCTGACGAAATTTCACCCCAGTGATACAGTGCCTGGGTATTTATTAAGTAACGCACGA
ATATCTTGCATAAACTCAATATTTTCAGGTTGGGTATTATTGTAATAGTGGTATTGAAATGCGTAAGGGTTGTCTTCACTGAAACCACGGCCT
TGGCGTTTTTCTTTTGGTTTTGCAGGGTTATCACGAAGCTGCGCATCATGATAACAAAAGTTAATGGCATCTAGTCTAAAACCATCAACGCCT
TTTTTAAGCCAAAACTCAACGTTATCTAGCACTGCTTTTCTAACATCTGGGTTATGAAAATTTAAATCGGGTTGTTCAGTTAAAAAGTTATGA
AGGTAGTATTGCCCTCTGCGTGGTTCCCATTGCCATGCACCGCCGCCAAAAATTGATAACCAGTTATTTGGTGCAGTGCCGTCTTCTTTAGAG
TCAGCCCACACATACCAGTCGGCTTTGTCGTTACTTTGGTTTTCTCGGCTGTCTAAAAACCATTGATGTTGATCTGACGTATGGCTTAATACC
TGATCGATAATAATTTTAATATCGCGGTCGTGTGCTTGTTCAATAAGCTCGTCGAAGTCGTTTAAATTGCCAAATAAAGGGTCAATATCACGG
TAGTCACTAATATCGTAACCAAAATCTTTCATAGGAGATTTAAAAAATGGCGAAATCCAAATAGCATCAACACCTAAGCTCTTAATATAATCA
ATACGATTAATTATTCCTCTTAAATCACCAATACCGTCGTTGTTGCT

Over the complete clone the blastx search addicted a homology of the DNA- sequence to the protein *alpha- glucosidase [Alteromonadales bacterium TW-7]* with an E- value of 0.0. This protein belongs to the Superfamily *cl07893* (family 13of the glycosyl hydrolases), based on the Conserved Domain Database. Alpha- glucosidase also belongs to the Multi Domain*trehalose_treC* (TIGR02403). Trehalose is a glucose disaccharide which serves in many biological systems as a compatible solute for protection against hyperosmotic and thermal stress.

Clone 3)

Sequenced: 1762 bp

Sequence length according to restriction digest: 1.5 kb

Missing: not determined

In this case there are more bps sequenced than expected after analysis restriction digest. Reasonably it can be expected that the missing section of the sequence contains EcoRI cleavage sites. The resulting small fragments were not visible on the gel.

GGGATCTATTAGTTGTTGATGAAGCGCACCACTTAAGCATTAATAAGGACACCAAGTACTGAATATCAACGTATGGCTGAGCTAAGCCAAGAT
ATTCCGGGCCTTATTTTACTTACAGCAACGCCTGATCAACTAGGCCATAGCAGTCACTTTGCTCGCTTACAATTGCTCGACCCAGACCGTTTT
TACGACTACGACGTATTCAAAGAAGAAGAAGCCAATTACAAAGATGTAGCGCAAGCGGCAAATCAGTTATTGCAAGAGCAAGCTCTTGACGAC
AATGCAAAAACAACGCTAATTGAGCTATTAAAAGAAACCGACATTACCGTCATTCTTGAAAAAGCACAGCAAGGTGATTTACCTGCGCGTAAA
GAAATACTTAACATGCTACTCGACAGACACGGTACAGGGCGTATATTGTTCAGAAATAGCCGCAGTGGCATTGATGGCTACCCAAGTCGTAAA
TTGCATGCCTATCCAATGGCATTACCAAAGCAATACAAAACGGCCATGTCGGTATTAGGTAACATGAGTGGTATTCAAAACGCTGAGCTGAGC
GCGCATCGTGCTCTTTTTCCTGAAAAAATATTCCAAGAGTTTGAAGGTGAAAGCGCAAGCTGGGGCGCCTTTGACCCTCGCGTAGATTGGTTA
ATTGAAACACTTAAAACGCTTAAACACGAAAAAGTACTCCTTATTTGTGCCAAGGCCGAAACGGCTATTAGCCTTGAACAAATACTGCGTGAA
CGTGAAGCGATTAAAGCTTCGGTTTTTCACGAAGGTATGTCGATTATAGAGCGTGACCGCGCGGCGCTTTTTTTGCAGACGAATACGATACGC
GCAATATTATTGTGTCAGAAATGGCTCGAAGNNNNNNNNAATTGGCATGCCTATCCAATGGCATTACCAAAGCAATACAAAAACGGCCCATGT
CGGTATTAGGTAACATGAGTGGTATTCAAAACGCTGAGCTGAGCGCGCATCGTGCTCTTTTTCCTGAAAAAATATTCCAAGAGTTTGAAGGTG
AAAGCGCAAGCTGGGGCGCCTTTGACCCTCGCGTAGATTGGTTAATTGAAACACTTAAAACGCTTAAACACGAAAAAGTACTCCTTATTTGTG
CCAAGGCCGAAACGGCTATTAGCCTTGAACAAATACTGCGTGAACGTGAAGCGATTAAAGCTTCGGTTTTTCACGAAGGTATGTCGATTATAG
AGCGTGACCGCGCGGCCGCCTTTTTTGCAGACGAATACGATAACGCGCAAATATTATTGTGTTCAGAAATTGGCTCCGAAGGTCGTAACTTTC
AGTTCTCACATCACTTAGTGTTATTTGATTTACCATTAAACCCAGATTTACTAGAGCAACGTATCGGCCGATTAGACCGTATAGGGCAAACAC
AAGATGTAAACATTCACGTGCCATACTTTGAAAACACGGCACAAGAAGTGTTACTACGTTGGTACAACGAAGGCCTTGATGCGTTTGAAACCA
CATCAACGACTGGCCAGCTATTGTATAAAGAATTTCGTGATGACTTACTCGAGTTTATTTCAGCCCATAACTGTGATGAAGACGAACTTGACC
CACTCCTTGAGCAAGTAGCTCAGCAAAATACTGTGCTACGTAAAAAAATGGAAAGTGGACGAGATCGTTTATTAGAACTGCACTCATCGGGAC
AAGGCGCCGCCGACTCATTAGTTGCTGATATTGAAAAGCTCGACAACCAGTTTGAGCTGCCCAGCTACATGATTAACGTGTTCGACACCTTTG
GTG

Over the complete clone the blastx search addicted a homology of the DNA- sequence to the protein *ATP-dependent helicase HepA* [Alteromonadales bacterium TW-7]with an E- value of 5e-160. Searching in the Conserved Domain Database resulted in two specific hits. The first one is a diverse family of proteins, the *DEAD-like helicases* （cd00046), involved in ATP- dependent unwinding of DNA or RNA. This domain contains the ATP- binding region. The second specific hit is the *Helicase superfamily c-terminal domain* (cd00079). This domain is found in a wide variety of helicases and helicase related proteins with the ability to unwind nucleic acid duplexes with a distinct directional polarity by utilizing the free energy from nucleoside triphosphate hydrolysis to fuel their translocation along DNA.

Clone 4)

Sequenced: 460 bp

Sequence length according to restriction digest: 1.4 kb

Missing: 940 bp

AAATTAACGCGTCTCTGGTGAAGAAAGGCTTGAATTCGCCTATTTAAAAAATAAAAATTACCAAAAAAAAAGTGAAGAAAAAAGAAATAGGG
GGGCCCTGTGCCTAATTTGAAAAAAAAAAGAAACATACAAAAAAAAAAAAAAANNNNNNNNCTCTGTTGTATAGCTGGGCTTTATATTCATGTA
ATTGTTAAGTTTTGTAATAATAGCCCACAGTTTGGCAAACTAATACAGCCCCCCCCCCCCCCCGGCATTCACACTGTATATAAGCCAAACCT
TTATGCAATTACTTATTTATAGAGTGTTATTTCCAGAGTGGATACTTAGCTTTAATAGAATAGATTACTGGTTAGTTGCTGCCCCCCCTTTTT
TGCTGTGCAGTTATTATTTACGTTATTTTATAGTCGTCGCTTAACAAGGGCCGCTTGAAGTGCTGTGGCGCAAATTAACCGACATTAAATCGG
CGG

Over the complete clone the blastx search addicted a homology of the DNA- sequence to the protein *putative unknown integral membran protein [Alteromonadales bacterium TW-7]* with an E- value of 6e-31. This protein belongs to the Superfamily *Probable integral membrane protein* (cl12135) with unknown function.

Clone 5)

Sequenced: 1157 bp

Sequence length according to restriction digest: 1.8 kb

Missing: 640 bp

TGATGATTCAGCTTGATTCGTCCCTTAGTGGGGCTTTATAAGGCACTATGAGTTCTGCATTAATCGCAACAGTGTTGCTTGCATCAATTAACA
CTCCAAACTATGAAGTATCAGCTTGTGACAAGTCTAAGTTTTCTGTTAATCATGCTGTTGCCGCCAAGTGGCCTTTGATTGAAGATCATCTTC
GCTTAGATGGAGGGTATGTAAAAATAGAAGCAATAGTAAACTCTGCAGGCTTTGTAACTTCACAAAAAATAGTAGATACAAAACCTCGGCGTG
TATTTGATCGCTCAGCTAAAAAAGCTATGAAGCAGTGGTCATTCTCGGAGAGTAACAAAGAAGAAAGATGCTTTAATGTTGTTTTTAAATTCA
AACTAGTGCGTTAAAGTACCTTATAACAAGCTAATAAATAAGGACTATGCTCCTCACCCGTTTTAGTGGACACCTCCTCATCACTTTGAGGAG
GCTAACATGCCTAGATATACCCAACCCAGAAAGACATGGTTTTACCCCGTTAATTTCAAAATCAAAGCAGTCGAACTAAGCTTACGAGACGAT
GTCATGTCAAAGGACGTTGCCGAGGCGCTGGATATTCATCCGTTAATGCTTAGTCGTTGGCGAAAAGAATATCGAGAAGGTAAGTTTAAAAAC
ACGCCGCGCTATCAAAGCAACAACAAAACAATGAGTAAACTCCCAACAAAAAAAGAGCTGGATAACATTCAAAAGCTTAAGAAAGAAAATGAG
CGTCTCAAGCAGAGAATGACCTGCTAAAAAGTGGCAACGGTATCTGGCGGAAGTACATCAGAACGATTTGGATTCATCCAAAAGTTCGGACAG
CCTAGCGTCAATACTGTGTCCTGGGCCTATGTGTCGCGTAGTGGGTTTTATGCCTGGNNNNNNNNTACAATAAACTCTCTATAGCCTTACACT
GGCTTATGTTTATTTTAATTGTCACTGTTTACGGCAGTATTGAACTTCGCGAATTATTTGATAAAGGCACTGAAGCCCGTGATGCCTTTAAAA
TGTGGCACTTTATGTTGGGTTTATCTGTGCTAGCTTTAGTTAGTGTGAGGGTAGTTGCGCGTTTAGTGAGTGGTGCAGCACCTGCCTTACAAC
CAGAGCCTGCAAAATGGCAAAATAAATTAGCTACATTTGTTCATATGCG

The blastx search addicted a homology of the DNA- sequence to the protein *Transposase ISCps9 [Alteromonadales bacterium TW-7]* on the left region (E- value: 8e-52) and to the protein *Cytochrome b561 [Pseudoalteromonas haloplanktis TAC125]* on the right region (E-value: 8e-17). The protein ISCps9, transposase orfA belongs to the domain *Helix-turn-helix domain of Hin and related proteins*, a family of DNA-binding domains unique to bacteria and represented by the Hin protein of Salmonella (superfamily cl01116). Functional the Hin recombinase induces the site-specific inversion of a chromosomal DNA segment containing a promoter, which controls the alternate expression of two genes by reversibly switching orientation.

Cytochrome b561 belongs to the domain *Cytochrome b (N-terminus)/b6/petB* (superfamily cl00859). Cytochrome b is a subunit of cytochrome bc1, an 11-subunit mitochondrial respiratory enzyme.

Clone 6)

Sequenced bps: 1323 bp

GTCCGCAAATCACAAATATGTGACTATCAGATTTAGCTAAGACTTATTCAGTTAAACAACCGTAATATTTTACGGTTGTTTCTAAACTATTCG
CTTCACAGCTACATGGCGTAGCTGTGTGCGTAATCTACAATACGTGCATTTTGGCTTTTTAAGTATTCACGGCCTGCTTTTACACCAAGCTTA
TGATCTTTACGTAGATCGTCGTTTGTACTGCCTAACAATTTGCTGTGCAGTGTTTCGTCTGCAAAAATTTGTATAATTTCTACATCACTTGGC
GGGTTTGCCATAAAGTTTAGCTCGTCTAAGTAAGCTTGTTCGTGCTGAATTAGGTAGTCGAGTGTTTTTGGGCAGTAACCGGCAGCTGTAGCA
AGCGATCTAAGCTTTTGAACCCATGCAGATTGCGCTTGAAAATCAGCATCTACCGTTCTAATAACCACAATTTTACGTGCCCCACGGTTATAC
GCTTCGCGTACTGGTAATGGTGCTGCTAGCCCACCATCAAGGAAAAAAGGTCTTCTTGGACTTTGTTTATTTTCGTGGTTTCGTTAGCCCGCT
TGCGCATTAACCCAAGGGCGTTAATTTAACACCTTGTTTGTATAAAAAATGGTAAAAGCACTTGACGCTTTTAATAGCTCTCTCCATTGATGC
TCTTCACCCGTTGGTGATAAGTAATACGCTTTTCTGTCTCGTGCGTTTGTAGCAGTAATAAGTAACTCACGTTCACCTAAAGACGTTTTAGCT
GTTTTAAAGTCAATCGCTCTATTTACTTCTTTTGTTTTATCAAAGTACCAGTCTAAATCAACAATGTGTTTTCCCATTAGGCCGCGACCTAGC
TGAAAAAAACGTTTATTACGAGACAGCCCTCTAATTAGGCGTTTAGCGTAACCTTTTTGACGTGCTAAATAACTTGTTAAGTTTTGTGAACCT
GCCGACGTACCAATAAATAAATCAAATGGGTCGTAGTTTTGCTCTAACCACGCATCAAGTACACCTGCGGTGAAAATCCCTCGCTGCCCGCCA
CCTTCAGCAATTAACGCAACCTTAGGCTGAGCTATTTTAGTTACTGAAGAAGCTGTTGCGCTGTTTAGAGAGTTCACGGGGTACCTGCCTTTA
ATTTAATAATGTTTGTTTCAATAAATACATTTTATAGAATTAAAGTAATGCCTTAAAATTGATTGTTTAAATCAGTTTGATTGATAAAACAGA
ACGATAGATCACCTATTAATACACTTAACTAATAGTTACTAATGCGTTTTGTTTTATTTTAAATATTTAGGCATCACATCAATTAATTTAAAT
TGTAAATTACTGATTGATGCC

Over the complete clone the blastx search addicted a homology of the DNA- sequence to the protein *hypothetical protein ATW7_00545 [Alteromonadales bacterium TW-7]* with an E-value of 2e-167. Searching in the Conserved Domain Database resulted in a Specific hit*Patatin-like phospholipase similar to yjju protein of Escherichia coli* (cd07208). This domain (Superfamily cl11396) predominantly consists of bacterial patatin glycoproteins. Patatin is a storage protein, but it also has the enzymatic activity of a lipid acyl hydrolase, catalyzing the cleavage of fatty acids from membrane lipids.

Clone 7)

Sequenced: 1199 bp

Sequence length according to restriction digest: 2.4 kb

Missing: 1200 bp

AGTGGAAGCGTCTGAGATTCGTCCGTGAAAATCAAAGTTATCTGAAGGTTATTTTTTAACACTTAAAGATTTTTCTCTATCTAACCCAGGTAT
GGTTTCGGTTCCAGAGCAGTACCGTTTAGGTTCAGCTAGCTTAGACTTCATTGGTATGGGCGATATGATTGCCTACGATACTCGTGCCCTAAT
AAACGATGGTTACTATGATTTACTTCAAGAAAGCCTTACAGACCAAAAACATAAAACACAGTCGTGGACAGTCCAAGAAAAAGTAACTGCCTT
CTTTGTTCAAGCTGATATTAACGCAGAAATTGGATCAATTCCGGTAACAGGTNNNNNNNNGCTATAATAACGTGTGTGTAGAAGCCTTGTATT
TATTTACAAGGGCTTTTTTTATAGCTACTTATCTAAATTTATCAAACTTAACAAAGCTTGGCTTGTAAAAGTGGGCAATACTATGAAACCAAT
AAAAAACGTTGTCATTGCGGGCGGTGGGACCGCTGGTTGGATAGCCGCAGCGCTACTTAATAAAGTATTAGGTAAAGTAATTAACATCACCCT
TATAGAATCCCGCAGTATTGGTACTATTGGTGTTGGTGAAGCGACCATTCCGCCCATTATTCAGCTCAACAATGCGTTAGGTATTAATGAACA
AGACTTTATAAATGCTACTAATGCCTCTATAAAGCTTGGTATTGAGTTCGAAAATTGGAAAACACCATCACATAGTTACATGCATGCGTTTGG
CTCGTTTGGTAAAGACTTTCCGTTTTGTGACTTTTATAACTTTTGGGTAAAAGGGCAAATTAGTGGTAGTGAGGATTCTTTATGGGATTTTTC
ACTTAATTACCAAGCTGCTAAAAAGCACAAGTTTGCACCACTAAGCGCTATTCCTAATACCCAGCTACCTGGGATAAGTTACGCTTTTCATTT
TGATGCCACCTTATATGCTGAGTACCTTAAAAAACTGGCAGTATCACGCGGTGTAAAACACATCGATGCAAAAATAGAGCGTGTTACTCAGTG
CCCAAATACAGGCCAAATTAACAGTCTAACGCTTGATAATAATAGCCAAATAGAGGGGGATTTATTTATAGATTGTACGGGGCAACGTGCACT
TTTAATTGAGCAAACGCTTAATACAGGGTTTGTTGATTGGTCGCATTATTTACCGTGCGATAGCGCCGTAGCAGTGCAATCAACAGGAAGT

The blastx search addicted a homology of the DNA- sequence to the protein *putative TonB-dependent receptor protein [Alteromonadales bacterium TW-7]* (E- value: 4e-50) on the left region. The right region showed homologies to the proteins *Tryptophan Halogenase*

[Alteromonadales bacterium TW-7] (E- value: 2e-89) and *hypothetical protein ATW7_07434 [Alteromonadales bacterium TW-7]* (E- value: 4e-133). The *putative TonB-dependent receptor protein* resulted by searching in the Conserved Domain Database in a Specific hit (cd01347) *TonB dependent/Ligand-Gated channels*. Energy (proton-motive force) and TonB-dependent conformational alteration of channel allow passage of ligands. TonB preferentially interacts with ligand- bound receptors. This mechanism would prevent the free diffusion of small molecules through the pore. This domain is a member of the Porin superfamily (cl00284).

Tryptophan Halogenase is a member of the large superfamily (cl09931) of proteins that share a Rossmann-fold NAD(P)H/NAD(P)(+) binding (NADB) domain. Typically, proteins in this family contain a second domain in addition to the NADB domain, which is responsible for specifically binding a substrate and catalyzing a particular enzymatic reaction. The function of the hypothetical protein ATW7_07434 was already described above.

Clone 8)

Sequenced: 1329 bp

GCAATCACCGTTATTGATGAAGATCTGCAGCTTGTTTTTGGAGATGCATCATCTGACTTAAAAGTAACGGTAACAATTCACGACAAAGCGATG
TACAAGGCGTTTGCATTATCTGGAAGTGTTGGTGCAGGTGAGTCTTACATTTTAGGTCAATGGAGTTGTGACAACTTAACCCGACTAATTGAG
TTATTTGCTATAAATGAAAAACAGTTAGACGAGTTTGAGAAAAAATTCGCGTTTTTCAGCAACATTGCCCACAGACTAACTCATTTAAAAAAT
AAAAATTCTGAGTCAGGCTCTAAAAAAAATATAGTTGCTCACTATGATTTAGGTAACGATTTATACGAATCGTTTTTGAGTAAAGAAATGCTT
TATTCGTGTGCTGTTTATCCATCAAAAGATGCCAGTCTAGAGGACGCGCAGCAGTACAAATTAAAGCGCATATGTGAACAAGTAGAATTGGCA
ATCCTGGCGATTCCGTATAAGAAAATTGGCACTGGATGGGGCGCATTGCCATTTACGCAGCAACACATACGACTGCCACGTGACGACTACCAC
AATATCTGACGAGCAACATGACTTGTGCAGAAAAAATTAAAGAGTTAGGCTTAGAGAATAAAATTACGCTTCTTAAGCTTGATTACAGACTAC
TCACGGGCAAATACGACAAACTCGTTTCGATTGAAATGATTGAAGCAGTTGGACACGAATATTTACCTAGCTTTTTTACCCAGTGTGGTTCGC
TACTCAAAGACGATGGCGCAATGTTGATACAAGCAATCACCATTAGCGATCAGCGATACAAGCACTACCTTAAAAATTCAGATTTTATTCAAC
AGTATATTTTCCCTGGTGGGTGTTTACCCTCATTAAACGAAATGAGTGAGCAAATCAAAAATAACACCGATATGGTTATCCATACTGTTAGTG
ATATTGGAACGCATTACGCACGAACGTTAGCTGACTGGCGTGAACGCTTTATCAAAAGTTGGCCAGATCTAGACCGCACGAAATTTGATGACC
GCTTTTATAGGCTATGGTTATTTTATTTTGCTTACTGTGAAGGCGCATTTAGAACGCGTGCAACCAGTACTGTACATTTAATGGCGCGTAAAC
CTCGCTTTAGTAGCAATGTAGATGAAATTGCACTCGCTTATTAATTTTGTCTTGTTTCAAGCAGTGTGGTTTTTATCATTACTGCTTGAAGGA
CAATCACTTTTATTTAGCGCTGGCATCATTGTCTTAATGTTTTATTTGTCAAAGCAAAAAAAACAAGATGCATTATTGCTATTAAAAGCACTT
CCTATTGCTTTGCTACTTGAATACATA

Over the complete clone the blastx search addicted a homology of the DNA- sequence to the protein *putative cyclopropane-fatty-acyl-phospholipid synthase [Alteromonadales bacterium TW-7]* with an E- value of 0.0. Searching in the Conserved Domain Database resulted in a Specific hit (cd02440) to *S-adenosy-L-methionine-dependent methyltransferases* (SAM or AdoMet-MTase) *class I*. These are enzymes that useSAM or AdoMet as a substrate for methyltransfer, creating the product S-adenosyl-L-homocysteine (AdoHcy).

Clone 9)

Sequenced: 1797 bp

Sequence length according to restriction digest: 1.4 kb

Missing: not determined

In this case there are more bps sequenced than expected after analysis restriction digest. Reasonably it can be expected that the missing section of the sequence contains EcoRI cleavage sites. The resulting small fragments were not visible on the gel.

ACGTTCAGAGATTCGTGGTAAGAGTATCAAGGTCGACCAATGACCTTAAACTTTCAAGATATTTCAGTCCGTGCAGCGTTACAAATTATCGCG
GGCTATAACGACTTTAACTTAGTAACAAGTGATTCCGTTACCGGTAACATTACCTTGCGCTTAGATGGCGTGCCGTGGGACCAAGCACTTGAT
GTCGTATTACGTATAAAAGGGTTGGACAAGCGTATGGATGGCTCTATCTTAATGGTAGCGCCAGCAGAAGAGCTTGCAGCCCGTGAAGCAAAA
GATCTAAAAGCAAAGCAGCAAGTTGAAGATTTAGAGCCGCTTTACAGCGAATATATGCGTTTAAATTATGCCAAAGCTGAAGACTTTGCTGAT
TTATTGAAAACTGATATTAATAGCATCATTACTCCACGCGGAAGCGTGTCGGTTGATCAGCGTACTAATACGCTACTCGTTAAAGATACTGTT
AAGAGTATAGAAAATATTAGACGCATGATTGAAACTCTAGACATACCGGTTCAACAGGTTGTTATTGAATCTCGTATGGTAACAGTTCGTGAT
AACGTAACTGAGGACTTAGGTGTGCGTTGGGGGTTTAGTGATCAGCAAGGTAGTGATGGCATATCAGGTTCTTTGGAAGGTGCTGAAACAATT
TCAAATGGGGTAATCCCTGACTTAACAGATAGATTAAATGTAAATCTACCTATTACAAACCCAGCGGCTAGTATTGGCCTGCACATAGCAAAA
CTCGCCAACGGTACATTAATTGATTTAGAGTTAAGTGCGCTTGAAGAAGAGAATAAAGGCGAGATTATTGCAAGCCCGCGTATTATTGCTGCC
ACAGCAAAGCGCGCATTGAACAAGTACTGAATACCTATACAGAATCAGCTTCTAGTGGNNNNNNNNCAAGGTAGTGATGGGCATATCAGGTTC
TTTGGAAGGTGCTGAAACAATTTCAAATGGGGTAATCCTGACTTAACAGATAGATTAAATGTAAATCTACCTATTACAAACCCAGCGGCTAGT
ATTGGCCTGCACATAGCAAAACTCGCCAACGGTACATTAATTGATTTAGAGTTAAGTGCGCTTGAAGAAGAGAATAAAGGCGAGATTATTGCA
AGCCCGCGTATTATTGCTGCCAACCAGCAAAAAGCGCGCATTGAACAAGGTACTGAAATACCCTATACAGAATCAGCTTCTAGTGGTGCAACA
ACGGTAAGCTTTAAAAAAGCAGTACTTAGTTTAGAGGTGACGCCACATATAACGCCTGATAATAAAGTGATTTTAGACTTAATTATCACGCAA
GATACACGGGGTGATGTTGTACAAACAGGTACCGGTGAGGCCGTATCGATAGATACTCAAGAAATTCAAACACAAGTATTAGTAGAAAACGGG
CAAACCATTGTACTCGGTGGTATTTTCCAACAACAAATAATCAACACCACAAATAAAGTGCCAGTATTAGGCGATATTCCTTATGTTGGTAGG
TTATTTAAAAGCACTAGCGAATTTAATGAAAAACGCGAATTGCTTATTTTTGTGACACCGAAAATACAAATAGATTAAAATAAGGTACTTTTT
TGTTAATCGACGGGTGATTATCTAGTTTAATTGCTCGTTATGTGGCTTTTTATTCTAATTGACTTGAAATACCCCTGCAATTACTGAGATAAT
CCCCTCCTTAATTTTGGGGCCAGGTCGTTAGGCGGGGTTTTATTTCAAATTTTAGAGTTCGTTTGTACTAAAAGATATGGCTGAGAAACGTAA
TATATTTCTAATGGCCAACGAATCAAGCTGAATCACAG

Over the complete clone the blastx search addicted a homology of the DNA- sequence to the protein *putative type IV pilus biogenesis protein PilQ (cytoplasmic ATPase) [Alteromonadales bacterium TW-7]* with an E- value of 3e- 137. Searching in the Conserved Domain Database illustrated that this protein is a member of once the superfamily cl04346and cl02829. The superfamily cl04346 is a short, often repeated, domain found in bacterial type II/III secretory system proteins. Cl02829 is also a bacterial type II and III secretion system protein. The protein is also a member of the multidomain *TIGR02515*, which is characterized by a number of proteins homologous to PilQ. These are involved in type IV pilus formation, competence for transformation and type II and III secretion.

Clone 10)

Sequenced: 1647 bp

AGTTGTTGRCCAACAKACKTAMKTCACGACTACTCCACGTTTTACCAARCATTAACTTGTCGTTTGCMATCTACRAAGAACAAACGTTACGTT
TTGGTGCGGCTAAAACTATTTCSCGCGCATTATTAAACGATATGAATGCGTCAATCAATGCATCATACGGGCAACAACCASATGATAATGGTA
ACTTTTGGAGTATTTCAGGTGGTAACCCTAAACTAGAGCCAAAAGAASCGACAGGCTTTGATATCARCTACSAAAATTACTTTAATGCRGAAS
GATACTTCTCTGCMSCGGTTTTCTATWMMSATATCAACCWKTGGATATTTGATGGTACTTACKCAKTCSATATGTCTGGCGTACCTMATCCTG
CMMCGGGTGAAATACCMKCAACTTCTTCAKGTACTGGTAATGGWAAAGTAAATGGCGGCRGCAGTGACCTATGGGGTTACSARCTTTCWCTTG
CKCTACCGTTTAACGTATTTCATTCTTCACTTGATGGCTTTGGTTTAATTGCAAGCCATACWGGCATTGAACRAGACGTTGAAGATCWAAATG
GGAATGAATATRAACTTGATGGCCTATCTGACCAMRTTGATAGCTTGACTGTTTACTACKARCKTWWTGGCTTCCWCKCSCGTACWWGTATGC
GTRRACRTAGTGACTTTMAAKGTAATGTAAATGGATTASGTCTTGCTGCCACTCAWGTTGACATWARMTGTGAAACWATTTGKGATGCACATT
GRGCTKTGWTWTTGCGCKAGCWCGTGATTGATARCTCATAKSGGCTKARAATGTTACCCTWCCYAAGTMTCANNNNNNNNGAAGCAGGTGTGA
TAGCTTAGATGGCTYAAATGTAACSTKCCAMGTACRAAMTATTACTGAAGARCCGTTTACCACTKTAAGTGGMGATAWCGCGYTGCAAGTAYG
TGACTACCAAGATTATGGTMGTACGTTMTTATTGGGCTTTAGTTACAAGMTATAATAAACGTTGTGTGTAGAAGCCMTWGTAATTTATTTTAC
AAGGGCTTTTTTATAGYTACTTTATMTAAATTTTATCWAWCTTAACAAAGCTTTGGCTTGTAAAAGTGGGCAATACTATGAAACCAATAAAAA
ACGTTGTCATTGCGGGCGGTGGGACCGAAGGTWGGATAGCCGCAGCTCTATTTATTAAAGTATTAGGTAAAGTAATTAACATCACCYTTATAG
AATCCCGCAGTATTGGTACTATTGGTGTTGGYGAAGCGTCCATTCCGCCCATTATTCAGCTCAACAATGCTTTCGGTATTAATGAACAAGACT
TTATAAATGMTAMTAATGCCTCTATAAAGCTTGGTATTGAGTTCGAAAATTGGAAAACACCATCACATAGTTACACGCAYGYGTTTGAMTYGT
TKGGTAAAGAAAATCAGTTTTGTGAYTTTTATAACTTTTGGGTAAAAGGGCAAATTAGTGGTAGTGAGGATTCTTTATGGGATTTTTCAMTTA
TTTACCAAGCTGTTAAAAAGCACAAGTTTCCACCASTAAGCGCTATTCYTAATACCCAGCTACCTGGGATAAGTTACGCTTTTCATTTTGCTG
CCACCCTACATGCTGAGTACCTTAAAAAACTGGCAGTATCACTCGGTGTAAAACACAKMGAGCAAAAATAGAGC

The blastx search addicted a homology of the DNA- sequence to the protein *putative TonB-dependent receptor protein [Alteromonadales bacterium TW-7]* (compare Sequence 7) on the left region with an E- value of 9e- 113.

The right region showed a homology to the hypothetical protein ATW7_07434 *[Alteromonadales bacterium TW-7]* (E- value: 5e-73). More detailed information about the functions of the proteins can be found under Sequence 7).

Clone 11)

Sequenced: 1549 bp

ATGATTCAGCTTGATTCGTTGGCATTTTACAACGTCTTCTAGGGCTAAGCCTTGGCGTTGTAATAATACAGTAAGGTGATAAATTAAATCAGC
CGATTCATTAGTAAGCTCATCGTTATCGTGCTTCATTGCGGCAAGCGCTACTTCTACGCCTTCTTCGCCCACTTTTTGACAGCTACGGCTTAA
GTCTTTAGCAAATAATGAAGCGGTGTAGCTTTTTGCTGGGTCGTCGTTTTTACGCTCAACAATCACATCTTCTAATTGCGCTAAAAAGCTCAG
GCTAGGTTTAGCGTCATCACCAAAGCAGCTTTGTGTGCCTAAGTGACATGTTGGGCCTTCTGGATTAGCCAGTACTAAAATTGAGTCGTAGTC
ACAATCGGTATGCACCGATACTACGTTTAAGTAGTTTTCTGATGACTCACCTTTGGTCCATAAACGTGACTTTGAACGCGAGTAAAAGGTTAC
TTTGTTACTTTCAAGCGTCACCTTTAGTGCTTCGCTGTTCATAAACCCTTGCATTAAAATAACACCAGAGCGAGCATCTTGAACAATCGCAGG
GATCATTTCGCTTTTAGCAAAATCTACTTGAGTTTGGTTTTGTTGTGTTACTTGCATAGTCTTGCTGCCACTTGGTTATCGGTTAAAAATTGT
TTTAATTCGCCAATGTTAATCACATTTTGTGGAAAAACGCTGCTGCTAATGCGCGGTCGACTTCGCTTTGTTTTAAATACATCAACAAAGTCTT
GCATACTGCTGCACCGCTGATGCGATTAAAGGGAATATTACATAATGCGCGCATTTTACTTAGCTGCTCATTGTCGTAACCGTTACGTACGCC
ATCTTGGTTCATACAGTTTAAAACAATCTCGCCTGCGCCTAAGTCTTGTACGCGTTTAACCCATTCCTCGGTTTTATAACGTGTACGGCTTGA
TGCATTAGGATCGCCGGTTAATTGATACACTAAATACTCGCCGGTCACCTCATCGTAAAAGCTATCAATACCTACAACAACACATTGCTTACC
AAATTCATCATGTAGCTCTTTTATAAGCTCAGGGCGGGCAATAGCGGGGCTGTTAATACTTATTTTGTCAGCGCCACGCTCTAGTACACGTGC
TGCATCGGCTACCGATTTAATGCCGCCAGCCACACAAAATGGAATATCGATGTGGCGGGCAATACTCTCTACCCAATTTACATCAAGTAGGCG
TTTTTCAACACTTGCGCTGATTTCGTAAAAAACCAGTTCATCGGCACCGGCATCGCTGTAGGCTTTTGCCATTGTTAAAATATCGCCAACAAC
TTCATGGCCTTTAAATTTAACGCCTTTTACTACTTGGCCGTCTTTTACATCTAAACACGGGATTATGCGTTTTGATAACATGCCAACGCCTCC
TCAACACTAAATGCGCCATCGAGTAGTGACTTACCTAAAATAACGCCACCTACGCCCAGCTCTTTAAGCTTTTTAATGTCATCAAGTGAGCTT
ACGCCGCCCGAAGCTTGCACTTTAATGCTGCTGTTATGGCGAGTTAAATCTTCGTAAAGCT

The blastx search addicted a homology of the DNA- sequence to the bifunctional protein *phosphoribosyl-AMP cyclohydrolase (N-terminal)*; *phosphoribosyl-ATP pyrophosphatase (C-terminal) [Alteromonadales bacterium TW-7]* on the left region with an E- value of 1e-100.

The right region showed a homology to *imidazole glycerol phosphate synthase subunit HisF [Alteromonadales bacterium TW-7]* (E- value: 4e-120). Searching in the Conserved Domain Database gave following results: The bifunctional protein *phosphoribosyl-AMP cyclohydrolase (N-terminal)*; *phosphoribosyl-ATP pyrophosphatase*is a member of the superfamily cl00344 and cl00345. This enzyme catalysis the third and second step in the histidine biosynthetic pathway and requires Zn ions for activity. *Imidazole glycerol phosphate synthase subunit HisF* (member of the superfamily cl09108; *TIM barrel proteins*) is the cyclase subunit of imidazoleglycerol phosphate synthase (cd04731), which catalyzes the fifth step of histidine biosynthesis. Steps 4 and 6 of the histidine biosynthesis pathway are contained in the *pfam00977* family (Multidomain).

Clone 12)

Sequenced: 713 bp

Sequence length according to restriction digest: 1.5 kb

Missing: 790 bp

CTCTGATGATTCAGCTTGATTCGTTGGAAATTATATAACGGTAAAGTTGAACTGCGCCTTAAACTACATTAACTATTTTACATAAACAAAAAG
CGCGAAAAACGCGCTTTTATAGTGACTAAATTCAAACCATTATACAGAGCCGTTAATAGGCCCGTGTTCGTGGTTTACATGCGGGTTTTGCCC
GCGCTGGCGCAGTAAATGGTCCATTAATGTAATAGCCATCATAGCCTCAGCAATAGGTATTGCTCGAATGCCCACACACGGATCGTGACGACC
TTTGGTGATCATCTCAACCGCTTCGTTGCTTGTATTAATGCTCTTACCAGGGATAGTAATACTTGACGTCGGTTTTAATGCAATAGACGCTAC
TATGTCTTGCCCTGTATAAATACNNNNNNNNTCTGATTGGATAGGCAGTACTGGATCACTTAACATGTAGTCATTAATGTTGATATCAGTTAC
TTCAAGCGCTTCATACGAAATATCAACAGCATCTACTTGCGCTTGCTTAAATGCTTGTGCAAGTGCGATGGCTATACAACCTGACCCTGTACA
TAAATCTAAAATACGATTTACCGAGTTTGGCTCAACAAGCCAAGGCGTAAAACGATTGCTAATAAGTTCTGCAAATGGTGAACGAGGAATTAG
TACACGTTCATCTACGTAAAACGGCATACCTGCAAACCATGCAATGTTAGTTAAATAAGGAACTGGAGTG

The blastx search addicted homologies of the DNA- sequence to *chorismate synthase [Alteromonadales bacterium TW-7]* on the left region with an E- value of 4e-41 and *N5-glutamine methyltransferase [Alteromonadales bacterium TW-7]* on the right region (E-value: 8e-53).

Searching in the Conserved Domain Database resulted in a specific hit (cd07304) in terms of *chorismate synthase*. It catalyzes the seventh and final step in the shikimate pathway. Since shikimate pathway enzymes are present in bacteria, fungi and apicomplexan parasites, but absent in mammals; they are potentially attractive targets for the development of new therapy against infectious diseases such as tuberculosis.

N5-glutamine methyltransferase (cd02440) is an S-adenosylmethionine-dependent methyltransferase. These enzymes use S-adenosyl-L-methionine (SAM or AdoMet) as a substrate for methyltransfer, creating the product S-adenosyl-L-homocysteine (AdoHcy).

Clone 13)

Sequenced: 1278 bp

GCAAGGTGTGATCATATTATCAAACTCATCGTTTAATGCTTTGCATAAACCAATGAACGCGCTGTTTTTAAGTATTATTCGGTTGTTTGTTTT
CTATGTTCCGTTTGCTTATTTAGGTAGTCATTTTGCAGGGTTAACGGGATTATTTATAGGTGCTGCTGTCGGTAATTTATTTACCGCATTGGT
AGCGTATAAATGGTTTATAAAAGAGCTTGAGTCTTTGAGCAGTCAGTCGTTACAGGAGTGCAATAGTTGAGTGATCATTTTCAGTTAAAATCA
CAGTTTAAACCAGCAGGGGATCAACCCACCGCAATAAAGCAGTTGTGCGAAGGTTTAGAGGCCGGGCTTGCTCATCAAACATTGTTAGGGGCT
ACAGGTACGGGTAAAACCTTCACCATGGCTAATATTATTAGCGACTTAAATCGCCCACAATATTATGGCCCACATAAAACGTTGGCTGCGCAG
CTTATGGTGAAATGAAAGAGTTTTTTCCTAAAAATGCCGTTGAATATTTTGTTTCTTATTACGACTATTACCAACCAGAGGCGTATGTTGTAT
CGAGCGATACGTTTATCGAAAAAGATGCTTCAATAAATGAGCACATAGAACAAATGCGTTTAAGTGCTACTAAAGCACTTCTTGAACGCCGCG
ATACCATTATTGTTGCATCGGTTTCAGCTATTTATGGTTTGGGTGACCCCCAGTCATACATGAAAATGATGTTGCTTTTAAAAGTAGGCGAAA
AAGTTGACCAGCGTGACATGCTTCGCCGCTTAGCCGAAATACAATATACCCGTAACGACATAGAGTTTAGCCGAGGTACTTACCGAGTTCGCG
GCGAAGTGGTCGACATATTCCCGGCAGAGTCAGAAACATACGCAGTGCGGGTAGAAATGTTTGACGACGAAATAGAGCGCTTGAGTATTTTTG
ATCTATTTATCCTAAAACGCACTATGTAACTCCGCGAGAAAAAATTCTAGGCGCAATCGAAAATATTAAAATAGAACTAAAAGATAGAAGAGC
TCAATTACTCAGTGCCAATAAATTGGTAGAAGAGCAGCGTATTGCGCAGCGCACTCAGTACGATATAGAAATGATGACCGAGTTGGGTTATTG
CTCGGGAATAGAAAACTACAGTCGATATTTATCGGGGCGAACCCCTGGCGATCCGCCACCAACATTGCTTGATTACCTGCCTGATGACGCATT
AATGATTATTGATGAATCCCATGTAACGGTGTCGCAAATTGGCGCTATGTATAAAGGCGACAGAAGCCG

The blastx search addicted a homology of the DNA- sequence to *excinuclease ABC subunit B [Alteromonadales bacterium TW-7]* over the complete clone with an E-value of 4e-55. Searching in the Conserved Domain Database resulted in two specific hits. The first is named *DEAD-like helicases superfamily* (cd00046). This is a diverse family of proteins involved in ATP-dependent RNA or DNA unwinding. This domain contains the ATP-binding region. The second *Helicase superfamily c-terminal domain* (cd00079) is no autonomously folding unit, but an integral part of the helicase. All helicases share the ability to unwind nucleic acid duplexes with a distinct directional polarity. This family is the C-terminal region of the UvrB protein which conveys mutational resistance against UV light to various different species.

Clone 14)

Sequenced: 1494 bp

ATTCAGCTTGATTCGTTGTTAATCTACCGCTATTTTATTTGGTCGCTTTGACCGCCATATCATTTTTATATTAAATGCTCTATTTATCGCACT
TGTTGCTTATATTGGGTTTATTAATCAGTTAAACATGGCTTTTTGGGCTGGATTACTTATAGCCACAGCATTACTCGCTTACCAACAAGTATT
AATTTTAAAACGTGATAGAGACGCATGCTTTAAAGCTTTTTTAAATAATCATTATGTGGGTATGGCTATTTTTATTGGTTTATTGCTATCGTA
CCCCATTAGCATTTAGTTATATTAACGAGGTGGATCAACTATTCCGCTAATGGATGCGTTTTTTCTAAACCAGCCAGCAATTGAATACCGATT
ATCTACTGCTGGTAATACTTCGTGAACAAAGCGTTCGCTTTCAAATAAAACTAATGTCCCCGCTTTTGGCGAAATGCTCACTTCAACCACTTT
GCTTTTGGGTTTATAAATAACAAGCTCACCGCCCTCATTGGGCGTGTTTAAGTATAAAACGCTAGTAAATACCCTATTAGAGCGCCCTTTAAA
TGCATCGACATGTTTTTTATAAAAAATCACCGTGAGTGTATTTTGCGTAATGACTTTCTTAATCAAACANNNNNNNNTTTATAAAAATCACCG
TGAGTGTATTTTGCGTAGTGACATTCGTAATCAAACAGCCCCATAAAAAAAAAAGTGCGATTGAGCGTTGTGCGAATACTCTCCATAACAGCTA
GGTAGGTTAATTGTGCTTGGGATGAACCATCAAACCAATAAGTTTGATCTTTTCGAATATTATCGTCAATTTGTAAATGATTTAAGCGGCCAA
TACCAGCATGGCTAAAATGCGGATTTATGCGATAACAATCAGCCATTAAATCAGCTATTAATTCTTTGTTTAATGCATTAGGAATAACTGTAT
AACCGCGGCTTTCAATGTCGTTATAAAACGGGCCATCGGCTGAAAAGTTGATCATTTAGGAGGTTCTTTTTTTGAAGGTGCGCCAGTTTAGCC
GAAAAAGAGTAAAAAGCTATTGTTTTGTGTCGCACCTGTCTCAAAAAAGAACGCTGAATTAATTAAGGGTTGTGGCTGCTTTTTTAATAAGTG
AAAGCAAATGTGCTAAGTGCTCCGTAACCGTGGTTTGTTGGTTTGTTTGAACAATTAGTTGCTCATGGCTTAAATCTTTATTTGTTTTTAACT
GAACAAGGTCGGTGTGTTCTGCCACAGTGTTTGCATGCTCAATGCTGGCGATTGCAAAACTATCGGATTTAATAATGCCAGAGATAACCTGTT
CAATACTGCCCATTTTGATAGTTGATGATTTATTTAAAGAATCAATGGCCTCGTTCAGCATGCTTTGAACTTGTTTTGATTGAGAGAAACAAC
CAAAGGCTGAATAGGAGTCTAGTTGGTGTTTGCTTATGTCATTTTTTTGGGCAAGCGGGTGATGCTTAGATACAAATAAAATAAGCTGATCAG
GCAAATGAATATCA

The blastx search addicted homologies of the DNA- sequence to *putative prolyl 4-hydroxylase, alpha subunit domain [Alteromonadales bacterium TW-7]* with an E-value of 3e-101 on the left region and to *putative transcriptional regulator, LysR family protein [Alteromonadales bacterium TW-7]* with an E- value of 3e-70 on the right region.

In accordance with the Conserved Domain Database *putative prolyl 4-hydroxylase* is a member of the superfamily cl01206. These enzymes catalyze the reaction:

procollagen L-proline + 2-oxoglutarate + O_2 ⇌ procollagen trans-4-hydroxy-L-proline + succinate + CO_2

Putative transcriptional regulator, LysR family protein (cd05466) iscomposed of two functional domains: an N-terminal HTH (helix-turn-helix) domain, which is responsible for the DNA-binding specificity, and a C-terminal substrate-binding domain, which is structurally homologous to the type 2 periplasmic binding proteins. The genes controlled by *Putative transcriptional regulator, LysR family proteins* have diverse functional roles: amino acid biosynthesis, CO_2 fixation, antibiotic resistance, degradation of aromatic compounds, oxidative stress responses, nodule formation of nitrogen-fixing bacteria, synthesis of virulence factors, toxin production, attachment and secretion, to name a few.

Clone 15)

Sequenced: 1240 bp

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CACGTCTCCGAGACAGATATTCAGGCGCAGGGTAAAAAATTATGTGACGATAGCGCATCCGGTACTTTATTTGTAGATGAAGGCAATACGCCT
CAGGTTTTGGCCGATGCAAAAAACACATTTGATGCAACTTACACCACGGCGACAGCGAGTCACTACCAATTAGAGCCACTTAACGCCATAGCT
GAATTTAAAAACGGTAACTGGATTGTACATACGGGTAACCAATGGCAATCACTCACGTTACCTGCTTTAGCCAAAGCCCTCGATATAGAGCAA
AATAAAGTCATTATTCGTCCTTACTATTTGGGTGGTGGCTTTGGACGTCGTTTATTTGGTGATTGGACAGTCCCTGCTGCATTAACGGCAAAA
GCAATAGGTAACCCGGTAGCTGTATTTACCCGTGAAGATGACAGTTTATTTGATCAGTCTCGCTCAGCGTCTACGGCTAAATTAACCGCTTCG
TTCGGTGAAAAGGGAGAGCTTAGCGCATTAGAGCATGCGTTTGCTGCAGGCTGGCCAACTAAAGCAATGGCGCCAGGTTTTTTGGCTCCTGGT
GTTGATGGTAATGGTAAATTTGATTCGTTTTCTGCCTCAGGTGCTGATCACTGGTATAGCATGAATAACCATCGTGCTCGTGCTATCAATAAC
GAGTTAGCACAAAGCACTTTTACGCCGGGTTGGTTACGTTCAGTTGGACCGGGTTGGATTGTATGGAGCCTTGAGTCTTTTATAGATGAAATA
GCGCACAAGCAAGGTATTGATCCGGTTAAGTTTCGTTTATCAATGCTTGACGGTAAAGGAAAACAAGCGGGTAAAGCACCTGAAAGTGTGGGT
GGCGCGCGCCGTTTACATGACGTACTTAAAAAAGTGTCAGACAAAGTTAGCTCAATAAAGCTTAACGAAAACGAGGGCATTGGTTTTTCGGTT
AGTTCAGGGCAAGAGCGCCACATGCCAGCATGGATTGGCACTGCAGCCCATGTTCACGTTAACAAAAGCGATGGCAAAATCACCCTTAAAAAG
CTATACGCTGTTGTTGACGCTGGTTTAATTGTACATCCTGATGGTGCGATGGCTCAATTAGAAGGGTCATTATTATGGGGAGCGAGTATGGCC
TTACATGAGTCCAACTCTTATAAAAATGGCCAAGTAGCTGCTACAAACTTTAATACTTACCTTCCACTGAGAATGCAAGATGTACCCGAAATG
GACATTGAGTTTGTGCAAAGTGATGAGTTCC
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The blastx search addicted a homology of the DNA- sequence to *Membrane-bound aldehyde dehydrogenase, large subunit [Alteromonadales bacterium TW-7]* with an E-value of 0.0 over the complete clone. This protein is a member of the superfamily (cl10595) *Molybdopterin-binding domain of aldehyde dehydrogenase*. Its function is energy production and conversion.

Clone 16)

Sequenced: 1454 bp

GTTAATGTATCGTCAAGTAAGGCGCTATTTTTAACTTGCTGACAGCTTACTACTTTAATGCGTTGATTAGCTGCTAAGCCTAATGTGTTAATG
CTTGCTAGCACGCCGTGGGCGATAATGTCATTCGCACACACAATGGCGTTAATAGCAGGGTTGCTTTTGTAAGCCTGTGCAAAAGCATTTTTG
GCACTAAGTGCTGAGCTTTCATCACATTCGATTGCGTTTAGTGGCGATATACTGTGTTGGGCACATGCGGTATTAAAGCCATGTTGAAGTGTT
AAGCAGCTGCTTAATTGTAAATTGCTGCCTATAAACGCAATGTTTGTAACCCCTTGAGAAAGTAAATGCATGCTAACTATATGGCTAGCTTTT
TTGTAGTCAGGTAACACACAAGGTGCATTACTAAATGCAAGCTCTTGCATTAACGTAACAACGCTATGCTTAGAACTTAATGTGTCTAACCAT
TGGGTGCATGTATTAGTCACAGGGCAAATTATAAATCCGGCGGCGTTGTATTCTTTTAGTTTATTAATTGCCTGTGTTTGCTGCTCAACGTTA
TTGTTACTATCTTGCTAACAATAACGGCTGAAAACCTTTCATGTTTTAATATTAGCTCAAGCTCGGTGGCCCAACTGTGCCATACAGGGTCAG
TTAAATCATTTATAACCACAGCGATTAAATTAGAGCGCTTTGAGCGCATAGCTGCGGCATCGCGATTATAAACGTAGCCTAACTCATCAATTG
CTTTTAATACTTTTTGTTTGCTTTTATTACTGACTTTTTTACTTTGAGTCAGCACCAAAGACACCGTTGATTTAGACACACCTGCAAGGTCGG
CAACATCAAAAATTGTGACAGATTTAGCTTTGTTATGCATAGTTGTGTTTGTCGCAGTGATAAATTAAAAACGCGTTAGTGTAGCTTAAACTG
GGCTGTTAAGTCATCTTTTGCCATGTGTTGAGCCACCTTAATGCTTTATGAACAAAGCAAAACGTGTTTATATACACTTAATTGTGTAAAAAA
TAACAAAAGGATAAAAACATGAACGCTTTTAGGCTGTTAGTTGCCTTTATTTGTTGTGTTGTATTACTACCATTTAGTCATGCAGAGCAGGGC
TACCAAACTCCTTCGCCTGATTTAGCGGCAGTGGTAGATGCTAAATTGGCGCCCACAAGTTACTTATCAAGTGATGGTCAGTGGCTTGCGCTA
TTTGAACGCAGGCGTGTTGTATCGCTTGATGAGCTTGCAATTGAAGAGTTAAAGCTTGCGGGGATTAAACTAAACCCGGCTAATTTTTCACGC
ACACGGGTGCGCGCAAATTTTAGTGCTGTAGAACTTAAACATGTGCAAAGTGGCACTGTTATTAATATTAATAACTTACCAAATGGTATTATT
CGCTCGCCTAAGTGGTCTTCTAATAGCAAATACTTAGCGTTTATTGTGAACAAACAAAC

The blastx search addicted homologies of the DNA- sequence to *transcriptional regulator, LacI family protein [Alteromonadales bacterium TW-7]* with an E-value of 2e-136 on the left region and to the *hypothetical protein ATW7_02277 [Alteromonadales bacterium TW-7]* with an E- value of 9e-69 on the right region. The *transcriptional regulator LacI* (specific hit cd01392) represses the expression of enzymes involved in lactose catabolism. When lactose is present in the bacteria's environment the repression is released and enzymes for lactose catabolism are expressed. The structure of LacI has two domains: a helix-turn-helix (HTH) DNA binding domain (superfamily cl02583) and a ligand binding domain (superfamily cl10011).

The *hypothetical protein ATW7_02277* is a member of the superfamily *Esterases and lipases* (cl12031). These enzymes act on carboxylic esters.

Clone 17)

Sequenced: 656 bp

Sequence length according to restriction digest: 1.4 kb

Missing: 740 bp

CGCGTTGGGTAGTCGTTGACGTAACCTACCACCAACATTTGAATAAACTGGTTAGCTTAAAATCTATAAAAGCAAACGACAGTATTACAGAGT
TGGCACTAAAAAAAGCAGGAAGATTATCGGTTATGCCCGTAACAGAACACGATTGGAATGAAATTATAAAAATGGCCAAATAGTAAAGGTCGA
GTTGCGCTTATTTTTTTACTGCTTTTGTCGTGTTGTGCGCGTGTTTGGGCACAATGGCAAAATACATAATAAGCCCTAACCACGAAAAAAAGA
TGATACCGATGAGCCAACCGATTTTTTCGTGGACTTGTGAAGGTTTACTNNNNNNNNTGGGCTTGGTCAGTGCAGTTCGAATGACGCGCAAAA
TCAACCTGCTCATTTGCTAAAAACGGATGATCCATCTGCTCTGTTACCGCGAGTAAAATACGACGTTCAGTGCGCTTTGTATCCGCCAAAATG
TAATCAATCATTTTTTTAGTTCTGGTATCGTGCCAACCAAAAATAATGATGTGGTTATTACTGTGAGCAAAATCTTTATCACCAGTCATAGCG
TGCCTAATTAAATAAGTAACAGTTTGCCCTGCTTTTCCAAGCAAAACACCAAATAAAGCTAAACCAAACGGAATTTGTATAAAGCAACACGAA
TCTCAGATCGCTA

The blastx search addicted homologies of the DNA- sequence to *hypothetical protein ATW7_15371 [Alteromonadales bacterium TW-7]* with an E-value of 1e-24 on the left region and to the *putative potassium channel [Alteromonadales bacterium TW-7]* with an E-value of 7e-48 on the right region. Searching in the Conserved Domain Database provided no results.

Clone 18)

Sequenced: 1308 bp

Sequence length according to restriction digest: one fragment at 1.0 kb and second fragment at 250 bp, caused by an EcoRI cleavage site within the sequence (labeled red)

ATTCGTTTGCCTAATCCCTTACACATATAAAAGGCAAGCCAGTGAGCTAACTTGCCTGATGACTGATCCATGCTCTCTCCTAGAGCACCGACT
CGCTACTTCTTAATGAATAACTGCAATATCTCCAACGCGAATTGGGTGCTGGTTTTTTGTTATTAATGCATAACTTACTTTGTCATACACTTT
GAATACGATTAACTCGCCTACTTTTTCTTTAGGCATTTTCCATGTTGTATTATCTTCGTCAGCTTTATTACTAAACAACTGACTTGCATCAGA
TAACAGTTTTTCAAATTTACTTGAATCCTCTGGGTAACGAGGACCACGAGGGCCATCAATAACCGTAGGTGACTCACGCTCTAAATCTAAAAC
ATGACCTGGTTCAATATTTTGCTCGCTGCCTAAGTTTAGCACTACAACATCCATCGTGCTGAATTCTCTTACTTGGCGAGAGACGCTATAACT
TGCCGTTCTACTGGCTCTAGTGGGCGGTGAATATCAAAATAGGGCTGGCAGCAAACTGCCCCTCCATTGCTGGCATTAAAAAAATCACCTTTG
TTTAATTTCTCGTTTTACTGACTCAACACGTAATGACGACGGCACACCATTACTTTCATCGCCACTTCTAAACGCTCGCGCCATGCCAACGTA
CGATGCACGCGTTGCTAATACTTCGCCTGTGTTTAAATCTCTGTAGGGTTCACCCTCGTTGTAAATCGCATAAGCACCATGTACTTTTAAGTC
ACCTTTAACGTATAAAATATGGCCTAAAGTTTGGGTTTTTGTGTTTTCGTTAGCACCTAACACATACGGCATATTTTTTATATCGTCGCTGTT
AATCGCTTGCTCGTAGCTTAAATAAGGTTTAATCATTTGCAAAGGTAAGGTGGTAATTGCATTCAACCCTTTTGGGGTAATACGTCCTTGAGG
TGACAGCTTTCGGTACGCTTTATTAATAACTAAACGTGGATTTCCGTCAGCATCGTATATAAGTGCCAACGCATCACCCGGATAAATTAGGTG
AGGGTTATCGATTTGCGGGTTCATTTGCCATAGTTCTGGCCATAACCATGGCTCATCTAAATAAATGCCAGATATATCCCACAACGTATCACC
TTTTTTTACAACATACTGTTTTGGTGCATCTTTTTTTATTTTTAACACATCAGCAATCGCCCCAAAAGAGGTGCTTAGTGCCAGTATGATTGC
AACTAACGGAAATTTCATTGAGCTTCCTTGTATTGTTTTTTCATTATTATTGCCCAAAACCTGTTAAAGGTGAACGTGAATGGCTAAAATAAG
AGGACG

The blastx search addicted a homology of the DNA- sequence to *hypothetical protein ATW7_16418 [Alteromonadales bacterium TW-7]* with an E-value of 0.0 over the complete clone. According to the Conserved Domain Database this protein is a member of the superfamily *Lysin domain* (cl00107). This domain was found in a variety of enzymes involved in bacterial cell wall degradation.

Clone 19)

Sequenced: 1750 bp

Sequence length according to restriction digest: 1.9 kb

Missing: 150 bp

ATGATTCAGCTTGATTCGTATTATTATTTGCCACTAAGTCAGCAAACGTTTTTGCTGTTAAAAACTGTATACGCTGGGTTATTTTGCCTTTCA
TTGTGTCGGGCGACGATACATTATAAACATGGCTATACAACTGCTTTAATAACGCATCTAAACTGTAAAACTGGTTATTACGGGCTTGTTGTT
GCGCTAAACGATTTAAGCGCTGTGGATTAAGCAACAACGATAAACTATGCTGCACTGCCACCTCTGGTAAAGCCATTGCATCAAGTGTTAACC
CTGTACGCCCCACTACGCTTTCGCGCGATTTAGACTCACCATATGCTTTTGGCGGAATCAACGATAATACCGATTCAGGTATAACTAAATTTT
TGGCAGTAAGCGTTGCTAAAATGGCATTAACTGCATCTTGTTGCACTTGCTTATCAACAACCTGAGCGCCTTGGACTGGCTCATCACCGCGTG
TTTCATATTCGTAATTTACACCTGCAATTAACTTAACCACAGACTCAACTTGAAAACGATGAAATAAATAAAGTGGTACAAGCTTTTCTTCTA
ACTGAGATAGCGCAACACCCAACTTAATATTATTAATACCAAACTGCGAGAGTGCCTTTTTACGAACATCTAAAACGCGTAAAAGCTCTTCTG
ATGGATTAGCGCCGTTATCCCATAAGTGAGCAGTTGGATGAGCGCCCCCTTTAGCTCGCGCGTCAGAGTCTGAAATAAACGCTAGCCCTTTGG

CTTTATTCTCTTTTAAAATTGCTGCCAGCTCTGCGTTTTCATCTTGATTAGTAAAGTCGCTATAACCGTATTTAATTACCTGGTATCCCACAC
GCCCATACCTGTTGCATAGCCTCGTGTTACATCAGCTTGCTTGTNNNNNNNNTATTTAATTACTGGGTATCCACACGCCCCATACCTGTGCAT
AGCTCGTGTACATCAAGCTTGCCTTGTTCATTAAAGCTCAATAATGGGTGCGGGTAATCCATTACTGAGGCTCTATCACTTACTGATGCCGCA
AAGTTATGGGCAATACCTAATGTATGGCCAATTTCATGGGCACTTAACTGGCGAATACGATCAAGCGCCATAGCGTGTAAGTTTTTAGCAACT
TCATCGCCTTTAAAATAAGGAGCAGCCAGCGCTTCTGCAATTAAAATATCTTGGCGAACGCGAAGCGATCCCAGCGTCACATGGCCTTTTAAT
ATTTCACCGGTACGCGGATCAATAACTGAGCTTCCGTATGACCAGCCACGTGTTGCCCTATGCACCCACTGAATCACGTTGTAACGAATATCC
ATCGGGTCAACATTGGTTGGTAATACTTTAACAATAAACGCGTTTTTATAACCTGCAGCGCTAAATGCATCATTCCACCACTTACCGCCTTCT
AGTAAGGCGCTTTTAACTGGCTCGGGTACACCGGGATCTAAATAATAAACAATAGGTTCAACCGGCTCGCTAATAGGTAAGCTCGGATCTTTT
TTTGCTAAACGGTGACGCGGTATATAACGAACATACATCGACTCGCCTAAAGGCGCTGCGTAATCTTTGTGTTCAATGCTCCAAAATCCCGAT
TGCGGGTTAAATTTACGTGGTATGTAATTATCATCAGGTAATTCAATAAGTGAATGGTGCATATGCACGGTAACAGTGTATGGGTCGGCGCTA
ACTTGGCGCACAAACTCACCCGGCTTTGCGCCTTTAAATGTAAGCACGGCTTCAAGTTCGGTGTTTTTTAAAAAAGCTTTGAGC

The blastx search addicted a homology of the DNA- sequence to *hypothetical protein ATW7_18525 [Alteromonadales bacterium TW-7]* with an E-value of 0.0 over the complete clone. Searching in the Conserved Domain Database resulted in a specific hit *Zinc-dependent metalloprotease* (cd04276). This specific hit covers a group of bacterial metalloproteinase domains similar to matrix metalloproteinases and astacin.

Clone 20)

Sequenced: 1336 bp

Sequence length according to restriction digest: 1.9 kb

Missing: 560 bp

ATGATTCAGCTTGATTCGTATATGCGGACTCACATCTGGGCGTCTAAACTTAACCGCATTCGAGAGTAAGTTTAAAAAAAGTTGCTGCATTTG
GCTTTTATCTGCTTGAATAACAGGCAACTCTGCCACTTCAATTTGTGCATCTGACTCTTTTATTGCAATCTCTAAGTCGCTAAGAATATCGTC
AACTACTTCATTTAAGTTAGTATCATCAAAGTCTTTACCACGCGTAGTAACACGCGAAAACTCTAACAAGTCGTTAATTAAGTTCGACATTCG
CTGTGCGGCGTTTTTCATACGGCCAATATAATCTAAGCCTTTTTCGCCTAAATCATCTTTGAACATGGTTTCTAACCGGTCACTAAATGCTTG
AATTTTACGCAGAGGTTCCTGCAAATCATGGCTTGCCACAAAAGCAAACTCTTCCAACTCCCGGTTACTTCTGCTTAACTCGTCTGAATACAA
GGTTAGCTCTTGCGTGCGTTCAGATACTTTACTAGCCAATGTTTCGTTTTGTTTCTCAAGATCAAACCGATATTGAGCGGCATTTCTGAGGTT
TAAGCGTGTCATTACAAACATGCCTATAATCAGTATTGCACTAGTAATAGCGGTAAATCGCAAACGTAATTTTGGCCTCGTTTTTGATTTTGA
GCAAGCTTTGAAAAATGGCTCACTTTTAAACAGAAATTCACGGGTCTTGAATATCATCTTACGCGTNNNNNNNNACCACAATTATAAAAGCCG
CCCACGTAACATTCGCTTTACCTTGTTGTTTTTTGCATGCTTATTTACTTTCCTGTTTGCGCATAAAACACGCTTTCATCATTGTGAATCAAC
AATTTTTTGTAAGCTTTCACTGCATTGTTGGCAATTGCTAATAATTTTATTTAGTGCCTCGAGTGCCTTTTCTTTTGGCATATCGTTTTCAAG
TACTAACTTAATTAGCTCAGCATTCATTGAAATACGATTTAAAGGGGCTCTTGCATCATGAACTAATTTATTAAGCGGCTCCGATTGATTGTT
CTGTGTCATGTTACCTCTCAACTTATTTATACTCGCCAATACCACCGTAAGCATTAAGGCGGTTATAAAGTGTTTTAGTGCTTATACCTAGCA
TTTGTGCGGCAAGTGTTTTATTCCCTTGTACCTTGTCGAGCGTTGCATGAATGAGCTCTTTTTCAACTTCTTCTATGGTTTGCCCAGCCGACA
CTGCGTTATCAGCACTTTGATTATTGACACGTGAAAATGGCGACTCTAGATTATCAGGCAGTGTCACTTCGCCTGATTTAGGGTCACTCATGA
TAAAGGCACGGTGAATAGCATGGCGAATTTCACGCACATGCC

The blastx search addicted homologies of the DNA- sequence to *putative two-component sensor histidine kinase [Alteromonadales bacterium TW-7]* with an E-value of 9e-108 on the left region, to the *putative orphan protein [Alteromonadales bacterium TW-7]* with an E-value of 1e-29 and *putative sigma-54 interacting response regulator protein [Alteromonadales bacterium TW-7]* (E- value: 5e-47) on the right region. The *putative two-component sensor histidine kinase* belongs to the *Histidine KinaseA (dimerization/phosphoacceptor) domain* (specific hit cd00082). These proteins subsequently

transfer the phosphoryl group to the Asp acceptor residue of a response regulator protein. Two-component signaling systems, consisting of a histidine protein kinase that senses a signal input and a response regulator that mediates the output, are ancient and evolutionarily conserved signaling mechanisms in prokaryotes and eukaryotes. The *putative two-component sensor histidine kinase* is also a member of the family *Histidine kinase-like ATPases* (specific hit cd00075), which includes several ATP-binding proteins and of the superfamily (cl05000) *CHASE3*, an extracellular sensory domain, which is present in various classes of transmembrane receptors that are parts of signal transduction pathways in bacteria. *CHASE3* domains are amongst others found in histidine kinases.

The *putative sigma-54 interacting response regulator protein* belongs to the (specific hit: cd00009) AAA+ superfamily (ATPases Associated with a wide variety of cellular Activities). Members of the AAA+ ATPases function as molecular chaperons, ATPase subunits of proteases, helicases, or nucleic-acid stimulated ATPases.

This protein is also a member of the *Signal receiver domain* (specific hit: cd00156). This domain receives the signal from the sensor partner in two-component systems.

No conserved domains have been identified for the query sequence of the *putative orphan protein*.

Clone 21)

Sequenced: 1536 bp

Sequence length according to restriction digest: 1.7 kb

Missing: 160 bp

CTTAGTAAGGCGTTAGGTGAAATGAGAATGCGGCTAATATTCGCTATATTTATATCTATAACAATAAACTCAGGTTGTAGCCTGCTTGAGACC
TCAGCTACTGTAGGATTAGCCTCTGGTCATGTTGTATCTCAATCAAGTAAAGAAGCAGCTGCACAGATAGCACCTACTTTATATGCTGATGAG
ATTAATATAACGGAATTAAAAAGCACTATCTGTACAACACTAGATTCTAAATTTGGAAACCGTTGGTATTATATGGGCAGTGACGATGAGTAT
GATTATGTGGTTACTTATCCATTTATAAAGTCACAGCAGATATATAAAATACTACGAGGTGCATTGTTTTTAAATCAGACGTTTGAAGTAACC
CAAAACAAAAATGATTGGATAACGATTGTAAAATACAATTATATGAACCCAGCAAACAATCACTTCCATTGGCAGTTAGAATGCAATGCTTCT
ACTAGCAGTAATTTAAAATTTGGTGATTTTTCACCTAACAAGTCATTTCAGCAGGACAAATAACTGTTGGCTTTGCTCCTGCGTCGCTATTTT
AGCCAACAATTATTTGCCTCTGAATGAGGCGTTATGTTGCTGGGTAAGTTATGGCCTCTTTAAAATCATTAAAAGGTGCGGCGTACGATATTG
CGCACCATGCTCAGAGTGGATGAGTATTTACACCCATATATGGCCCAACTGTGCGAAGAAAAAGGATTAAATTGAAGCTAGCGTCAATTTATT
GGATAGCAATCCTTATCCCATGTGAACTTCGCAGAATTGATCAATTGGAATCGTCGTGTATTGCATTGCAAGAGAAGTTCAAGGACATTTTAG
CCAAGTTAAACTTACTACTATCTGAAGTACGTGGTTTGCATTTGAAATTTAGCTTTTCTTCAAGTATGGATGATTATTATACATGCTCTGTTG
AGAGCACACTATTGAAGCAAGATGGAGCAAAGTTTGTTCAAAATGTTAGGTAGGCAACATAACAAAAACATAAAGACGGATTTGGCGGAAAAG
CGCCGCCAAACCGCTTATGTAAAGTGTTAGCAGTACAGGAAAGTTCAGTGTTAGAAATAGGTGTCGAGGAAAAATCAGGAAATTTTATTGGGA
GTGTAATTCACCTTCCATCTAATGCCTTGGTCAAAAGTTCATCTAAAACAGCCGAGAAAGCCGTATCTTTGTGTTTACGAGATCTTAGTAATC
AACTTTCTAAATTACAGTGGTTTTATATCCAAGTATCTGAAGGTGTAATAACTGAATTATCAATAAATGGTCTCTTTGATTTTTCTTGTTCTG
CCGTGAGTGTTACCTCATTTGAGGAGTATCTAGATAGTCAAAAAGTTATGCTTTCTTCTCTCCATCAATTTAGTTTCAATAACAAAAACTATT
CTGGTTATGTTTGTAATCCCAATAGCTATCAGAAACTTATCGAGAGTATTTTAAATAATTCAGAGTTTGAGTTATCGTACTGCTAACAAGGCA
TTCAACAGGACTAAAAACAGCGGGCTTTTCGCTTCGCTCCATTTAGCC

The blastx search addicted a homology of the DNA- sequence to *ATP-dependent DNA helicase [Blattabacterium sp. (Blattella germanica) str. Bge]* with an E-value of 0.034 on the right region. This protein is a member of the Rep family helicases (cl14126). Rep helicases catalyse ATP dependent unwinding of double stranded DNA to single stranded DNA. *ATP-dependent DNA helicase* also belongs to the Multi Domain *ATP-dependent exoDNAse,* which is involved in DNA replication, recombination and repair.

Clone 22)

Sequenced: 1364 bp

Sequence length according to restriction digest: 1.5 kb

Missing: 130 bp

TATTAAATCACTTTCACTTTCTTGGTTGTCACCTTGGTCAATGCCCTGATAAGTGCCTCCGTCACCAACCCAATGCTTTGCGGTAGAGTAAAT
ATGATCGTCATCTAAAAAGTTAGCTGTGTTTACTTTCCCCTGAAGGCCTTCAATCATCTCTTTAGCATAGCTGGCAACTATTTCAGGGTCTTC
AGAATAACTTTCGTAGGTTCTCCCCCATCGATCATCCCTTGCAACGGCAAGTGTTGGGGCAAATGTCCAGTCTATACCTGCAAGTAAAACTTC
TTTCGCTGTGGCTTCTCCAATTTTATAAATTAAATCTGGGTTACGTGTTGCACCTAATCCAATGTTATGTGGATATAAAGTAGCACCAATAAA
TTTGTTATTGCCGTGTACTGCATCAATGCCATATGTAACGGGAATAGCTAAACCGCCTTGTGATGTATCCATTGAGGCTAAATAAAGCTCGTC
CATAAAAGACACCCAATCAGATACACTCGCATTACGGTTATTGTGTAAAATGTTCCACCACCATTCAAAACCGATCTAAATGATATTTTTTTT
ACATCTTGAGGCGTTACCAGGTTATTTCTGCTTGTACCATTTGACCCACTTTTTGTTCTAGTGACATTTGGCTTATTAAATGGGTAATACGTG
TTTCCAACTCTGCATTTTTTTTTATTGCTGGTTCAAGAGTTGGCCAAAGTGTTATATCGTTTTTAGCAACTGTGTTAACAGACGCAAAGCTCA
TAAAAGAGCCAAGTAAAAATGAAGAATAAGTTAATTTCATTAATCTAATAACCTGATACTTTGTTAGTGTGGCAAACAGTTTTATAACAAACG
CGGCATAATGTCGTTCAAATAGAGGTTTTAAGCGTATAAATGCACGCAATTTACTCTATTTGAACGCCCGGCTATTTTAAATATTAGAGATTA
TTAGATAACATACAACGACAATGAGAACACACGTGTACCTAACAAAAATTTTAATAAAGGTGTATTAAATTGTGAAAGAAGTACTTTTTAACA
CTCACGATCTCGCTTTAATTTTTACCATTTTTATATGCTTATCATTTAGTGTATTTTTAGTAACATTAAAAAAGGGTAATAAACTTAGTAACT
ATTTACTTGCTTGCTTTTTATTAACTCAAGCTGCAATTCCTACGGATAACCTTATTAACTTTGGTGAGGCATTCCGAGCGTTTGCGTTGAGTT
TATCCCCAAATCTATTTTACACATTTGGATTAGCTTTTTGGTTGGAAGTCCCTTTGCTTCTGCTTTATATCAAATCGTTAGTTAACAAAAACT
ATCAGCTAAAAAAACGCGATTCGCTATTATTCATACCGTTCATTTTTTATGCCGCTTATCAC

The blastx search addicted a homology of the DNA- sequence to *1,4-beta-D-glucan glucohydrolase [Microbulbifer hydrolyticus]* with an E-value of 3e-70 over the complete clone. This protein is a member of the superfamily cl03393. This domain is involve in catalysis and may be involved in binding beta- glucan.

Clone 23)

Sequenced: 1402 bp

Sequence length according to restriction digest: 1.5 kb

Missing: 100 bp

ATGAWTCAGCTTGATTCGTGCATTTGTTGATTACGGTGCTGATCGTCATGGTTTCCTTCCTTTAAAAGAAATTGCCCGCACTTACTTCCCTGC
TGGTTACACCTTTAATGGTCGTCCGAATATTCGTGACGTGATCAAAGAAGGCCAAGAAGTAATCATTCAGGTTGATAAAGAAGAACGTGGCCA
AAAAGGCGCAGCGTTAACAACTTTTATTAGTGTTGCTGGTAGTTACTTGGTACTTATGCCGTATTGAAGGTGAKGAGCGTACCKAACTTAAAG
AAGCACTCRGCCGTTTAGAACTACCTAAAGGTATGGGCTTAATTGTGCGTACTGCGGGTGTTGGTAAATCGTTTGAAGAATTAAATTACGAKT
TAAAAGCGTTATTAGTTCACTGGGAAGCTATTAGACAAGCTGCCGACAGTGCTRAAGCGCCGTTTTTAATTCACCAAGAAAGCAACGTAATTT
TCCGTGCGATTCGTGATTATTTACGTCGTGATATTGGGSGAAATTTTAATTGATAAGCCACGTGTTTTTGAAGAAGCTAAGCTCACATGAGCGT
TTCGTCTGACTTATGAGCCGCGTAAGCTTTACCAAGGCGACACACCGTTATTTACGCATTACCAAATTGAAAGCCAAATTGAGTCTGCGTTCC
AACGTGAAGTGCGTTTACCATCAGGCGGTTCAATTGTTATTGACCCTACTGAAGCACTTACGTCTATCGATATCAACTCGTCTAAAGCTACAA

AAGGCGGCGATATTGAAGAAACAGCACTTAATACTAACCTTGAAGCAGCTGACGAAATTGCACGCCAATTACGTTTACGCGACTTAGGTGGTT
TGATTGTTATAGATTTCATCGATATGACACCGCCACGTCACCAGCGTGAAGTAGAAAACCGCTTAAAAGACGCTGCGCGCCCAGATCGTGCTC
GTGTACAAATTGGTAAAATTTCTCGCTTTGGTTTACTTGAAATGTCGCGTCAGCGCCTACGCCCTTCATTAGGTGAAGCAAGCCAAGGTGTGT
GTCCTCGTTGTAGTGGCCAAGGTACGGTTCGTTCTAACGAGTCTATTGCTCTATCTATTCTTCGTTTAATTGAAGAAGAAGCAATTAAAGACA
ACACTTCTCAAGTAAATGCACAAGTACCTGTTGCTGTTGCCGCCTACTTGTTAAACGAACAACGTCGTAGTGTTCACCGTATTGAAAAGCATC
ACAAGTGTGACGTAGTGATTATTCCTAATCAACACATGGAAACACCACACTACGAAGTAATGCGCTTACGTAAAGACGAAACACTTGAAACGG
TAAGTTACGAGCAAGTTGTTGCGCCTGAACCAGAAGCGTTTGAAATGTCAAAATCGCCTGCAGCACCTGTTCGTGAAGAGCCTATGCTAAAAG
GTGTTGT

The blastx search addicted a homology of the DNA- sequence to*RNase E: endoribonuclease for rRNA processing and mRNA degradation; member of the degradosome; involved in the production of deoxyribonucleotides via the formation of NDPs [Alteromonadales bacterium TW-7]* with an E-value of 0.0 over the complete clone. This protein is a member of the *S1_RNase_E: RNase E and RNase G, S1-like RNA-binding domain* (specific hit cd04453). RNase E is an essential endoribonuclease in the processing and degradation of RNA.The N-terminal region of RNase E contains the catalytic site.

Clone 24)

Sequenced: 1301 bp

Sequence length according to restriction digest: 1.4 kb

Missing: 100 bp

TGCTCGTACTCTACTTTTATTTGCTTGAATGCATTCTCGCAGCCAGTAGGTTTGTCTGGGTGGTGCGCCAGTGCAAGCTGTCGCCAACGTTTT
TGTAGTTCTTTAAGCGAAAAATCATTTGTTAGCTGCCACTTTGCCAACAGTGCGTCAAAGTGATCTTCTGTTAAAGCTGACGCGCTATTTACC
TTCTTAAACTTTAGCCAAAAACTTGTGAGTAGCTCATTAATTTGCTCTTCGCTTGCTTCGTAATTCCCCCAGTCTAAATAATAATCTTTTAAG
TTTGATTTAACCGTTGCAGGGCTTTCTATGTCTGTTTCAATAAGTTCTATATGAAGTGTTGATATTAAGAGGCTTTTAGGGTGAAGTTGTTCC
TTTAGTTGATACAATGCATTCATAATTAAAAAATTACGCTTAAATAAATCACGTTCAGGGTTTTTATCAAGTGCTAAATTGCGCCACAATAAT
AAGCTCAGCTGCTAGATATGAACTTGCCACCACTTTTTGGGTTAATATTAAGTCAAATATTCGGTCAATCAGTGGGTTAAGCATTGTTTAGGT
CTAGTATTTAGTGTATCTTCAGTACAGATTACATTGTTATACGTAACAATTTGAAGGATTAAAATTATGCATGGCAAGCTCAAGTGCCTCTTA
TTCATTGTATGCAGTTTGTTCTATTTGCCAAGTTATGCTCAAAAATCAGAACCATTATTAGAAGAGTTTAAACAACAACAACAATGGGATGCA
AAGCTTGAATTAGGTAACGCCTTACTTAATAACCAACGTATTACAACTCAAAGTCGTGCTGTAATATATGCAGGCCTTGCCGATACTGCGCTG
TCGAGCGGCCACTATAGTGATGCTTTTAATTACTTTAAATCATTAGAGCAAGAAACTTCTGGATCACTTCTTCCTAGCGAGCACTTTCGTGCG
ATTAAAATGCAAGGTGTTGCACTTTTTTATCAAGGCTTGTTTAAACAAGCAATTACTCAATATACCCGTGCACTTGATATTGCAAAAATTAAT
AACCAACCATTAGAGCAAGCAAATTTATTGAATAATATAGGTTTGGTTTATTTTAATATGCATAAATTGGTAGCAACGCTTGATTATTATTTA
AAAGCAAAAGAAATTTACGAAAAAGTAGGCAGTGAACAAGATAAAAGCGATATTTTACTTAATATAGCAGGCGTTTATATACGCTTATCTCGT
TATGAAAATGCCATGAGTATTTATCAAGATGTGTTAAAAATTTTTCGCAAGTTAGATGATAATTTGGGTATTGCTTTGGTGCATAACAATAT

The blastx search addictedhomologies of the DNA- sequence to*putative DnaJ-class molecular chaperone [Alteromonadales bacterium TW-7]* with an E-value of 3e-79 on the left region and to *putative membrane bound sensor or esterase [Alteromonadales bacterium TW-7]* with an E- value of 2e-122 on the right region. *Putative DnaJ-class molecular chaperone* belong to the superfamilycl02542, *DnaJ domain*. Proteins counting to this domain are highly conserved and play crucial roles in protein translation, folding, unfolding, translocation, and degradation.

*Putative membrane bound sensor or esterase*belongs to the *Tetratricopeptide repeat domain* (Specific hit cd00189).These proteins are involved in a variety of functions: protein-protein interactions, chaperone, cell-cycle, transciption and protein transport complexes.
Further functions are identical with those described in Sequence 20) according to the membership to specific hits cd00082 and cd00075.

Clone 25)

Sequenced: 1268 bp

GGATTAATTTTAGGCCCTGTGCTGTTAATTTGGTTTGCTATTTTTATTTACTCAATTCGACTCGGTTATTTGCTTATTTATAAAAATATGGCT
TTAGAGATCACGCTAATTACTTTCACCGTTGCTTTAGTTGGTGCTATTGCCTTTGTATTTTATGGTTACAGGCAATTTATAAATGAAACGTCG
TTGTGGGCATTTGAAATACCCAGCTATTTTGTATTCAACAAATTTGCAATATTCACAGCTGTTTTGGCTTTATGTTTACATTTTTTTTCTACT
AGTATGCAAACAAGCGCAGCCGTTAATTGTGTGGTGTTTATACTTTTGTTTATGTTTTCTGCTGCAGTGTTATTAAGTGTGGGGTTACATGAC
CGTTTTATAGGTTATAACAATATCCAACTTACTCATTAATTCAAGGGTAGAAGATGAGATCGTTATTTGTGCTGTACATTGTGTGGTGCTCAA
TGGCTAAAAAAGTTGACCCAAATAATACGCAAACGGGGTACAAGGCGAGTGTCTAATCGGTATGGTGCTATCTATTTATATTGGTTTATTTGC
TCTTTTCTTTTATTTTGATTATGTAAAATTACTTACTGATTCCGTTAGAAATAATTTAATCGTGTTCAGTACACTTTATTTAGCGTTAATTTT
AGGGGGTATGCATTTAGCGTTGGGTAACGCTGTTGATTATAAAAGCAGAGTTCGTGCAAGGTTGAATACAACAGTGCTTAGGCATTACACGCT
TGGGTTAAAACTTTGGATATTTGGTCTGATACCTTACTTCATAACTTTGGTAACTATTTTTATATTGAACTCGAATTAGTACTATTCTTTTCT
GCGCTTTAGCGTGCTTATTTTCGTATCTACCTCAAATTAATTGCGATATACAAATACATAGGTAATTGTAAACCAATAAAAATAAGGAGCTAC
ATAAATGCTTAAACCATCAATCAAATTATTAAAGCTAAGCATGCTTGCGTTTGCTTGCTCAGCATCAAGTGGCGTTATTGCGGCTGATATGCA
ATATAAAGATCAACAAAATCTGCATGATATTGCGGCAAGTGTGTCAGCAGAGCGAATTGGCAGCGATATTCAAACGCTGGTGGACTTTGGTAC
ACGTCATACACTTTCAGAGACTAAATCTGACACTCGAGGTATTGGTGCTGCTCGACGTTGGATTAAAGCTGAGTTTAAAAAAATATCCGCACA
ATGTGGTGGGTGCTTAGAGATTATTGAGGTATCAGACACTATTTCAGGCGAAAAACGCA

The blastx search addicted homologies of the DNA- sequence to *hypothetical proteins ATW7_13613 and ATW7_13608 [Alteromonadales bacterium TW-7]* with E-values of 3e-66 and 2e-47 on the left region (no conserved domains have been identified for this query sequences) and to *leucine aminopeptidase-related protein [Alteromonadales bacterium TW-7]* with an E- value of 2e-53 on the right region.
The *leucine aminopeptidase-related protein* is a member of the *Fibronectin type 3 domain* (specific hit cd00063), which is found in bacterial glycosyl hydrolases.

Clone 26)

Sequenced: 1727 bp

Sequence length according to restriction digest: 3.0 kb

Missing: 1280 bp

GTATCTATGCCTATTAATTTATCTTGAAAGTTAAATTGATAGTAATGTTCTCGAGGAGTATCAAACGCAATAGCTTGCTGGTTATGAAATACT
TGTACTATTTGCTGCCCAGAAACCATTAATGAAAATGGCTGGTCAGCCAAGGTTAGGTCTATTCTTATCATAATTAAAGCCATTTAAAATAAT
CGCGCTAGTATAACGTTTGGCTAAAAAAACACGAGGGCAGGCGTTAACTATTGTATTAAATTTAAGTACCTAACAATAGCTAACGCTGCGAGT
GAAAGGTTACTTTTCTTCTTCAGGATGTTCGTCTTCTTCACTTACAATATGCGCACTGCTGGCCAAACGCTTACGCCAGCGTTGCTTTGCCAC
TGCACGCATATTGGTTGACTGATCGCGTTCGTCGACTATTTCTAAGCCCATTAGTGATTCAATCATATCCTCAAGCGTTACTATGCCGCGCAC
TTCTCCATATTCATCCACGACTAAACATATGTGCGTACGCTGAGTTAGTAGCGTTTGCAATAATGACGATACATTTTGAGATGTGGGTACTGT
ATAAATTGGTTTGATCAGCTTACTGATTTTATAATCGGTGCCTAAGCGATGATACGCCAACATAATATCGTTTTTATGCACGAAACCGACAAT
GTCGTCGGCATCTTTATCAAATATTAATACGCGGGAAAACGATGACGAACCGTGCTCATTTAAATATTGGTTTACTGTTAAGTCTTTATGTAC
TTTAAAATAACTGTGCGCGGCGTCATTAAATCAGACATTTTGCATGCCTAAATGTAAAGGATTACGATAATTTCAGACTCATCTTCGTGCAAA
GCGCCCGATTCAGTGCCATATCGGCNNNNNNNNCAAGGGTGTTGCGCCAAGGGCTTGCTGCTACATGAGCAAAGCGAGGGTCTAATCGCCTAT

AACTGCTCGCAAGCGATAAAAACACATACGGCAGTACAAACAATAAATGGGTAAATACCACATTAAAAAACGCGGCTTGGTTATTTACTAGTT
GTTGCAGCCACACTAGGCCAAATAAAAACGCAATACTTGGTACAAGCAGCGGTAAATAAATAATTAAGCTGGTAAAGCGCGACATGGGTTTTG
CACTTAACTGCTCTGACTCTAAGCACAAAAGGGTAATAATAATGGCAAATAACGTAGTAACTAGCCCTATAGCAAGGGTATTAAAAAGCGGGC
TTTGCATTTGCATTAGCGCACTTTTAAAGTGAAGTAGCGTGAGCTGATCGGGCAGTGCCGCCGGAAAACGCCAAAATCCGGCAACCGACCACA
TAATTAAGCCAATTAATGAAAGCAAAATAAAGCCAATTACAAATACTGTTAATACGGTTGTTATTTTTTGCCAATAAACACCGCCATATTCTC
GCTCGCCATTGGTGAGTAAATCACTAAATAATGCTTTTATGGTTTTTTCGCCGCCAAGCCAAAGTGCTAGCAAACCGCCAGTAAGGACAAGCT
GCAATAACGCCCCCGCAGAGGCTTTAATACGCAGGTTTAAATCTACATCGTTAAACCAGTGCATAATAGCCACTGCCAAGGTGGGTGGTGTAT
TTGGCCCTAGAATAAGTGGCATTTCTACACTTGCGCTGGCATAAGCCAATACGGCTAAAATGGGTAGGCGCAGTAATGGGTAAAGGCTTGGTA
ATACTACTTTAAAAAAAGCAGTCATAGGGCAGTAGCCTAAATTAAGCGCGACACGAATCTC

The blastx search addicted homologies of the DNA- sequence to *hypothetical protein ATW7_15406 [Alteromonadales bacterium TW-7]* with an E-value of 6e-75 on the left and to *putative ABC-type transport system, permease component [Alteromonadales bacterium TW-7]* with an E- value of 5e-131 on the right region. Searching in the Conserved Domain Database resulted in a specific hit (cd04590) for *hypothetical protein ATW7_15406*. This specific hit comprehends two tandem repeats of the cystathionine beta-synthase domains associated with the CorC_HlyC domain (transporter associated domain). The function of the CorC_HlyC domain is uncertain but it might be involved in modulating transport of ion substrates. The cystathionine beta-synthasedomain may play a regulatory role, although its exact function is unknown.

Putative ABC-type transport system, permease component is a member of the superfamily cl00427. This family includes domains with a transmembrane subunit (TM) found in Periplasmic Binding Protein (PBP)-dependent ATP-Binding Cassette (ABC) transporters which generally bind type 2 PBPs.

Clone 27)

Sequenced: 1348 bp

TGATTCAGCTTGAATTCGTCCGTATTTCTAACCTTCAAGCGTTTAAAATTAACGTAATACCACGCAAAATGCTGGTTGATAGTATCGATACTA
TTATCAGCACGTCGCATCAGTTTGAGCAAGGCGACACCCTTGATTTATTAAGCCAAGAACCTTACAGCGAAAGCGCGGTAAACGAAGCAAAAG
ATCACCTAATAAAACTCGCCTCAGACAAAGCAGGCCTGACATTAACTGACTTGTTCGATATTCGTTTTGAAGTGGTAAACCGCGCTGGCGAAA
CCGAACACTTTGAAAAAATAGACTCAGCCGGCTCAAATGGCACACGTATTACTATTAAGTTATTGTGTGGCATGTTGTTTATTCGTTACTTAT
TAAGCGATCAGGAACAAAACCTGTACCGTATACCTATTTATATTGATGAAGCGGCTGACATTGACCCGCAAAACCAAAAAGCAATTATTAGAA
ACCGCATTAGCTTTGGCTTGTGCTATATTTGCCTCAGTTAAGCCGCAAATAAGTTGTGACTACATTGTGCCTATTCGTTCGGTAAAAGATGGC
GCACAAAACTGGGTAGATGAAAAAGATTGGATAGAAGTAGAGCATTAAATTTTTGAGTGGCTAATTAGTATTAATTAGTCATTCATATTCATC
ACACTAAACTAATTTTTAGAACCTTTTAATAATACACATTTATATAAGGTGTGTTATTTTAAGGTAATGCATTTTATTGGAGCAAACGTAATG
AAAAATATACTTATTGTTGCAGCACTAGCACTACTTAGCGCTTGTACTCAAACCCCTGATTGGGATTACGATAAATCAGCTAACTTTAGTAAT
TTTAAAACATTTGCTTGGGTAGAAAACGCGAGTTTAACTAAAGACACCAACAACTACCAAATTAACGGTTTAATGGAAAAACGTGTGCGTACT
GCGGTAAATAATGAGCTTACAAACACCCTTGGTATGACATTAGTAGATCCAGCCCAAGCTGATGTACTAGTTAACTACCATGCGTCGGTAGAT
AAAGAATTAGAAGTAGATACATTTAATACTGGTTACAGTAACCGTTGGGGTTACTGGGGTACTGGTTTTCAACGAGACACAACAGCGCGTGAA
TACGAAGTGGGTACATTGGTTATCGATATTGTAGACAGTGCATCTAATCAGCTAATATGGCGCGGTGCTAAAGAAGGCCGTTTAAAGAAAAAA
CAATCGCCAGAGCAACGCGAAGCGTCAATTAAAAAAACAGTCGCAAATATTTTAAGCAACTTCCCGCCGCAAAAGCAGCAGTAATTTTAAAGT
TAGTTTTAAAGTTAGTTTTAAAGAGCTCACAGTATGTGAGCTTTTT

The blastx search addicted a homology of the DNA- sequence to*hypothetical protein ATW7_14201 [Alteromonadales bacterium TW-7]* with an E-value of 2e-98 over the complete clone (no conserved domains have been identified for this query sequence).

Clone 28)

Sequenced: 1270 bp

ATGATTCAGCTTGATTCGTGGCTGATACTCAATACCGCGTTGTTGGGCATTAAAATAACGTATTGGACGAATTTGTTTATTAGCTAAATGTTG
CCACAGCTCAGTAGTAACATCGCTTTGATGTGCGACTTGCTGGTTATTACGCCAAAGTGTAAGTACCTGACTACTTTGCTTATTTTTGTGGGT
GTTGGTAATTGTGTAGTCGGCTTTTAAATACTGGCTATTTAGGTTGCACTGAGTGGCAACTGCGCTGGCGCTTGTTAGTGTGGCAACCAACAT
GGTTGCGAGTAATGATAATGCTGTTTTCATGGTGCTCTCTATATTTAACGTCACTGTTATTGCCATAACATAACCACTAACAATGGCATTTTT
ATGACATAAAAAAGCCCGCAAAATAATGCGGGCTTTAATAGCCTCTATTTACTGAGCCACTGTTTCATCGGCTGTTTTAACTAGGTCTGCGGC
AAAGTCCATTACATGGAATAAAAAGCTTTGCTCGTTTGTGCCTGTTACTAAATGTGCACCTGGGCCCATTGCGTAAACACCTACATCTTCACC
CGCGTGGGTTTCTGAACTAAGTGGTACTAACGACTCTTGGTGAAAGCCCGGTGTGGTTGTATCAACATTTGTTAAATCAACACGCCCTGTAAC
CGGTGCAGCTAAGTAAGAAGCGTCGGCATTGGTTTCATCGCCTAAGTCTCTGTAGCCTAACCCATTGGTGTAACCTACTGTAGTATATGGCAT
GTCGTCGGCTGCAAGGCTTGGCTCGGTTTGGCCAACTGCAACCACTTTACCTAATATTGGGTTACCACGTTTTGGGTAGCCTGCAATAGTAAA
TACATGGCTGTGATCGGCAGTCACCACAATCAGCGTTTCTTCTGGGTTAGTGCTGTCCATAGCGGCTTTAACGGCTTTAGCAAATTCAATGGT
GTCGTTAAGCGCGTTGTATGCATTGCCTGCGTGGTGTGCGTGATCTATTCGCCCGGCCTCTACCGATAAGAAAAAGCCGTTTTCGTTATTGTT
AAGCACGTTGATTGCTTTTTCAGTCATTTGTGAAAGCGAAGGCTCGCCCGCTACATCGTTTGCACGGTCAGCTTCGTATTGCATGTGAGATTC
GTTAAATAAACCAAACACCTTTGTAGCATCGTCGCTAATTGCATCAAACCCTGTTTGGTCCATTACGTAGTTGCCATTAGGGTAAGTAGCTTG
CCACTCGGTTACTAAGTTACGGCCATCGGTACGATCCCCTTCAACTGCGCTGATTGCATCG

The blastx search addicted a homology of the DNA- sequence to *alkaline phosphatase [Alteromonadales bacterium TW-7]* with an E-value of 2e-161 over the complete clone. Searching in the Conserved Domain Database resulted in a specific hit (cd00016) for this enzyme, which catalyzes the hydrolysis reaction via a phosphoseryl intermediate to produce inorganic phosphate and the corresponding alcohol, optimally at high pH- values.

Clone 29)

Sequenced: 1358 bp

AGCTTGGTTTAGAACTCGACCTTGATGATATAAGCTTATCAGCACTCATGCCCGAAACGCTTGCTATAGGAAGTTGGGACGACTTTTTAGCTA
ATGAATCCACCCTCAATGACTTTATTGACGCTCATGCTGCAAATGCCAAAGCACAAAGTTCCGTACTTCGTTACACGGGTTTACTTGAAATTA
CGGAAAACAAAGTAACCGCTCAAGTAGGTATTACTTATACTCCTATTGGTGATGCGTTAGCTAATTTAACACCTGGCGATAATATTTTTGTCA
TTAATAGTAAGTGGTATAGCGCTAACGCTCTTGTGATTCAAGGGCCAGGTGCGGGTAAGGAAGTAACAGCGGCGGGTATTCATTCTGACTTAT
ATTGGCTGGTACAAAATATAAAATAAAGGTTACAAGGGCCTTGTATCAATCCGCAATAATACCTAATCATTTTGCGGGGCTAAACGTGTCGCT
ACATGCGTTAAAAAATTCTTATTAGAACAGCTAATTTGGTATTAACTAAAACGGCAGCCAATGGCTACCGTCTTTATTTAAAACAGTCTCTTC
TGTTTTAGTGTCTCTCAAATATAATCTGTTGGTTGAGACATTACGTATTTGTGGGGGCGGTGCCTTTGTCAAAATACTCAAAGCGACTGGTGT
AATCATTTTTATGACTTAATAAACCGGTTTCTAAATCAATCCGAATAGACACTAAACCTTCTGGCGGCTCTATGGGGGCTTCAGGTTTACCTT
CAAGCGCAACTTTCATAAACTCAACCCAAGCTGGCTGTGCTGTTTTAGCGCCAGATTCAGCCCCTGATATTTGCCCGCTATCAAGATTATTAT
TATATGAAGTTCTGCCTAGCGGGTTGCCAGGGTTATCAAAACCAACCCATACCGAGGTCATCACATTTCGATTAAAGCCACTAAACCAAGTAT
CTACAGAGTCATTAGTTGTGCCTGTTTTTCCTGACAGATCACGACGGCCTAATGCTTGAGCACGCCAACCTGTTCCTGACCAACCCGTTTTAT
GTGACCAGTTTCCACCTCCCCAAACAGCGCTATGCATTGCTTGAGCAATTAAAAATGCATTTTGTTTTGATATGACCTGAGGGGCACATTTTA
ATTGTGGCGTATCAGTATTTTGTTGTGGGAATAATCCAGTAGCATTATCTGGGGTTAATGGCTGAGCTGAAGTGTCGTCACACACTACTGAAG
GATTAGCTTTAAACAAACTGTTACCGTAAGCATCTTGGATTTCTGAGATAAAGTACGGCTCAATCAAATGCCCACCATTCGCAAATGCACTCA
TACCGCGAGCTAGTTCAATTGGAGTAATTGAAGCACTGCCCAGTGCTAAGGATCAC

The blastx search addicted homologies of the DNA- sequence to *bifunctional aspartate kinase II/homoserine dehydrogenase II [Alteromonadales bacterium TW-7]* with an E-value of 4e-66 on the left and to *Murein polymerase, bifunctional murein transglycosylase (N-*

terminal) and transpeptidase (C-terminal)[Alteromonadales bacterium TW-7] with an E-value of 5e-143 on the right region. The *bifunctional aspartate kinase II/homoserine dehydrogenase* is am member of the superfamily (cl00452) *Amino Acid Kinases* (AAK) (catalytic domain). The AAK superfamily includes kinases that phosphorylate a variety of amino acid substrates. The enzyme is also a member of the superfamily cl09931, whose function is already described under Sequence 7).

Murein polymerase, bifunctional murein transglycosylase (N-terminal) and transpeptidase (C-terminal) are known to belong to the the superfamily cl07896, which includes bifunctional penicillin- binding proteins consisting of transglycosylase and transpeptidase in the N- and C-terminus respectively. The transglycosylase domain catalyses the polymerisation of murein glycan chains.

Clone 30)

Sequenced: 1124 bp

CTTGACCTTTTGAAGAATGCTATTTTCAACATATTTGTGAATACGACGAAGATGCACAGGCATACGTAGGCGCAATGAAAACCCACTTTTACG
ATAACGAATATACTCCAGAGAGCTTAGTGCTGTTGTTGTGTCAGCGTTTAGAAAAATACGCGAGTGTTGCCGCACAAAATGCAGTTGAAACAC
CGCTTTCATTATTTAATTTATTTTTTAGCAAACGTTATTTTACGCTCATATGAGCATGCTTTAAAATAATGGCAGTAAGGCTAGTGCTGTTGA
TGCGTTATCGCTATGGAAAGAAGTGATTTTAGAGATCGCTTAAGTCACTTTATACTGAGTCAATTGCTGACTTAGAAGTGTTAAGTGGGCTTG
CAAGTGACAAAGTAGCAATGGGTAAAGACTATTTTACTTTATTGAAACAAGTATCGCCTGATGTTGATTCTGTGGTTTTACTTGAGCAAGCGC
TCCTAACACATTTACGTAAAAAAACGGCCAAGCAAACAATGGCTATGCGCTTTATTGAGCAAGGTGCAACAGGCACACTAGCTGATGATAACG
GTAAAACAGCATTCATGTGGGCGGCAGAGCGTGGTTTTGTAAACGTTGTGCAAACCTTATTACCTCAGCAAGATAAAAAAGCCCTTGATAGCC
TTGGTAATACTGCATTGCATTATGCTGTGCTCTCTAAAAATGAATCGTTAATGGTGATGCTAATAAAAGCAGGCTTTGATTTTAGAGCGCGTA
ATAACGAGGGCCTAAGTTGCTACCGATTGGCTGTGAGCATTAAAGCGCAAAACCTAGTTAAATGCCTAGAGCGTGATTTTGGAATTAAAGAGC
TCTCCCCAGAAGGGCAATTTGATCGTATTAAAAAAGTGCATGTTTTACATGCTATTGTGACTTTATTATTGCCATTACAGTTATTTTTCTTTT
TTGATGAAGCGCTGACCATAAAAAGTGAACTGACGCTTGGTTTAACTATGGTTACGGTGATTTGCTTTTTCTTTGCTATGAGCTTAAAGCGCT
GCGCTTTGTATCCACATATAAAACACCCTTGGGGGTTATTTGTATTGAGGGGCTTTTCGTTATTAAGCTTATTAGCTCAGCTAGGTTTAGCGG
GTATAGTG

The blastx search addicted a homology of the DNA- sequence to *putative membrane protein with ankyrin repeat [Alteromonadales bacterium TW-7]* with an E-value of 2e-167 over the complete clone. This protein can be counted among ankyrin repeats (Specific hit: cd00204), which mediate protein-protein interactions in very diverse families of proteins.

Clone 31)

Sequenced: 1177 bp

ATGAATCAGCTTGATTCGTGTGTATTTTTATTAGGTTACGGTGTGTTTAGGTTTGGTATTGAATACTTCCGCCAACCAGACGCACAACTAGGC
TTATTTGCCGACTTTATCTCTATGGGACAAATACTGTCACTGCCTATGATTATAGGTGGTTTAGGTTTATTAATTTGGGCTTATAAAAACCCG
CAGCAAGTAACAGCAGCAAAGGCATAATAAAAGCATGAAACAATATTTAAAATTATGTCAGCGTATCGTTGACGAAGGTCAATGGGTAGAAAA
CAAACGCACAGGCACACGTTGCTTAACCGTCCGTATAAACGCGGATTAGATATGATGTAGCAAACAATCAGTCCCGATGATCACAACGCGTAA
AAGTTACTATAAAGCGGCCATTGCAGAGTTACTTGGCTATTTACGAGGCTACGATAGCGCAGAACAATTTCGTGAAATTGGCTGTAAAACATG
GGATGCTAATGCCAACGATAACCAAGCATGGCTTAACAACCCAAATCGTAAAGGCGAAGACGATATGGGGCGGGTTTACGGCGTCCAAGGGCG
CGGCTGGCAACGCCCTGATGGCTCAACACTCGATCAGCTAGCTAAAGTAATTAACAATTTAAAAAATGGCATTGATGATCGTGGTGAAATAAT
CACGTTTTATAACCCAGGTGAATTTGAACTAGGTTGTTTAAGACCTTGCATGCACACGCATACGTTTTCACTACTAGGTGACACGCTTTACTT

AACATCTTATCAGCGCAGCTGCGATGTGCCGTTGGGGCTAAATTTCAATCAAATTCAATGTTTTGTATTGCTTGCATTGGTCGCCCAGATTAC
CGGCCATAAACCGGGCAAGGCGTACCATAAAATAGCTAACGCTCATATTTACGAAAATCAGCTCGAATTAATGCGCGACGTTCAGCTCAAGCG
CGAACCATTTGAATCGCCTACACTTACAATTAATCCAAATATAAAGTCACTTGAAGACATAGAAACATGGGTTACCCGAGATGATTTTGAAGT
AACAGGCTATCAATGCCATGACGCAATTCAATATCCATTTTCAGTATAACAACCGATTAAGGAAAACAACATGAAAGCAGTGATCCCAGTAGC
GGGACTTGGTACGCGCATGCTACCAGCCACAAAAGCAATCCCTAAAGAGATGCTTCCTGTC

The blastx search addicted a homology of the DNA- sequence to *thymidylate synthase [Alteromonadales bacterium TW-7]* with an E-value of 1e-157 over the complete clone. Thymidylate synthase (TS) catalyzes analogous alkylation of C5 of pyrimidine nucleotides. The enzyme is involved in the biosynthesis of DNA precursors and is active as a homodimer.

Clone 32)

Sequenced: 1770 bp

Sequence length according to restriction digest: 1.9 kb

Missing: 130 bp

ATGATTCAGCTTGATTCGTACGTTTAATATCATTGAGTGATATATCTAGGCTGCGACAATGCTTTACGAATTCCAATTGTTTAAGTGCTTTAC
TATCATACAGCCTGAAATTACCTTCTGAACGGTTTGGATTTAACAGTAATCCTTCTCTTTCATAATATCTAATCGTTTGTATTGAACAGCCCG
ATTTTTTTGACAATTCGCCTATTTTCATCAATTTCCCCCAGACATTTGACTCTATAGTTACTATAGGATCTATAGTATAGGTATGCAACGAAA
GGGATATATAAAAATGACTGAGAAATATTTAAACAATACGAATATCGTTAATTTTGAACACAAAGATATCCAATCCCTGATCGATCAACGAGG
GTGGAAAAATTTGGATAATTTCAACAAAATTGGTGCCGCTTATCAGTTTGTAAAAGATGAAATAATGTTTGGCTATAACAAGAGTGATGATAT
TTCAGCAAGCGATGTGCTAGCAGATGGTTATGGACAATGTAATACTAAGGGTAATCTGTTAATGGCACTATTGCGTGGACTAGGTATCCAGTG
TCGTTTCCAAGGTTTTACCATTGATCAGCAACTATAAAAAGGTGCTATCCCAAGCTTGTATTTTGGTTAGCACCTAAATACATCATTCATAGT
TGGGTAGAAGTTTACTTTGAAGGGCGTTGGATAAACTTAGAGGGCTTTATATTGGATGAGCAATACTTAAGCTCACTACAGCAGAAATTTAGC
CAAGCAAAAGATGAGTTTTGTGGTTATGGTGTTGCAACAAAGTGCTTATCTTCACCCGGTACGGATTGGCGTGGAGAGGATACCTATATTCAA
AAGAGTATTCATGACGACTTTGGGCTTTATGATTCACCAGATANNNNNNNNGTTCTATTCTCGGTGTAAACGTGTGTATCACGAGTGATCCGA
CATTGATATATGATGTATCAAAATAGAAATAAAAAAGTACTGGAGGTACAAAATGCACAACCATAGTCACAGTCATCAGCATGGTAAAGACGA
CCGGGATTGGTTGGGCATTTTTCCTTAATGTGATATTTACCATTATCGAATTTATAGGTGGTTGGCTCACAAACAGTACGGCGATAATGGCGG
ATGCCGTTCACGATCTTGGTGATAGTCTTTCAATAGGTTTTGCGTGGATTTTAAGTCGTTTTTCAGATAAAGAAGCATCAGACAAATTCAGCT
ATGGTTATCGCCGACTTACACTTTTTGGTGCATTGGTAAATAGTATTGTTTTGATTATCGGCTCTATTTGGGTTTTATTTGAAGCAATACCTA
AGCTGTCCAATCCTGAAATGCCTGTAGTTGAGGGGATGTTAGGGCTAGCCATACTCGGTGTTGCCGTAAATGGTTATGCGGTATTCAAATTAA
AAGCGGGTGAAACACTAAATGAAAAAGTGCTGACATGGCACCTACTTGAAGATGTACTCGGTTGGGTTGCCGTTCTTATAGTCTCTATTGTAT
TGTTGTTCGTTGAACTGCCGATATTAGATCCTCTATTATCAATAGGGTTTACGCTGTTTATCTTATTTAACGTTTTCAAAAATCTTAAATCAA
CATTGGTGCTTTTTCTTCAAGCTGCACCCGATAAAGAGACCCAAGAGCGTATTAAGCAGTCATTAAATGAATTTCCTGAAGTAAATGGTATAC
ACCATATGCATTTTTGGTCACTGGATGGTGAAAGTCACGTTTTAACAGCGCACCTTGAGCTGAGTGACAACTTTGACGTTAAAGAATTAATTA
AGCTAAACAAG

The blastx search addicted homologies of the DNA- sequence to *conserved hypothetical protein [Pseudovibrio sp. JE062]* with an E-value of 7e-61 on the left and to *cation diffusion facilitator family transporter [Kangiella koreensis DSM 16069]*with an E- value of 3e-96 on the right region. The *conserved hypothetical protein* is a member of the superfamily cl10469, which includes animal transglutaminases and other bacterial proteins of unknown function. The *cation diffusion facilitator family transporter* can be counted among the superfamily cl00316. Members of this family are characterized as integral membrane proteins that are found to increase tolerance to divalent metal ions such as cadmium, zinc, and cobalt.

Clone 33)

Sequenced: 1740 bp

Sequence length according to restriction digest: 1.5 kb

Missing: not determined

In this case there are more bps sequenced than expected after analysis restriction digest. Reasonably it can be expected that the missing section of the sequence contains EcoRI cleavage sites. The resulting small fragments were not visible on the gel.

GATCTTGTATACACCTTTACCAAATGAGCCTATGTTTGAAATAACGTAAACGTGACCTTGTTTAGTTTGCTCTGCCATTGACTTTGCGCGTTC
AGACAAATTATGTGCATTTTCAAGATCATTTTTAAGGCGAGCAATTTCGTTATTGAGGCTATCAAGCTTGTCTCCCGTTGCTTTTTCCGCACT
CCTTAGTGCTTTGTTGAGTAATTTCTGGTAATTTTCTTCTTCCTTCTGAGCTTTCTCCGCTTCTTGCTTGAGCCTTGCCTCTTCACGCATTTG
GCGTTTAATCTCTGCCTGTTCTTCTTTCTCTTGCTGTCTTTTGTCTTTATATTCGTGAGTTAGTCTCAATTCATTGAGTTTAAGTTCTAAATA
CTCGTTGCTGATAAGGGTATTGTTGGTTTCATTAAGCTTGTTAATAGACATAAATGCCTTAGTTATGCGGGACTCCATCCGAGTTACATTATT
CCAACTAACATTAGCCAACGCCGCATCACATTCGTTATTGAACGCTTTTGTTGTTAGCTTTATGTTTCGGTTAGTCATGGTTTTACCTTTGGT
CTTGCTACCTTCAACTACCCATTCAGTGTGACATGCAACAGCCCGTTTATCGGCTAGCATTTGTTTCTGCATAGCTCTTATTTTTTCAATCTC
TTTTTTATATCGTTCTGACGTGTCAAACGAAAAATGGGGTTTATAGAATCCCAGCTCCGCTAGTTCTATGGCTTCGTCATATATCGCAACTTG
TTGCAACAATTTATCTAAATAAACTTTTTTACTTGCGTACTCACGACTTAGTTCAGCCGTTTTTTCGTTTTGCTGCCTTGTTAAAGTTTTGCG
ATCGAACTCAAATTTTTTCTCTNNNNNNNNTTGCTCTCGAGTACTATTTCTCACGTGCGACGATGATCATATACGCGATCTAGCATGAGTATC
CCAATTTCTTTGTTAAATGCTAGCGCGATTTCTGGGCACTTACGCTTGATGACAGATCCAGCTTCAGTATATAAAGCTTCGCAATCAGAAAGA
TGAAAAGGTGTTGTACTGAAATTATAAAACGATTAAAACCGTTTAAACGTTTTTCCACAGCACATAAATGAGCACTTGCTACATCTCGAGCAT
CAATACCACGAGTCAGACGAAATACCGCCATTAAATCCGCAGCCTCAGGAAAACATCTCGACATTTGCAGCACAGTCACTGGAAGCTGAAACA
GGTTTGAAATTTCTTTTAGCTTAGTTTCAGCATCAATTTTACTTTTATGGTAAATCGACTTAGGTTGCGGAGTAACCTCCTCATTAATCCAAC
CTGAAATACCTTTCGGAGTCGAAGCATAACCGTACAACGCAGTTGTACTGGTAAAAACAAAGTGCTTAATACCTGCTTTTAAGCCAGACAAAG
CCAGTTTCTCAGTCGCGTCTACGTTGATAGATTTAAACTCAGAATCAGGCACTAAACCAACATGAGGAGCATGAAGAGCCGCTGTATGAATGA
TGACATCAATATTTTCAAGAACCCCATCAATCAGAGCACTATCACGAATATCACCAATGTAATCGGCTGTTGAACAAGGTGTTTTATCAATAC
CGACTACGTTATGTGTTCGCATTAATTTAATATAGATCGCACGCCCTACTCTACCCGCAGAGCCTGTTACCAATATTCTCATTTACAGCACCT
TCTTAATGATTTACCTCAAAGCAGCTAACAATTTAATAGTGTGCAAATAGCGTATTTATTTGCTATTGCTAGCT

The blastx search addictedhomologies of the DNA- sequence to*Chromosome segregation ATPase from phage origin,putative coiled-coil [Escherichia coli UMN026]*with an E-value of 2e-76 on the left and to*UDP-glucose 4-epimerase [Vibrio cholerae bv. albensis VL426]*with an E- value of 6e-104 on the right region. The *Chromosome segregation ATPase from phage origin,putative coiled-coil* is a member of the superfamily cl11176, which includes proteins of unknown function. The *UDP-glucose 4-epimerase* can be counted among the superfamily cl09931, whose function is already described under Sequence 7).

Clone 34)

Sequenced: 1171 bp

GAAAAATCGATGCAGCGCAAGTTGCCTCAAATTAAGCAGCAAATACCCATAACAACTGATGAATTAATGGACATTAATGATTTATTAGAATTT
GATAACCATATAACAGCCCCTTTGCATGGCTTTGAGAATGCACATGATTATTATCGTCAAGCCAGTGCTATGCCCTACTTAAAATATATTACT
GTACCTACCTTGATCATTCATGCGAAAGACGACCCAATGCTGTCGATTAAAGCGGTGCCTAGCAAGCAAGATGTGAGTGAAGATGTTACCCTG
TGCGTCTCTGAAAAAGGTGGGCACGTTGATTATAGGCGTAGTAATCGTTTAAAGCCCATTTTTTGGTAGAAAAAGCCGTACCCAGCTATTTGG
CCAACACATAATAAACAAAACGAGTAACAAGCAGTAATGATTATCCCTTTTGAACAACTTGATAAAGACACACTATACAACTTAATAGAAAGC
TATGTACTGCGTGAAGGCACTGATTATGGCGAGCAAGAGTTCAGTATTGAAAGTAAAGTAGCGCAAGTCAATCAGCAGCTAAAAAATGGTGAA
GCCATGGTATTTTTCTCTGAGCTGCACGAATCGGTCACTATCATCTCAAAGAGTGAATTTAAAGCACTGCAAAGTGAAGAGCACCAACAAGCG
TCGTATGATTAGAGTGTAATACTTGTAACTTTTTAGCTGAGCGTACACAATGGCTTTTTACCCAAAGAGCCAAAACATGATAAAACTCAGCAC
TCTAGTGGTAGCCGTATCACTAGCTTTAACAAACCAAGCCTTTGCAGAGCAACCTGTTAGCGCGTCAGACAAAGCGGTTAAACTTGCCAAAGA

CACTATTTTAATCGACACTCACATTGATGTACCTTACCGTATTCACGATACGTGGGCTGATGTAACTAAAGCCACTAATGATGGCGACTTTGA
TTATCCTCGCGCAGTAAAAGGTGGCTTAAACGCGCCATTTATGTCGATATATATTCCTGCCCATTTAGAGTTTGAAGGTAAAGGCAAAAGCTA
TCAGTTAGCTAATCAGTTAATTGATGGGATGGAAGCGATTGCACAACGCGCACCCGCTAAATTTGCAATGGCAAGCTCGACAAACGACATAGA
GCAACAATTTAAAGATGGCAAATTGTCTATTGCTATGGGGATGGAAAACGGCTCC

The blastx search addicted homologies of the DNA- sequence to *putative enzyme with alpha/beta-hydrolase domain [Alteromonadales bacterium TW-7]* with an E-value of 5e-55 on the left and to *putative periplasmic dipeptidase [Alteromonadales bacterium TW-7]*with an E- value of 3e-74 on the right region. The *putative enzyme with alpha/beta-hydrolase domain* is a member of the superfamily *Esterases and Lipases* (cl12031). Enzymes of this family act on carboxylic esters which includes proteins of unknown function. Searching in the Conserved Domain Database for *putative periplasmic dipeptidase* resulted in a specific hit (cd01301), which describes it as a membrane-bound glycoprotein hydrolyzing dipeptides.

Clone 35)

Sequenced: 1262 bp

GTAAAGTAATACCATTCTCTTTTGCTTTAGCTAAAAAGTGAGCTCTATTTTTTATGAGTAAAGGGTAGCGCCAATACGTTACGTCAGCATCAA
AACTCGCTTTTACAAAAAGAGAATTAATGATCTTTTTTTTGCAGATAATCCACATTTTTTTTTTCGAACTTGCATTTGCTTATTTAAATTTAAAA
AGCGGTCATTTAATATTTCTAATTGTTTTGAGCCACTAAATTGAGCCATTTTATTATCATTCAAAAACCCCTTAGCACATTCGAATAAACGCT
CAGAGTTAGTCAGAACAGCTCCGCCATATTGATTATCAATAGGTTTATCATAACCAAAACTCAAAATAGAAAGGTCGCCAAAACTACCTAATA
GTTTGCCTTTATATATACTGCCATACGCTAACACGCATCCTCTATAAATGTAACGTGTGCTGTTTGCAAAACTTGCTATTTCATCAATTTTAC
AATAGTGCCCGTAACCCATGAACGGCCCAATATTACTGATACAGAATCATTAAAAACCGGCTCTATATTATTGACTTCCATAGATAAAGAGCA
CAACGAAACATCAGCCAGAATAACGTTGTACCCTGCCATTTGAATAGCAAACAAAACAATCGGGCAACAGCTTGCCGGCATTATTACTGAGCT
CCCCTTTGGAGCCTGTGAGGCATTTAGCGCAGCTATAAGCGCAAAGCTGCCAGAGCTAGTCAATAATGCATGCCGACGGTCAAATTTAGTAGC
AATAAAGCTCTGTAATTGAGTTAAACCCATAACCTCAAATCCTTGCGATAACGAGATAAAGGAAAGCTCTCTGGCTGAGTAAGTATTTCTACT
TTTTTGACACAGTGCTGTAAATCATTTTCTAGTTGCTCAAAAGAAAAAAAGCGCCATAGAGAATAACGATGTATTAACGGAATCGTTTTTTCA
TATTCCAGATCACTTGAACTTTTTATATCATGCTCAAAGTGTTTATCTAGTATATCGCCAATCAAAATTACGCCACCGGGCCTGCATACTCGT
ATCATTTCTTTAATAACATCAAGTGCATATTGATAGCTTGGGAAATAGTGAAAAATACTGTAACTCAGCACTCTTTCAAACTCATTGTCTGCA
AACTGAAGATCACTGGCTTGTGATTGATAAAAGACGCCTTGAGGAAGCAATTTTTTTGCTTCATCGATCATTGCTTGAGAATAGTCAACACCT
GCATATTGTGAGCATTTTTGTAATTTTGATAGTAGTAATCCGTTGCCACAGCC

The blastx search addictedhomologies of the DNA- sequence to *perosamine synthetase [Alteromonadales bacterium TW-7]* with an E-value of 5e-75 on the left and to *membrane-associated protein [Alteromonadales bacterium TW-7]* with an E- value of 2e-88 on the right region. The *perosamine synthetase* is a member of the superfamily *Aspartate aminotransferase (AAT) superfamily (fold type I) of pyridoxal phosphate (PLP)-dependent enzymes* (cl00321). The combination of PLP with an alpha- amino acid results in a compound called Schiff base, which is the substrate in four kinds of reactions (transamination, racemization, decarboxylation, various side-chain reactions).Searching in the Conserved Domain Database for the *membrane-associated protein* resulted in a specific hit (cd02440), whose function is already described under Sequence 8).

Clone 36)

Sequenced: 1342 bp

GATGATTCAGCTTGATTCGTGGGCGCTAAAACAAAGTTAATTGCCACCGATGGCGTGTTCTCTATGGATGGCGTAATTTGTAACTTAGAAGCT
GTGTGCGATTTAGCTGATAAGTACGATGCGCTTGTTATGGTTGATGACTCTCACGCTGTAGGGTTTGTTGGCGAAAATGGTAAGGGCACACCT
GAATACTGTAACGTACTTGACCGTGTAGATATAATTACTGGTACATTGGGTAAAGCGCTGGGCGGCGCATCAGGTGGTTACACTTCAGGTAAA
AAAGAAATTGTTGAGTGGTTACGTCAGCGTTCGCGCCCTTACTTATTCTCAAATTCATTAGCACCTTCAATTGTAACTGCATCAATTAAAGTA
CTAGAGATGCTTGAAAATGGTGGCGAACTTCGCGCTAAGCTTTGGTCTAATGCTAAATATTTCCGTGAGAAAATGGAAGCGGCAGGCTTTACC
TGCGCAGGTAAAGATCATGCCATCATTCCTGTGATGCTAGGTGACGCTAAAGTGGCTCACTTATGGCTGATAAATTACTTGCAGAAAGGTATT
TATGTGACTGGTTTTTCATTCCCTGTTGTACCAAAAGGCCAAGCACGTATTCGTACGCAAATATCAGCAGCGCACAGTATAGAGCAGCTAGAT
ACAGCTATTGCAGCCTTTACTCGCATTGGTAAAGAAATGGGCGTTATTTAATTTTTAAAACTAAGAAATGACTTGGTTTTTGTGAGCGTAAAT
TATGAAAGCATTATCTAAATTAAAAGCAGAACCGGGTATCTGGATGACAGATGCACCTAAGCCTGAAGTGGGCCATAACGATCTGTTAATTAA
AATACGTAAAACAGCGATTTGTGGCACCGACGTTCATATTTATAAATGGGACGAGTGGGCGAGTAAAACAATTCCTACACCAATGGTTGTTGG
TCATGAGTACGTTGGTGAAGTGGTTGATATGGGTCAAGAAGTTCGCGGCTTTTCAGTGGGTGACCGGGTATCTGGCGAAGGTCATATTACCTG
TGGGCATTGTCGAAATTGTCGTGCTGGGCGTGTGCATTTATGCCGTAACACCACAGGAGTTGGTGTTAATCGCGAAGGCGCGTTTGCTGAGTA
CTTGGTGATCCCTGCATTTAATGCATTTAAAATTCCAGACAACATATCTGACGAATTAGCCTCTATTTTTGACCCATTTGGCAATGCTGTACA
CACAGCCCTTTCGTTTGATCTAGTAGGAGAAGATGTACTAATTACTGGTGCCGGTCCTATAGGTATTATGGCTGCAGCTGTTGCAAAACATGT
AGGCGCGCGTCATGTAGTAATTACTGATGTTAACGAGTAC

The blastx search addicted homologies of the DNA- sequence to *2-amino-3-ketobutyrate coenzyme A ligase [Alteromonadales bacterium TW-7]* with an E-value of 7e-118 on the left and to *L-threonine 3-dehydrogenase [Alteromonadales bacterium TW-7]* with an E- value of 3e-113 on the right region. The enzyme *2-amino-3-ketobutyrate coenzyme A ligase*can be counted among the superfamily cl00321, which is described under Sequence 35). The *L-threonine 3-dehydrogenase* is a member of the superfamily cl12011 described under Sequence 8).

Clone 37)

Sequenced: 1527 bp

GATGATTCAGCTTGATTCGTGCTACAGAGTAGTGGCCGTAACGTTCAACTTCGTCACGAAAAGGACGGATCATTTGTGAATGACAGTTATAAC
AACCTTCACGCACGTAAATATCGCGGCCTTCCATTTCTAATGCATTTAGAGGACGTAAGCCATCTACTGGTTTTGTTGTGTCGTCTTGGAACA
TAAGTGGAGTAATTTCAACTAATGCACCAAAGCTAATAGCAAAAACAGTCAGTATGGCCATAAGGCCAACGTTTTTTTCTACTATTTCATGTT
TGTTTTGTGAATTTTTATTGCTCATCATCTCACTCCGTTATGCTAATTGTGCTTGCGCGTCTAACTTCAGTGATTCTTTTTCAGCTGAAATTG
TACGGAAAACGTTATAAGCCATAACTAACATACCTGTTACGATGAATACACCGCCAACAAAACGCATTATGTAGAAAGGATGTGATGCTTCTA
AAGACTGAACAAAGCTATACATTAATGTACCGTCAGCATTTACTGCACGCCACATAAGACCTTGCATTACACCTGAAATCCACATTGCAACAA
TGTATAAAACAACACCAACAGTGTGTAACCAGAAGTGTGTGTTAACAAGCTTAATGCTGTACATACGACCTTGTGCAAATAGTGCAGGGATAA
GTGATAAATAGCACATTAGAAATCATAGCTACCCAACCAAGTGCACTGAGTGAACGTGCTACAGTCCAATCTGTGTAATGAGATAACGCGTTC
ACAGATTTAATTGCCATCATTGGGCCTTCAAACGTAGACATACCGTAGAAAGAGAGTGATACAACTAAGAAACGAAGTACTGGGTCAGTTCGC
AGTTTATGCCACGCACCCGATAAGGTCATTATACCGTTAATCATACCGCCCCAAGATGGAACGAATAAGATAATTGACATAACCATACCTAAA
CTTTGCGTCCAATCTGGAAGTGCAGTGTAGTGAAGGTGATGAGGACCAGCCCAAATGTATAGAGATACAAGCGCCCAAAAGTGAACGACCGAT
AAACGGTAAGAGTATACAGGGCGACCTGCTTGTTTAGGTACAAAGTAGTACATCATACCTAAGAAACCAGCTGTTAATAAGAAACCTACAGCG
TTATGACCGTACCACCATTGAACCATCGCATCTACTGCACCTGCGTAGATAGAGTATGATTTAGTTAACGATACAGGCACTGCCATGCTGTTC
ACAATGTGCAGTACTGCAACAGTGATGATAAAGCCAGCATAGAACCAGTTAGCAACATAGATATGCGATACTTTACGTTTAATTAAAGTACCA
AAAAATACAACTGCATAAATAATCCAAACAACAGCGATTAAAATATCGATTGGCCATTCTAGTTCAGCGTATTCTTTAGAGCTTGTAATACCT
AATGGTAAGGTAATTACTGCTAATACAATAACTAGCTGCCAGCCCCAAAATGAAATTGCGGCAAGTTTGTCTGAGAAAAGGCGCGTTTGACAA
GTACGTTGCACGACGTAATAAGACGTCGCAAAAAGTGCA

The blastx search addicted a homology of the DNA- sequence to *Cytochrome c oxidase, cbb3-type, subunit I [Alteromonadales bacterium TW-7]* with an E-value of 0.0 over the complete clone. Searching in the Conserved Domain Database resulted in a specific hit (cd01661). This Cytochrome cbb3 oxidase is the terminal oxidase in the respiratory chains

of proteobacteria (multi-chain transmembrane protein located in the cell membrane). This enzyme catalyzes the reduction of O_2 and simultaneously pumps protons across the membrane.

Table 5

Sequence	**Protein**	**E- Value (Sequence-Protein)**	**E- Value (Protein-specific hits)**	**E- value (Protein-Superfamily)**	**E- value (Protein-Multidomain)**
1	Phosphoglycerol transferase	4e-52		cl10460 2.18e-72	MdoB 2.86e-39
2	alpha-glucosidase	0.0		cl07893 1.28e-67	TIGR02403 2.87e-119
3	ATP-dependent helicase HepA	5e-160	cd00046 3.30e-13 cd00079 2.47e-14		
4	putative unknown integral membral protein	6e-31		cl12135 2.15e-22	COG 2311 1.29-23
5	Left region Transposase ISCps9 Right region Cytochrome b561	8e-52 8e-17		cl01116 1.11e-04 cl00859 1.96e-30	
6	Hypothetical protein ATW 7_00545	2e-167	cd07208 1.19e-62		COG4667 1.15e-55
7	Left region putative TonB-dependent receptor protein Right region Hypothetical protein ATW 7_07434 Tryptophan Halogenase	4e-50 4e-133 2e-89	cd01347 1.60e-29	 cl09931 8.62e-176 cl09931 9.13e-180	TIGR01782 1.31e-128
8	putative cyclopropane-	0.0	cd02440		COG2230

	fatty-acyl-phospholipid synthase		2.53e-08		8.72e-77
9	putative type IV pilus biogenesis protein PilQ (cytoplasmic ATPase)	3e-137		cl02829 7.68e-45 cl04346 5.36e-09	TIGR02515 1.55e-148
10	Left region putative TonB-dependent receptor protein Right region Hypothetical protein ATW 7	9e-113 5e-73	cd01347 1.60e-29	 cl09931 8.62e-176	TIGR01782 1.31e-128
11	Left region bifunctional: phosphoribosyl-AMP cyclohydrolase (N-terminal); phosphoribosyl-ATP pyrophosphatase (C-terminal) Right region Imidazol glycerol phosphate synthase subunit HisF	1e-100 4e-120	 cd04731 9.88e-78	cl00344 8.70e-34 cl00345 5.97e-31	PRK02759 6.37e-87 pfam00977 2.57e-62
12	Left region chorismate synthase Right region N5-glutamine methyltransferase	 4e-41 8e-53	cd07304 1.88e-151 cd02440 2.52e-08		 TIGR00536 9.53e-68
13	excinuclease ABC subunit B	4e-55	cd00079 1.80e-19 cd00046 4.24e-07		PRK05298 0e+00

14	Left region putative prolyl 4-hydroxylase, alpha subunit domain Right region putative transcriptional regulator, LysR family protein	3e-101 3e-70	 cd05466 1.83e-08	cl01206 1.00e-23	 COG0583 6.15e-27
15	Membrane-bound aldehyde dehydrogenase, large subunit	0.0		cl10595 1.97e-05	COG1529 6.95e-52
16	Left region transcriptional regulator, LacI family protein Right region hypothetical protein ATW7_02277	8e-136 9e-69	cd06289 3.02e-78 cd01392 3.22e-08	 cl12031 6.32e-22	COG1609 3.09e-50 COG1506 5.97e-41
17	Left region hypothetical protein ATW7_15371 Right region putative potassium channel	1e-24 7e-48			
18	hypothetical protein ATW7_16418	0.0		cl00107 1.06e-04	
19	hypothetical protein ATW7_18525	0.0	cd04276 6.98e-57		
20	Left region putative two-component sensor histidine kinase Right region putative orphan protein putative sigma-54	9e-108 1e-29 5e-47	cd00075 2.27e-20 cd00082 3.26e-06 cd00009 8.76e-16		 COG2204 3.01e-120

	interacting response regulator protein		cd00156 7.14e-13		
21	ATP-dependent DNA helicase	0.034		cl14126 1.90e-13	COG1074 3.70e-44
22	1,4-beta-D-glucan glucohydrolase	3e-70		cl07971 2.68e-60 cl03393 6.79e-36	COG1472 5.75e-68
23	RNase E: endoribonuclease for rRNA processing and mRNA degradation; member of the degradosome; involved in the production of deoxyribonucleotides via the formation of NDPs	0.0	cd04453 3.28e-24		PRK10811 0e+00
24	Left region putative DnaJ-class molecular chaperone Right region putative membrane bound sensor or esterase	3e-79 2e-122	cd00156 9.81e-20 cd00082 2.03e-13 cd00075 1.05e-12 cd00189 7.64e-05 cd00189 6.65e-03	cl13736 4.77e-27 cl02542 1.34e-05	
25	Left region hypothetical protein ATW7_13613 hypothetical protein	3e-66 2e-47			

	ATW7_13608 Right region leucine aminopeptidase-related protein	2e-53	cd00063 1.15e-04		COG2234 8.71e-13
26	Left region Hypothetical protein ATW 7_15406 Right region putative ABC-type transport system, permease component	6e-75 5e-131	cd04590 2.24e-20	cl00427 3.22e-13 cl00427 1.03e-05	COG4135 9.20e-79
27	hypothetical protein ATW7_14196	2e-98			
28	alkaline phosphatase	2e-161	cd00016 4.42e-107		smart00098 3.54e-110
29	Left region bifunctional aspartate kinase II/homoserine dehydrogenase II Right region Murein polymerase, bifunctional murein transglycosylase (N-terminal) and transpeptidase (C-terminal)	4e-66 5e-143		cl00452 1.83e-76 cl02955 5.50e-39 cl09931 5.74e-03 cl07896 9.62e-68 cl01039 4.90e-24	PRK09466 0e+00 PRK11636 0e+00
30	putative membrane protein with ankyrin repeat	2e-167	cd00204 3.98e-13		
31	thymidylate synthase	1e-157	cd00351 1.29e-56		

32	Left region conserved hypothetical protein Right region cation diffusion facilitator family transporter	7e-61 3e-96		cl10469 1.05e-11 cl00316 2.11e-60	
33	Left region Chromosome segregation ATPase from phage origin, putative coiled-coil Right region UDP-glucose 4-epimerase	2e-76 6e-104		cl11176 1.17e-13 cl09931 2.94e-05	 COG0451 2.84e-27
34	Left region alpha/beta hydrolase fold Right region putative periplasmic dipeptidase	5e-55 3e-74	 cd01301 2.69e-78	cl12031 4.55e-13	PRK10985 4.79e-106
35	Left region perosamine synthetase Right region membrane-associated protein	5e-75 2e-88	 cd02440 1.22e-08	cl00321 2.11e-39	
36	Left region 2-amino-3-ketobutyrate coenzyme A ligase Right region L-threonine 3-dehydrogenase	7e-118 3e-113	cd06454 2.77e-132	 cl14614 2.20e-131	COG0156 1.29e-123 PRK05396 0e+00
37	Cytochrome c oxidase, cbb3-type, subunit I	0.0	cd01661 1.06e-167		

The lower the E-value, or the closer it is to zero, the more "significant" the match is. However, virtually identical short alignments have relatively high E- values. This is because the calculation of the E- value takes into account the length of the query sequence. These high E- values make sense because shorter sequences have a higher probability of occurring in the database purely by chance. Within this study 50487 bp were sequenced. All sequences were exclusively of bacterial origin, so no viral or eukaryotic dues were detected.

4.4 Antibacterial activity of *Pseudoalteromonas marina* and derived VLPs

In this study the antibacterial activity of *Pseudoalteromonas marina* and derived VLPs was tested by a disc diffusion assay. The results of all arranged tests are summarized in table 6.

Table 6

	L. monocyto.	B. subtilis	B. cereus	S. aureus	V. cholera	V. algino.
			Supernatant			
70 (7)	-	-	-	-		
(8)	-	-	-	-		
72 (3)	-	-	-	+	-	-
92 (7)	-	-	-	-		
(8)	-	-	-	-		
96 (3)	-	-	++	-	-	-
(4)	-	++	-	-		
(5)	-	-	-	++		
(6)	-	-	-	++		
(7)	-	-	-	-		
(8)	-	-	-	-		
120 (1)	-	-	++	+	-	
(2)	-	-	++	+	-	-
(3)	-	-	-	-	-	-
(4)	-	+	-	-		
(5)	-	-	-	++		
(6)	-	-	-	+		
144 (3)	-	-	++	-	-	-
(4)	-	-	-	+		

(5)	-	-	-	++		
(6)	-	-	-	+		
			Pellet			
70 (7)	-	-	-	-		
(8)	-	-	-	-		
72 (3)	-	-	-	++	-	-
92 (7)	-	-	-	-		
(8)	-	-	-	-		
96 (3)	++	++	++	++	-	-
(4)	+	+	-	-		
(5)	+	-	+	+		
(6)	-	-	-	++		
(7)	-	-	-	-		
(8)	-	-	-	-		
120 (1)	-	+	-	+	-	
(2)	+	-	++	-	-	-
(3)	+	+	-	+	-	-
(4)	++	+	+	++		
(5)	++	+	+	++		
(6)	-	-	-	+		
144 (3)	+	+	++	+	-	-
(4)	++	+	++	++		
(5)	++	+	++	++		
(6)	-	-	-	+		
			VLPs			
70 (7)	+	+	-	-		
(8)	+	+	-	-		
92 (7)	+	+	-	-		
(8)	+	+	-	-		
96 (6)	-	-	-	-		
(7)	+	+	-	-		
(8)	-	+	-	-		
120 (2)	-	-	-	-	-	-
(6)	-	-	-	-		
(8)	-	-	-	-		
144 (6)	-	-	-	-		
(8)	-	-	-	-		
			Kontrollen			
A.d. (2)	-	-	-	-	-	
(3)	-	+	-	-		

Tris/HCl (1)	-	-	-	-	-	
(3)	+	-	-	-	-	-
(4)	-	-	-	-	-	
(5)	-	-	-	+		
(6)	-	-	-	+		
(7)	-	-	-	-		
(8)	-	-	-	-		
514+4% (1)	-	-	-	-	-	
(2)	-	-	-	-	-	
(3)	+	-	-	-	-	
(4)	-	-	-	-		
(5)	-	-	-	+		
(6)	-	-	-	+		
(7)	-	-	-	-		
(8)	-	-	-	-		
TBT (2)	-	-	-	-	-	-
(6)	-	-	-	-		
(7)	-	-	-	-		
(8)	-	-	-	-		

Table 7

Optical density of the pathogenic bacteria for testing antibacterial activity of *Pseudoalteromonas marina*						
	L. monocyto.	**B. subtilis**	**B. cereus**	**S. aureus**	**V. cholerae**	**V. algino.**
Test 1	0.6	1.2	2.7	2.9	2.8	-
Test 2	0.4	2.4	6.4	5.2	4.1	3.1
Test 3	0.6	1.3	2.3	2.3	2.2	1.7
Test 4	0.4	0.6	0.6	0.8		
Test 5	0.8	0.8	1.3	1.7		
Test 6	0.6	1.2	1.4	1.6		
Test 7	0.3	1.1	1.2	1.4		
Test 8	0.4	1.0	1.1	1.2		
Test 9	0.7	1.1	1.7	1.5	2.2	1.7
Test 10	0.8	1.0	2.5	2.1	1.9	2.1

Testing the antibacterial activity of *Pseudoalteromonas marina* three different constituent parts were determined for containing the antibacterial factors:

1) Supernatant of the bacterial culture

2) In Tris/ HCl buffer resuspended pellet of the bacterial culture

3) Isolated VLPs resuspended in TBT buffer

5 Discussion

The strain used in this study belongs to the genus *Pseudoalteromonas* of the class *γ-Proteobacteria*. The bacterium was cultivated in a *514.Bacto Marine Broth (DIFCO 2216)* at 26°C and 250 rpm. The species *Pseudoalteromonas marina* is featured by its widespread occurrence in the marine environment. It is already well known that some species of the genus *Pseudoalteromonas* have antibacterial activities (Holmström et al. 1999, Longeon et al. 2005, Bowman 2007, Soiza et al. 2008, Gram et al. 2009, and produce particles (VLPs) (Nevot et al. 2006, Chiura et al. 2002; 2009). In order to determine the origin of their VLPs - either viral or bacterial - the VLP-DNA was isolated and partially sequenced. To investigate if the species *Pseudoalteromonas marina* exhibits antibacterial activity antibacterial assays were conducted.

5.1 *Pseudoalteromonas marina* starts producing VLPs by entering the stationary phase

Gram- negative bacteria have an outer membrane above a thin peptidoglycan layer. The periplasma separates the outer membrane and the plasma membrane. Those gram- negative bacteria produce particles, so- called "Outer Membrane Vesicles" (OMVs), which are released from the surface of the cell during bacterial growth in the environment.
OMVs contain OMPs (Outer Membrane Proteins), LPS (Lipopolysaccharids), phospholipids and periplasmic constituents. They are 50- 250 nm in diameter, spherical, bilayered and have a membranous structure (Beveridge, 1999). In comparison to the OMVs, "our" VLPs with a diameter of about 30- 160 nm also seem to be bilayered and spherical, but show also morphological similarities to viruses. Viruses inject their genetic material into a host cell, which then produces more viral proteins and viral DNA instead of its common products. Bacteriophages are viruses infecting bacteria, they exhibit two phases: the lysogenic (viruses latent inside a host) and the lytic phase (formation of new viruses, killing the host cell and infecting other cells). The investigated VLPs show also infecting mechanisms. This can be deduced from the fact, that they induce horizontal gene transfer (Chiura, 1997).

Evolutionary, viruses are best known genetic elements for inducing horizontal gene transfer, thus increasing the genetic diversity of the acceptor species, e.g. the bacterium.

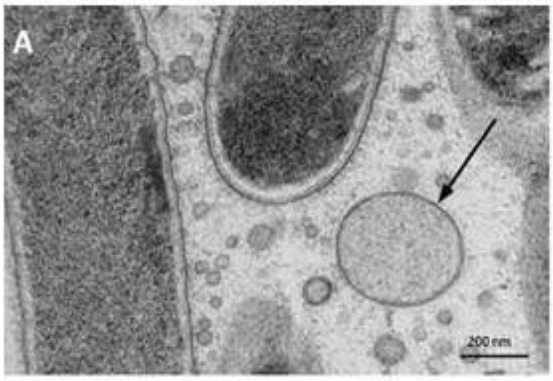
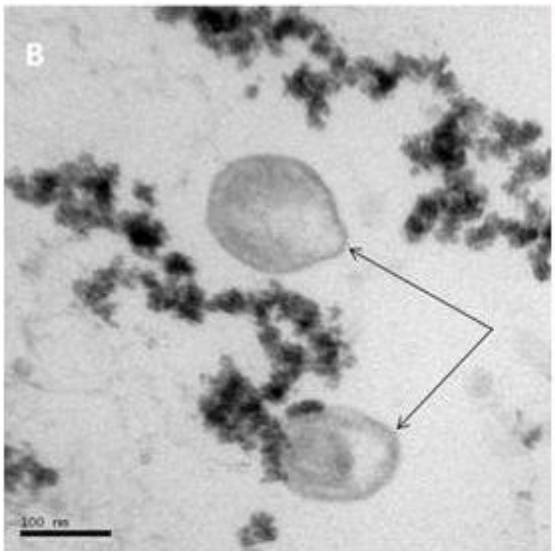
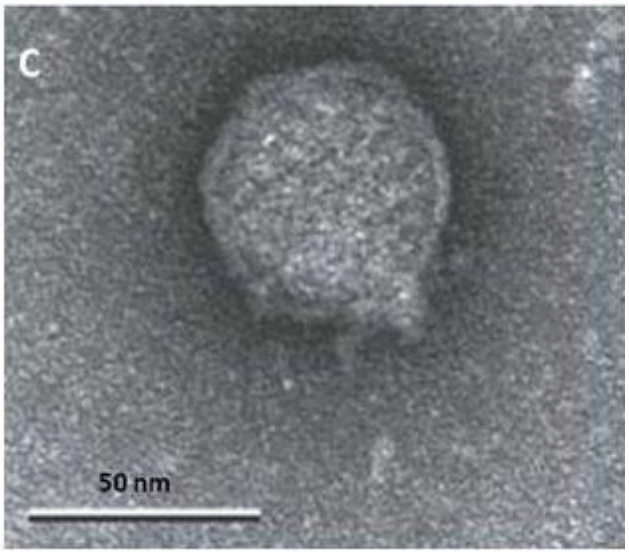


Figure 92: Membrane Vesicle of the Antarctic bacterium *Shewanella livingstonensis NF22*^{T} under Transmission electron microscopy **(A)** and VLP of the marine bacterium *Pseudoalteromonas marina* under electron microscopy; structural and size similarities visible; Membrane Vesicle/ VLPs labeled with arrows. **(C)** showsa Podovirus with a short non-contractile tail (typically lytic); in comparison **(B)** and **(C)** show morphological similarities.
(A) Frias et al. 2008
(C) Suttle C.A. 2005





Virus particles, also so- called *Virions* contain DNA, RNA or proteins, have a protein coat and in some case an envelope of lipids surrounding the protein coat. Because of some similarities of MVs/ OMVs, viruses (bacteriophages) and VLPs it could be hypothesized that they are somehow related, but our VLPs do not really fit into one of those groups. Thus they can be interpreted as new vehicle for mediating genetic exchange, nutrient sensing and bacterial survival (Frias et al. 2010)**.** The similarity of viruses and VLPs can be based on the characteristic of VLPs to induce horizontal gene transfer (Chiura, 1997; 2004; Chiura et al. 2002; 2003). Due to the fact that the structure of VLPs is nearly unexplored VLPs could also be membrane vesicle like structures (Figure 22). *Pseudoalteromonas marina* spontaneously releases VLPs into the media. This ejection of VLPs was first described by Chiura et al. (2009). He found out that the VLP production starts at the end of the logarithmic phase. In

our study VLPs could be detected in nearly every observed stage of bacterial growth, the difference was that in early stages no DNA could be extracted (Figure 23; see Results:

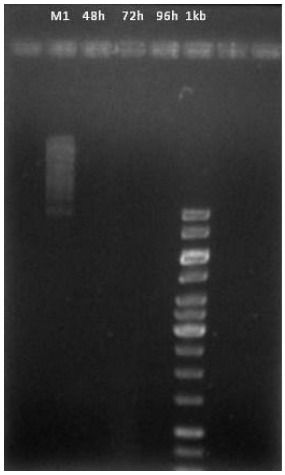
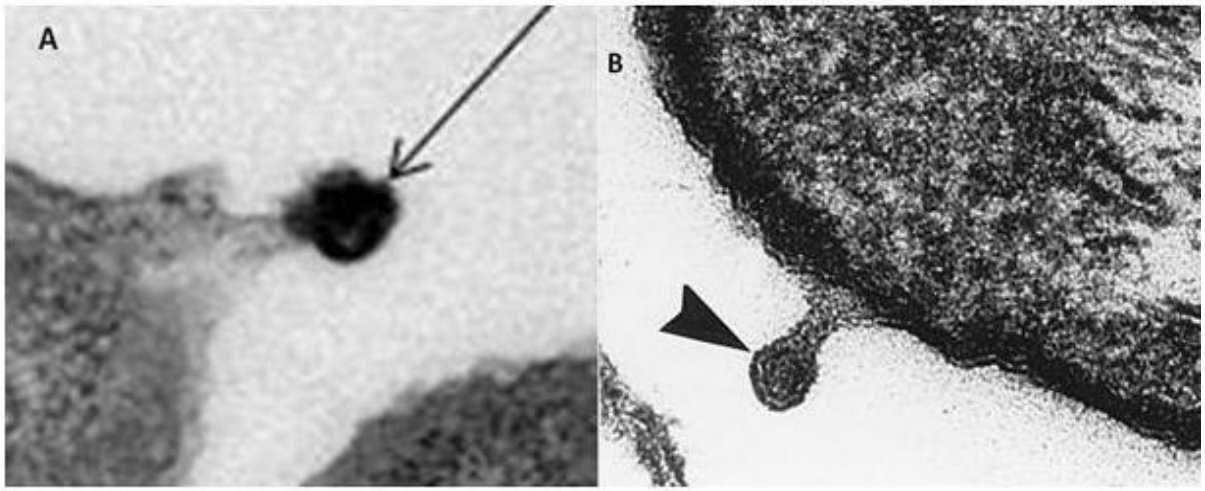


Figure 23: Extracted VLP- DNA using GES- reagent; VLP- DNA extracted out of isolated VLPs after 48 h, 72 h and 96 h of bacterial growth; no DNA detectable.
For length control two molecular weight markers were applied to the gel, the 1 kb Ladder- marker (1 kb) and the High Range Marker (M1).

Figure 12) and in some stages only RNA or proteins were detectable (see Results: Figure 17). Wether this phenomenon was due to a handling problem or can be taken as an indication of different particle contents in different growth stages must be investigated in further studies. In any way, DNA could be extracted from a specific growth stage with only one of the applied extraction methods (see Material and Methods 3.1.3.2). It is not surprising that the DNA-extraction from VLPs is much more difficult than the extraction of bacterial

Figure 24: Bacterial cells of Pseudoalteromonas marina under electron microscopy **(A)** and Pseudomonas aeroginosa under transmission electron microscopy **(B)** showing the release of particles; VLP in **(A)** has a diameter of ~100nm; MV in **(B)** has a diameter of ~50 nm; particles are labeled with arrows. **(B)** Kadurugamuwa and Beveridge 1995.



genomic DNA. The VLP- DNA could be seen in the agarose gels exclusively fragmented and not as high molecular weight DNA as expected. Probabely this fragmentation of the VLP- DNA was an effect of the DNA treatment during the extraction.

Analyses by fluorescence microscopy revealed that the VLP concentration at the beginning of the stationary phase was highest whereas in the logarithmic and later stationary phase particle concentration seems to be reduced (see Results: Figure 10). Particle production in general takes place in every monitored phase of growth (48 h, 72 h, 96 h, 120 h, 144 h), but the amounts seem to vary. Younger cultures (48 h, 72 h and 96 h) showed lower VLP production than after 120 h growth time. After 144 h the VLP production appears to decrease again. The fact that the VLP production seems to decrease after 144 h of bacterial growth cannot be traced back to the decreasing bacterial density in this phase (see Results: Figure 5). Therefore more investigations on the development of VLP production after 144 h of bacterial growth would be necessary. Moreover direct counts have to be accomplished to get significant results.

The structure of the VLPs was observed by electron microscopy. Electron dense structures (almost black under electron microscopy) could be detected within the bacterial cells and the size of the VLPs seems to vary. Due to the fact that the electron microscopy pictures are snapshots, the pathway of the VLPs out from the bacterial cell cannot be analyzed. In some cases the dark spots (VLPs) were visible in the centre of the bacterium near of an electron dense network structure (Figure 25), other pictures showed VLPs near to or almost integrated in the membrane (see Results: Figure 10) and finally the VLPs seem to be released from the bacterium into the environment (Figure 24; see Results: Figure 11). A comparison of VLPs, MVs (OMVs) and viruses is given in table 8.

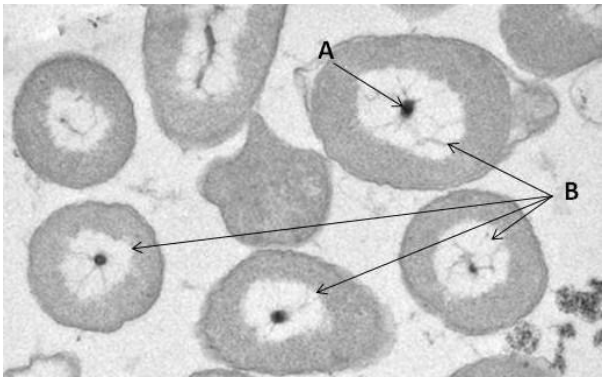


Figure 25: Pseudoalteromonas marina after 117 h of bacterial growth under electron microscopy; **(A)** shows dark spots (VLPs) visible right in the middle of the bacterium within a netted structure **(B)**;

Table 8: Comparison of VLPs, MVs (OMVs) and viruses on the basis of multiple studies (Lee et al. 2008; Chiura et al. 2009)

	VLPs	MVs	Viruses (phages)
Shape	spherical	spherical	wide diversity of shapes
Membrane		Bilayered proteolipids	
Diameter	30-160 nm	20- 200 nm	10- >100 nm
Constituent parts	DNA (transport receptor and binding proteins, biosynthesis proteins, catalytic enzymes, proteins involved in cellular processes proteins involved in protein folding and cell wall degradation) RNA	Outer membrane proteins and lipids, periplasmic and cytoplasmic proteins Lipopolysacchride, DNA, RNA and other factors associated with virulence	nucleic acid (DNA or RNA) surrounded by a protective coat of proteins (capsid); lytic or lysogen

The production of VLPs seems not to start at a specific growing phase and the variations in production frequencies are worth further investigations.

5.2 VLP- DNA packaging

Because of the fact that VLPs produced by *Pseudoalteromonas marina* are on the one hand similar to MVs (Beveridge, 1999) and on the other hand share some morphological characteristics with viruses (Chiura, 2001), the interest on the DNA packaged in those particles increased. Studies on gram- negative bacteria suggest that plasmids, chromosomal DNA and even bacteriophage DNA is packaged into MVs. The encapsulation of DNA in MVs has the advantages that the DNA would be protected from exonucleases and the MVs should enhance the efficiency of DNA delivery and uptake into a recipient cell. It is already known that MVs isolated from *Pseudomonas aeruginosa* contain DNA (Renelli et al. 2004). Two possible mechanisms of DNA packaging are discussed: the DNA could be inserted before leaving the bacterium or exogenous DNA could somehow enter the MVs (Renelli et al. 2004). According to the studies of Chiura (Chiura, 1997; 2004; Chiura et al. 2001; 2003)

the DNA packaging mechanism of MVs and VLPs is similar - thus it can be hypothesized that also "our" VLPs are membrane vesicles.

In many aquatic environments free virus particles play an important role, because of their biochemical nature, infectious activity, host specificity and gene transfer capability. The origin of those free virus particles is still unclear, but observations of some gram- negative marine bacteria showed that those VLPs were spontaneously released into culture media. The fact that both - viruses and "our" VLPs - are able to mediate gene transfer between bacteria (Chiura 1997, 2004; Chiura et al. 2009) could be taken as hint that the VLPs investigated in this study are somehow related to viruses. On the other hand their appearance in the experiments was neither related to plaque formation nor to cell lysis thus being similar to GTA particles (Solioz et al. 1975; Biers et al. 2008). Based on all investigations done with elements inducing genetic exchange the characteristics of VLPs seem to be mostly related to MVs and to GTAs, described as bacteriophage- like vehicles (Marrs, 1974).

Sequence analysis showed that the VLPs of *Pseudoalteromonas marina* contain exclusively bacterial DNA. Within the analyzed DNA fraction no eukaryotic or viral DNA could be detected. These results suggest that the bacteria package parts of their own DNA into VLPs - again a similarity with MVs. This packaging occurs in specific stages of growth: in this study DNA could be extracted from bacterial culture of 96 h, 120 h and 144 h (see Results: Figure 12). In earlier or later stages of growth no VLP- DNA could be extracted. In some experiments in this study only proteins or huge amounts of RNA but no DNA could be extracted. Thus it could be that in some growth stages *Pseudoalteromonas marina* produces RNA or protein containing VLPs and in others they contain DNA. Unfortunatelly, no regularity in which stages which molecules are packaged could be observed until now.

The VLPs contain bacterial DNA with gene-homologous coding for a broad spectrum of proteins such as transport receptor and binding proteins, biosynthesis proteins, catalytic enzymes, proteins involved in cellular processes (DNA replication, transcription, translation, recombination, DNA repair, ribosome biogenesis), proteins involved in protein folding and cell wall degradation. The blast search resulted also in sequences coding for putative proteins, that means for proteins with still unknown functions. A proteomic analysis of *Pseudalteromonas antarctica NF3*^{T} (Nevot et al. 2006) showed similar information by analyzing the DNA of the derived MVs.

5.3 The antibacterial activity of *Pseudoalteromonas marina* and derived VLPs

Based on findings that some marine bacteria produce antibacterial compounds (Burkholder et al. 1966; McCarty et al. 1994; Holmström et al. 1999; Long et al. 2001; Anand et al. 2006; Bowman et al. 2007; Hayashida- Soiza et al. 2008; Kennedy et al. 2008; Wilson et al. 2009; Gram et al. 2009), *Pseudoalteromonas marina* was tested for antibacterial activity.

Altogether 10 test series were accomplished using the disc diffusion assay, described in Results: Table 6. The first 8 test series were carried out by dropping the filter discs into the solution which were tested: into the supernatant of the bacterial culture, into Tris/ HCl buffer as negative control, into bacteria resuspended in TBT buffer and into VLPs resuspended in TBT buffer. Within the test series 9 and 10 the filter discs were first applied to the agar plates with the pathogenic bacteria and then 10 µl of the fractions were pipetted directly on the filter discs. It lastly turned out that the at first positive interpreted results of the first 8 test series can be based on swarm effects of over saturated filter discs and consequently are false positive results. This could be confirmed by test series 9 and 10, in which this swarm effects were eliminated by using another saturating technique.

It must be mentioned that within the used disc diffusion assay only very small amounts of VLPs (10 µl) were available for the test. Thus it could be that the amount of the VLP attached antibacterial substance was not enough to show an explicit antibacterial effect. It can also be that the pathogenic bacteria had a growth advantage over *Pseudoalteromonas marina*. Based on O.D. measurements (see Results: Table 7) the precultures of the pathogenic bacteria are in the logarithmic phase and consequently the quantity of eventually produced antibacterial substances of *Pseudoalteromonas marina* could have been too low.

It could also be possible that the antibacterial activity of *Pseudoalteromonas marina* and its VLPs only takes effects on other marine bacteria, which occur in their natural environment. Hence it could be that the chosen pathogenic bacteria were inappropriate as test candidates.

The sequence analysis resulted in the finding that the VLP- DNA contains sequences homologous to genes coding for *alkaline phosphatase* and *proteases*. These enzymes are known to contribute the pathogenicity of *Pseudomonas* infections and protease has also a proteolytic function (Kadurugamuwa and Beveridge, 1995).

Concluding, it is still unclear whether the bacterium *Pseudoalteromonas marina* produces antibacterial substances or the antibacterial activity is based on exported particles containing such substances. Our test series gave no information about these possibilities, although bacterial fractions and VLPs were tested separately. The negative results can also be a consequence of the fact that *Pseudoalteromonas marina* starts producing antibacterial substances only when an opponent occurs in its environment. In our study VLPs were isolated from a bacterial liquid culture according to standard methods (DIFFCO *2216*). Perhaps the bacterium has to be exposed to some kind of stress to initiate the production of antibacterial substances. Frias et al. (2010) found out that cold- adapted antarctic bacteria produce much higher amounts of MVs when the optimal temperature conditions were lowered. Also in our study it was observed that the VLP- production increased by entering the stationary phase, which is a starvation state, and so a kind of stress situation for the bacterium. Bowman (2007) described various *Pseudoalteromonas* species regarding to their production of bioactive compounds. Bowman differentiated between pigmented and non-pigmented *Pseudoalteromonas* species. Hence, *Pseudoalteromonas marina* is a non-pigmented bacterium and no bioactive compounds were identified. In general the pigmented species, such as *Pseudoalteromonas distincta, Pseudoalteromonas tunicata* or *Pseudoalteromonas denitrificans* produce high amounts of low and high molecular weight compounds with antimicrobial, anti- fouling, algicidal and various pharmaceutically relevant activities.

Because of the rapid increase of antibiotic resistant bacteria over the past decade, there is an urgent need to discover novel antimicrobial compounds. So it can be meaningful to further investigate the antibacterial activity of the marine bacteria e.g. of the genus *Pseudoalteromonas*.

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Abstract

Virus- like- particles (VLPs) are membrane vesicles derived from some marine bacteria. They are spontaneously released from specific bacteria during growth. In this study the production of VLPs of *Pseudoalteromonas marina* was investigated with special focus on the time when particle production starts, the morphology of the particles, their DNA and its functionality. PCR was used for the verification that the cultivated species under investigation is *Pseudoalteromonas marina.* For such purpose 16S rRNA sequences were used.

The actual state of knowledge is that VLPs were generated by -for the most part - unidentified marine bacterial strains, that they contain a large linear DNA molecule (20- 500 kb). Hence, the DNA of those particles was extracted and sequenced to determine the source of the particle DNA (bacterial or viral). Therefore the bacterium *Pseudoalteromonas marina* was cultivated and the VLPs were isolated by filtration and ultracentrifugation at different bacterial culture ages.

Collectively three DNA-fractions had to be extracted: bacterial genomic DNA was isolated to get a PCR-target for the verification of the species (*Pseudoalteromonas marina*), DNA out of the VLPs was extracted for further analyses and plasmid DNA in order to sequence the cloned VLP-fragments. To prepare VLP–DNA suitable for cloning in order to construct a shot-gun-library the original high molecular weight DNA was mechanically fragmented. Blunt-ends were created necessary for cloning the DNA. The "repaired" DNA was size fractionated and cleaned by agarose gel electrophoresis and eluted from the gel pieces. The achieved, eluted DNA was ligated in an appropriate plasmid vector (pSMART HCKan); for transformation the purchased competent "E.cloni cells" were used. Sequence data were analysed computer- based.

Based on the knowledge of recent studies that some marine bacteria are inhibitory to other bacteria, thus playing an important role in marine ecology, the antibacterial activity of *Pseudoalteromonas marina* and derived particles was tested. Therefore a disc- diffusion assay was conducted. Additionally, as producers of antibacterial compounds they were thought to be of high medical relevance, especially considering the rapid increase and spread of antibiotic-resistant bacteria. Because of this rapid increase over the past decade, there is an urgent need to discover novel antimicrobial compounds.

Zusammenfassung

Virus- like- particles (VLPs) sind Membranvesikel welche von einigen marinen Bakterien spontan während des bakteriellen Wachstums abgegeben werden. In dieser Arbeit wurde die VLP- Produktion des Bakteriums *Pseudoalteromonas marina* mit besonderem Augenmerk auf den Beginn der Partikelproduktion, die Morphologie der Partikel sowie die beinhaltende DNA und ihre Funktionalität untersucht. Zur Verifizierung des Bakteriums *Pseudoalteromonas marina* wurde die Polymerasekettenreaktion basierend auf der 16S rRNA durchgeführt.

Der aktuelle Wissensstand bezüglich der VLPs ist, dass sie in den meisten Fällen von noch nichtidentifizierten marinen Bakterien produziert werden und eine hochmolekulare DNA (20- 500 kb) beinhalten. Demnach wurde die VLP- DNA aus den Partikeln extrahiert und sequenziert, um herauszufinden ob diese bakteriellen oder viralen Ursprungs ist. Zu diesem Zwecke wurde das Bakterium *Pseudoalteromonas marina* kultiviert, um dann aus verschiedenen Wachstumsstadien VLPs durch Filtration und Ultrazentrifugation zu isolieren. Insgesamt wurden drei verschiedene DNA Extraktionen durchgeführt: bakterielle genomische DNA, um durch PCR die Spezies *Pseudoalteromonas marina* zu verifizieren, VLP- DNA zum Zwecke weiterer Analysen und Plasmid- DNA, um die klonierten VLP-DNA Fragmente zu sequenzieren. Die hoch molekulare VLP- DNA wurde für die Klonierung und Herstellung einer *Shotgun- library* mechanisch fragmentiert. Ebenfalls notwendig für eine erfolgreiche Klonierung war das Erzeugen von *Blunt- ends* der fragmentierten VLP- DNA. Die VLP- DNA wurde anschießend durch Agarose-Gelelektrophorese der Größe nach fraktioniert und gereinigt. Im Anschluss wurden die entsprechenden Gelstücke eluiert, die eluierte DNA in einen entsprechenden Vektor (pSMART HC_{Kan}) ligiert und in kompetente „E. cloni Zellen“ transformiert. Die Sequenzdaten wurden computergestützt analysiert.

Basierend auf dem Wissen, dass einige marine Bakterien inhibitorisch auf andere Bakterien wirken und somit eine wichtige Funktion in marinen Ökosystemen haben, wurde das Bakterium *Pseudoalteromonas marina* und die von ihm produzierten VLPs auf eine mögliche antibakterielle Aktivität hin untersucht. Zu diesem Zwecke wurde ein *disc-diffussion- assay* durchgeführt. Als Produzent von antibakteriell wirkenden Substanzen

würde dieser Spezies eine hohe medizinische Relevanz zugesprochen werden, insbesondere unter Berücksichtigung der ständig ansteigenden Diversität Antibiotika resistenter Bakterien. Aufgrund dieses schnellen Anstiegs innerhalb des letzten Jahrzehntes ist es von äußester Dringlichkeit neue antimikrobiell wirkende Substanzen zu entdecken.

CURRICULUM VITAE

Personal details	
Name:	Melanie Kappelmann
Address:	Schulgasse 18/18; 1180 Wien
Telephone:	0699/11798874
Email:	Melanie.kappelmann@gmx.at
Date/ Place of Birth:	18.10.1983/ Deggendorf; Germany

Education	
March 2010- present	Diploma thesis "Virus- like- particles of *Pseudoalteromonas marina* and their possible medical significance" Department of Virology, Microbiology and Molecular Biology, Medical University of Vienna, Austria
Since 2004	Studies in Biology (Anthropology: Main subject: Human genetics) University of Regensburg, Germany (2004/2005); University of Vienna, Austria (2005- 2010)
1994- 2004	Grammar school
1990-1994	Elementary school