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Virus like particles of *A. kielensis*
and their possible medical significance

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1 Introduction

1.1 Virus like particles (VLPs)

In the literature the term “VLP” is used to define an artificial biotechnological molecule on the one hand and very small microbiological unities which were not described precisely until now on the other hand.

1.1.1 VLPs in biotechnology

Virus like particles are biotechnological constructs, that look like viruses, but are not infectious. As they do not contain any DNA or RNA they are not able to replicate in the target cells. Currently they are used to develop new vaccines and gene therapy focuses on using them as gene delivery system (Shi et al 2005).

1.1.2 VLPs in environmental microbiology

Environmental microbiology uses direct counts to estimate the number of microbes (any life form that can only be observed via microscopy, including viruses) present in an environmental sample. Therefore the sample is typically pulled through a 0,02 µm pore sized filter. After incubation of the filter in an epifluorescent stain that binds to nucleic acids, the number of viruses and other, larger microbes (bacteria and other single-cell organisms) are determined by epifluorescence microscopy. Thereby viruses appear as small dots and because it is not absolutely known, if all small dots represent viruses or other pieces of nucleic acid, for example free DNA derived from lysed bacterial cells, they are designated as virus like particles (Breitbart and Rohwer 2005).

1.1.3 VLPs of *A. kielensis*

In the course of this study the term virus like particles is used to designate spherical structures which are released by *A. kielensis*. These structures appear as VLPs as defined

in chapter 1.1.2 when observed under an epifluorescence microscope but do show some features which are not typically virus-like.

To date very little is known about this kind of VLPs, which were discovered by Chiura more than 10 years ago. They are produced by – for the most part – marine bacteria and contain large, linear dsDNA fragments (~20-500 kb). Exposure of the amino acid deficient recipient *E. coli* strain AB1157 to a concentrated fraction of these VLPs led to the restoration of amino acid deficiencies in the recipient. Furthermore the recipient started to produce VLPs itself which were again able to restore amino acid deficiencies and induce particle formation in the next recipient bacterium. Thereby neither plaque formation nor cell lysis was observed which would be expected if the VLPs represented bacteriophages (Chiura 1997, 2004).

Additionally a decrease in the efficiency of plating (EOP) was observed after the exposure of the recipient strain to the VLP fraction, indicating that the VLPs did not only mediate horizontal gene transfer but also showed alethal effect on the gram negative recipient bacterium (Chiura 1997, 2004).

1.2 Other phenomena of encapsulated DNA released by bacteria

1.2.1 DNA bacteriophages

The term bacteriophage literally means “eaters of bacteria” and names viruses which infect bacteria. They consist of a protein capsid and therein encapsulated genetic material. With some phages an outer envelope is found. The genetic material can exist in circular or linear form, as ssDNA, dsDNA, ssRNA or dsRNA.

Bacteriophages – as other viruses – do not possess an own metabolism, and therefore rely on host cells and their metabolism for replication. Because of that it is still debatable whether viruses represent a form of life. Viruses are known to have a rather specific host range and for dsDNA bacteriophages their genome is typically ~20-600 kbp in length (Lang and Beatty 2007).

Various forms of bacteriophages can be distinguished depending on their mode of replication. Phages with a lytic cycle inject their genome into a host cell where the replication of the virion is started immediately. In order to release the virions the host cell has to be lysed. In some countries lytic phages have been used in bacteriophage therapy as an alternative to antibiotics since the 1930s. In a recent publication Kutateladze and Adamia (2010) once more pointed out that bacteriophages constitute a promising alternative in the battle against antibiotic resistant bacteria.

Temperate phages are able to carry out a so called lysogenic cycle. Thereby the injected viral genome gets integrated into the host genome. The endogenous phage is called prophage and replicates together with the host DNA. It stays dormant and therefore harmless for the host cell until the conditions of the host bacterium change for the worse. Then the prophage switches from the lysogenic to the lytic cycle, phage replication starts and finally results in lysis of the host cell.

Temperate phages are able to mediate gene transfer via a process called transduction. Transduction occurs when the functional prophage switches from the dormant to the activated state and enters the lytic cycle. Typically virus replication would lead to the package of phage DNA into capsids but it also occurs that mistakenly a part of the host genome gets packaged into the virion. This virus particle then infects another bacterium but is no more capable to induce virus production by the host cell and subsequent cell lysis and plaque formation. Via recombination the foreign, bacterial DNA gets integrated into the host genome. The process of transduction is completed. The transduced cell is called transductant. The prophage is called defective or cryptic.

So with both, lytic and temperate phages, the release of virus particles from the host cell usually goes hand in hand with cell lysis, and when cultivated on agar plates, with plaque formation. This was not observed with our VLPs (Chiura 1997, 2004) and therefore they can be clearly distinguished from these kinds of phages. But some very few phages get released from the host cell by budding without causing cell lysis. According to Wommack and Colwell (2000) this process is limited to only a few rare phage groups and they believe that most of the aquatic bacteriophages are released from their host cell via cell lysis. The best studied group of phages, which is released from the host cell via budding or extrusion without causing cell lysis or plaque formation, is the group of the Ff

phages. Ff phages are filamentous bacteriophages which have a circular ssDNA genome and are known to increase the generation time of their host bacterium. They are male specific phages, as infection with this kind of phages starts with the attachment of the virus particle to specific types of pili which are only found with male cells that contain a conjugative plasmid (Birge 2006).

So, if our VLPs constitute bacteriophages, these bacteriophages would have to show a set of specific features. They would have to carry a linear dsDNA genome. Their host range would have to be very broad. They would have to be capable of mediating gene transfer and therefore be temperate phages which are able to integrate into the host genome. And, they should not cause lysis of the host cell, but would have to be released by budding.

1.2.2 Gene Transfer Agents (GTAs)

Lang and Beatty (2007) defined the term gene transfer agent as virus like particle that only carries random pieces of the bacterial donor genome (instead of viral genes). This DNA is transfered to other bacteria due to a process similar to generalized transduction. Thereby no negative effects occur for the recipient bacteria such as cell lysis or plaque formation. GTA particles are typically 50-70 nm in size (Biers et al 2008) and contain relatively small DNA molecules (<14 kbp; Lang and Beatty 2007).

Gene Transfer Agents were first discovered and most extensively studied with *Rhodobacter capsulatus* (RcGTAs). Ultrastructurally RcGTAs resemble a small, tailed bacteriophage with a head of ~30 nm and a tail of ~50 nm in length (Lang and Beatty 2001). They contain random linear dsDNA fragments of the genome of the producing cell, which are 4,5 kbp in length (Lang and Beatty 2000). The RcGTA genes are arranged in a cluster of 15 genes. These genes include homologs of phage structural genes but no host lysis or self replication genes (Biers et al 2008). GTA production is growth phase dependent and regulated by a cellular signaling pathway which also controls motility (Lang et Beatty 2002). Therefore the authors suggested that both mechanisms (GTA production and motility) serve as alternative strategies to cope with poor environmental conditions.

Lang and Beatty (2007) found that GTA genes are widely spread in α-proteobacteria. But with focus on marine bacterioplankton, Biers et al (2008) noted that RcGTA homologs were surprisingly infrequent in marine metagenomic sequence data.

Prophage like GTAs have also been reported – amongst others – from *Brachyspira hyodysenteriae* which are called VSH-1 (Matson et al 2005). These particles contain random fragments of the host genome that are 7,5 kbp in length. The VSH-1 prophage is inducible by mitomycin C and its genes span 16,3 kbp of the host genome. Additionally to structural genes also lysis genes have been identified which were not found with RcGTAs. Nevertheless the release of VSH-1 particles was not observed to be associated with cell lysis of the producing bacterium. Similar to RcGTAs VSH-1 particles structurally resemble small, tailed phages.

As our VLPs, GTAs are able to mediate gene transfer and contain linear dsDNA molecules, but these are significantly smaller in size (<14 kbp). They are as well released from the host cell without causing cell lysis or plaque formation but have not been reported to exhibit a lethal or inhibiting effect on the recipient cell.

1.2.3 Outer membrane vesicles of gram negative bacteria

It is commonly known that gram negative bacteria constantly produce and release outer membrane vesicles (MVs) during bacterial growth and thereby entrap periplasmic compounds. These vesicles are about 20 to 250 nm in size and probably result from budding (bulging and pinching off) of the outer cell membrane of gram negative bacteria (Mashburn-Warren et al 2008). Therefore it is not surprising that membrane vesicle formation is not reported from gram positive bacteria which do not possess an outer membrane.

The cell wall composition of gram negative bacteria differs significantly from that of gram positive bacteria. Both types of bacteria possess a plasma membrane and a peptidoglycan layer. But while the peptidoglycan layer of gram positive bacteria is relatively thick and comprises the outermost structure of the bacterial cell, that of gram

negative bacteria is rather thin and underlies an additional outer membrane which is rich in proteins and lipopolysaccharides. With gram negative bacteria the periplasma is located between the outer and the plasma membrane, while with gram positive bacteria this gel like matrix is found between the plasma membrane and the thick peptidoglycan layer.

The molecular mechanisms underlying the formation and release of MVs by gram negative bacteria are not known yet. Several models have been proposed by various research groups concerned with this matter. In a recent publication Deatherage et al (2009) supported a model, wherein bacterial growth and division invoke temporary and localized reductions in the density of associations between different components within the envelop structure and thereby allow the release of outer membrane as MVs.

MVs of different gram negative bacteria were shown to exhibit a lytic effect on a variety of gram negative as well as gram positive bacteria. Thereby the extent of their lytic activity depends on the composition of the cell wall – e.g. the peptidoglycan chemotype – of the target bacterium (Beveridge 1999). Armed with virulence and antibacterial factors (proteases, toxins, surface adhesions, autolysins and other components) it is possible that they play a predatory role in the natural ecosystem. Membrane vesicles might be released by the bacterium in order to lyse surrounding cells which compete for the same nutrient resources.

But most interestingly, relating to our VLPs, MVs of some gram negative bacteria have been reported to contain DNA as well. To date such DNA containing MVs were only found with some gram negative bacteria, namely with *Neisseria gonorrhoeae* (Dorward et al 1989), *E. coli* O157:H7 (Kolling and Matthews 1999) and *Pseudomonas aeruginosa* (Kadurugamuwa and Beveridge 1995). Membrane vesicles of *Haemophilus influenza*, and *Haemophilus parainfluenza* were reported to be able to take up extracellular DNA and protect it from DNAses (Kahn et al 1982).

Kolling and Matthews (1999) found that MVs of *E. coli* O157:H7 contained DNA, in addition to proteins, which was protected from extracellular DNAses. Yaron et al (2000) were able to show that MV DNA of this *E. coli* strain consisted of plasmid, chromosomal (including specific host genes) and phage DNA. One 3,3 kbp long plasmid

was found within the vesicles that contained all genes necessary for its replication, stability and mobilization, but did not contain the tra genes. These tra genes are needed for efficient conjugation, as they mediate close cell contact between bacteria. Therefore the authors suggested that MVs might mediate the non conjugative transfer of the plasmid from donor to host cells. Furthermore MVs of *E. coli* O157:H7 are able to deliver genetic material which then gets expressed in the recipient cell.

DNA containing vesicles from *P. aeruginosa* PAO1 were investigated by Renelli et al (2004). It was found that they harboured chromosomal, surface bound DNA and were also able to take up exogenous DNA. But MVs of *P. aeruginosa* PAO1 were not able to mediate gene transfer. Generally MVs encapsulate periplasmatic compounds, but as they contain chromosomal DNA as well, also a cytoplasmatic compound is present inside these MVs. According to Renelli et al (2004) the DNA is either moved from the cyto- to the periplasma where it gets entrapped during MV formation, or the DNA is derived from lysed cells and is taken up exogenously.

Summing up, as with VLPs of *A. kielensis* MVs of some gram negative bacteria entrap DNA molecules and some of those are even able to mediate gene transfer. Furthermore MVs have been reported to exhibit a lytic effect on other, even distantly related bacteria. Their lytic potency seems to depend on the cell wall composition of the recipient bacterium and this might be true for VLPs as well.

It is possible that VLPs of *A. kielensis* are MVs with the capability to encapsulate and transfer genetic material as well. As the structure of the VLP envelope is still unknown, it cannot be said for sure, whether our VLPs are derived from budding of the outer membrane and therefore represent outer membrane vesicles or not.

1.3 Possible medical significance of *A. kielensis* and its VLPs

Antibiotic resistant bacteria are a serious health concern and reports of hospital-acquired infections have accumulated over the years.

Fischbach and Walsh (2009) mentioned three classes of antibiotic-resistant pathogens that are emerging as major threats to public health, namely methicillin resistant

Staphylococcus aureus (MRSA), multidrug resistant (MDR) and pan drug resistant (PDR) gram negative bacteria and MDR and extensive drug resistant (XDR) strains of Mycobacterium tuberculosis. To pick one example, MRSA is supposed to cause about 19000 deaths per year in the United States of America and to cause additional public health care costs of 3-4 billion dollars.

Having in mind that bacteria are only very distantly related to humans it should be easy to find novel substances which are able to kill these organisms without being toxic to the patient. But the reality of antibiotic development draws a different picture: antibiotic resistance is increasing while the discovery of novel antibiotics is declining (Fischbach and Walsh 2009).

Nevertheless reports of novel antimicrobial compounds isolated from marine organisms have accumulated over the last years and the marine environment is commonly regarded as a promising source for the discovery of further substances with antimicrobial activity.

The first antibiotic isolated from marine bacteria was pentabromopseudilin. Burkholder et al (1966) isolated this antibiotic from the marine bacterium *Pseudomonas bromoutilis* and found that it was capable to inhibit strains of *Diplococcus pneumoniae*, *Staphylococcus aureus* and *Streptococcus pyogenes* as well as *Mycobacterium tuberculosis*. In a recent study Gram et al (2010) tested 876 cultivable bacterial strains for antibacterial activity against *S. aureus* and *Vibrio anguillarum* and found that the most prominent producers of antibacterial substances belonged to the orders Vibrionales, Alteromodales and Rhodobacterales. Most of the bacterial strains which are known to spontaneously produce the VLPs of our interest belong to one of these three orders.

As mentioned above VLPs of *A. kielensis* and other marine bacteria have already been shown to exhibit a lethal effect on the recipient *E. coli* strain AB1157. Thus *A. kielensis* and its VLPs might show antagonistic activity against human pathogenic bacteria as well.

1.4 Aims

VLPs were first observed by H. Chiura over a decade ago. They are released by – for the most part – marine bacteria without causing cell lysis or plaque formation and contain dsDNA molecules which are ~20-500 kbp in length. Exposure of VLPs to the amino acid deficient recipient *E. coli* strain AB1157 resulted in restoration of the amino acid deficiencies and to a decrease in the efficiency of plating of the recipient bacterium (Chiura 1997, 2004). Thus, VLPs are believed to mediate horizontal gene transfer to even distantly related bacteria and to exhibit a lethal effect on some bacterial strains.

The aim of this study was to determine the origin of the DNA contained in VLPs of *A. kielensis* and to test whether the bacterium or its VLPs exhibit an antagonistic activity against different pathogenic bacteria. Therefore *A. kielensis* had to be cultivated and a method had to be established that allowed the reliable separation of VLPs from bacterial cells. The obtained cell-free VLP fraction was processed for either DNA extraction or to test for antibacterial activity against various pathogenic bacteria.

As the composition of the VLP envelope is not known, an appropriate protocol for the extraction of VLP DNA had to be found. The obtained VLP DNA was then used to construct a shotgun library and some clones were sequenced. Analysis of the obtained sequences should reveal whether the VLP DNA is of viral or bacterial origin. The shotgun library was prepared for long term storage to allow its usage for further research.

The test for antibacterial activity of *A. kielensis* and its VLPs was carried out against various pathogenic bacterial strains. As it had already been demonstrated that VLPs of *A. kielensis* have an antibacterial effect on the *E. coli* strain AB 1157, it was reasonable to expect that those particles will also show a similar effect on some of the tested pathogenic bacteria.

2 Material and Methods

A list of the suppliers of the used materials is provided at the end of this chapter.

2.1 The species *Ahrensia kielensis*

A. kielensis is a gram negative, aggregate forming, marine bacterium. It is rod shaped (0,6-1,0 x 2,0-4,0 µm), motile and strictly aerobe. The bacterium grows best at 20-30 °C, can also grow at lower temperatures (5 °C) but not at 37 °C (Garrity 2005).

The bacterium is named after R. Ahrens, a German microbiologist who isolated a new species from brackish water of the Baltic Sea and classified it to the genus *Agrobacterium* as *Agrobacterium kieliense* (Ahrens 1968). The taxonomic position of the bacterium was soon questioned (Rüger and Höfle 1992) and phylogenetic analysis of 16S rDNA sequences confirmed that marine *Agrobacterium* species were not related to terrestric species of the genus *Agrobacterium* (Uchino et al 1998). Thus, it was proposed that the marine *Agrobacterium* species should be reclassified which led to actual classification of *A. kielensis* (Uchino et al 1998).

Classification of *Ahrensia kielensis*	
Domain	Bacteria
Phylum	Proteobacteria
Class	Alphaproteobacteria
Order	Rhodobacterales
Family	Rhodobacteraceae
Genus	*Ahrensia*
Species	*Ahrensia kielensis*

Table 1 systematic position of *Ahrensia kielensis*

To date very little is known about the species *A. kielensis*, and the little information available is often contradictory. To mention an example, Rüger and Höfle (1992)

described the bacterium as having peritrichous flagella, while Uchino et al (1998) described it once as having a single polar flagellum and once as having peritrichous flagella in the same publication.

2.2 Laboratory work

2.2.1 Cultivation of bacteria

In order to study *A. kielensis* and its VLPs the bacterium had to be cultivated first. To determine whether *A. kielensis* and its VLPs were antagonistic to other bacteria several pathogenic strains were chosen and grown as well in our laboratory. Cryo cultures of all strains were either available in our laboratory or purchased from DSMZ (German Collection of Microorganisms and Cell Cultures).

The following procedure was used for 200 ml test cultures of *A. kielensis* and for cultivation of pathogenic bacteria.

Procedure and materials

- A frozen cryo culture was stroked out on an appropriate agar plate
- Incubation in the dark at 26 °C
- A single colony was picked for inoculation of liquid medium
- Incubation in the dark at 26-31 °C and 150-250 rpm

For cultivation of *A. kielensis* 514+Y+P medium was used. For cultivation of pathogenic bacteria LB medium was used.

514+Y+P medium: for 1000 ml: 40,1 g medium 514, 5 g pepton, 1 g yeast extract; pH value adjusted to 7,55-7,64; medium autoclaved; for solid medium: 15 g agar added prior to autoclaving;

LB medium: for 1000 ml: 10 g peptone, 5 g yeast extract, 10 g NaCl; pH value adjusted to 7,2-7,6; medium autoclaved; for solid medium: 15 g agar added prior to autoclaving;

2.2.1.1 Preparation of a glycerol stock

Glyerol is added to bacterial liquid cultures prior to long term storage at -70 °C. Glycerol depresses the freezing temperature of water and thereby protects the membrane of bacteria from damage by ice crystals.

In this work a glycerol stock of *A. kielensis* was prepared and stored at -80 °C. Aliquots were used for inoculation to ensure standardized conditions of *A. kielensis* cultures.

Procedure and materials

- A 6 ml preculture of *A. kielensis* was incubated for 48 hours in the light at 26 °C and 125 rpm (inoculated as described above)
- 60% glycerol was sterilized by autoclaving for 20 minutes
- 1,5 ml tubes were filled with 100 µl sterile 60% glycerol
- The preculture was vortexed properly and 300 µl were added to each tube
- The tubes were vortexed again to make sure that the glycerol was evenly dispersed and frozen in liquid nitrogen
- Storage at -80 °C

2.2.2 OD measurement

The optical density of a bacterial culture can be measured in a spectrophotometer. Visible light passes through the bacteria suspension and thereby gets scattered depending on the cell concentration of the sample.

Procedure and materials

- 1 ml liquid culture as probe
- 1 ml 514+Y+P medium as blank

The optical density was measured using a SmartSpec™Plus Spectrophotometer from Bio-Rad.

2.2.3 Epifluorescence microscopy

In epifluorescence microscopy a specimen containing a component labelled with a fluorescent molecule is exposed to light of a specific wavelength. This results in the emission of light of a different wavelength and thereby allows the investigation of the labelled component.

Samples of liquid cultures and VLP suspensions were stained either with SYBR Gold or acridinorange and examined under a Leica DMRB epifluorescence microscope.

2.2.3.1 Staining with SYBR Gold

Procedure and materials

- 10 µl sample were mixed properly with 990 µl of A. dest.
- Filtration through a 0,02 µm or a 0,05 µm pore sized filter
- Staining for 30 minutes in 60 µl SYBR Gold solution in the dark
- Repeated filtration of 1000 µl of A. dest. through the filter
- The filter was dried in the dark
- The filter was placed between a microscopy slide and a cover slip putting one drop of Citifluor beneath the filter and one above it

SYBR Gold solution: 10 µl SYBR®GOLD nucleic acid gel stain (10000x in DMSO, Invitrogen), 390 µl A. dest.;

2.2.3.2 Staining with acridinorange

Procedure and materials

- 10 µl probe were mixed properly with 990 µl A. dest.
- 500 µl dilution were pipetted onto a 0,1 or a 0,2 µm pore sized filter
- One drop of acridinorange was added
- The rest of the dilution was added

- Incubation for 1 minute
- Filtration
- The filter was dried in the dark and placed between a microscopy slide and a cover slip using paraffin oil instead of Citifluor

Acridinorange solution: 0,3 g acridinorange, 100 ml A. dest., 3 ml formaldehyde;

2.2.4 Electron microscopy

An electron microscope focuses an electron beam onto an object and thereby achieves a resolution that is a thousand times higher than that of a light microscope due to the small wavelength of electrons. Transmission electron microscopes (TEM) are used for investigation of inner structures of cell thin sections. Scanning electron microscopes (SEM) are used for imaging the surface of a sample.

Samples of liquid cultures and aliquots of VLP suspensions were kindly prepared by Ingrid Gerstl und Regina Wegscheider for examination under a Zeiss EM902 (Zeiss Inc., Germany) transmission electron microscope which was done by ao. Univ. Prof. Adolf Ellinger.

2.2.5 Sonication

Sonication means the agitation of particles of a sample via exposure to ultrasound energy (> 20 kH).

In this work sonication was done to dissolve bacterial aggregates prior to particle harvest and to randomly fragmentise DNA. A Branson Sonifier 450 was used.

2.2.5.1 Dispersion of bacterial aggregates

Procedure and materials

- The bacterial culture was placed on ice

- Repeated sonication for 30 seconds at 100 W with 1 minute break

The harvest of VLPs was started immediately after sonication.

2.2.5.2 Sonication of VLP DNA for shotgun library construction

A shotgun library is characterised by the circumstance that the insert DNA fraction was randomly sheared prior to construction. Therefore VLP DNA was treated as described in the following.

Procedure and materials

- 45 µl DNA were filled up to 200 µl with A. dest.
- 100 µl DNA were placed on ice and sonicated for 7 seconds at 50 W
- The residual 100 µl DNA were placed on ice and well and sonicated for 10 seconds at 50 W

2.2.6 Harvest of VLPs

Various centrifugation and filtration steps were performed to separate the VLPs from the bacterial cells.

2.2.6.1 Separation of bacterial cells from VLP containing culture medium

The first centrifugation step was performed to pelletize the bacterial cells. The supernatant was used for the following filtration steps whereas the pellet itself was either discarded or used for the test for antimicrobial activity of *A. kielensis* and its VLPs.

Procedure and materials

- The culture was transferred into 3-4 50 ml conical centrifuge tubes
- Centrifugation for 40 minutes at 7500 g and 4 °C using a Sigma refrigerated centrifuge 3K30
- The supernatant was separated from the bacteria pellet

2.2.6.2 Filtration

In order to remove remaining cells and cell debris the supernatant was filtered several times.

Procedure and materials

The probe was filtered through the following Rotilabo® syringe filters in the indicated order:

- 0,45 µm pore sized filter
- 0,22 µm pore sized filter
- sterile 0,22 µm pore sized filter

2.2.6.3 Ultracentrifugation

In order to concentrate the VLPs the sterile filtrate was ultracentrifuged resulting in a cell-free VLP pellet that was resuspended in different solutions depending on the protocol chosen for DNA extraction.

Procedure and materials

- The sterile filtrate of each culture was transferred into 3-4 quick seal tubes using a 10 ml sterile syringe and a sterile needle
- Centrifugation for 4 hours at 4 °C and 42000 rpm
- The supernatant was either discarded or stored at 4 °C
- The pellets were resuspended in either 1x TBT buffer or Cell Suspension Solution provided with the Gentra® Puregene® Yeast/Bact. Kit from QIAGEN

1x TBT buffer: for 1000 ml: 5,84 g NaCl, 12,1 g Tris, 0,94 g $MgCl_2$; pH value adjusted to 7,6; autoclaving;

2.2.7 Disc diffusion method

The disc diffusion method was used by Hayashida-Soiza et al (2008) to detect antibiotic compounds released by a *Pseudoalteromonas haloplanktis* strain. Filter discs were charged with different solutions and suspensions and placed on a bacterial lawn. If the charged filter contained any antimicrobial compounds the area around the disc became clear.

Following this method it was tested whether *A. kielensis* and its VLPs were antagonistic to the following bacteria:

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

Procedure and materials

Overnight cultures of the pathogenic bacteria were plated. For each plate filters were charged with the following suspensions or solutions and placed on the plates:

- Bacteria pellet resuspended in either 50 ml Tris buffer (10mM; pH 7,5 or 10,1) or A. dest.
- Supernatant from the first centrifugation step
- Sterile filtrate
- VLP suspension
- As controls filters were charged with 514+Y+P or LB medium, with Tris buffer (pH 7,5 or 10,1) or A. dest., and TBT buffer

The plates were incubated for 48 hours at 26 °C.

10mM Tris buffer: for 1000 ml: 1,21 g Tris; pH value adjusted to 7,5 or 10,1; autoclaving;

2.2.8 DNA extraction

In this work DNA was extracted from *A. kielensis* (genomic DNA), from VLPs of *A. kielensis* (VLP DNA) and from transformed bacteria (recombinant plasmids).

Genomic DNA was used as template for amplification of the 16S rRNA encoding region via PCR and VLP DNA was needed for the construction of a shotgun library.

2.2.8.1 Extraction of genomic DNA using SDS

SDS denatures proteins and therefore can be used for cell lyses. Proteinase K was added to degrade proteins. Phenol/chloroform (1:1) was used to precipitate them and chloroform to remove the remaining phenol. Sodium acetate stabilizes the DNA and ethanol absolute precipitates it.

Procedure and materials

- 1,5 ml sample was taken from liquid culture under sterile conditions
- Centrifugation for 1 minute at full speed (16100 g) using a Eppendorf Centrifuge 5415 C
- the supernatant was discarded, the pellet resuspended in 400 µl 1x TE buffer
- 50 µl 10% SDS were added
- 50 µl proteinase K (20 mg/ml 1x TE) were added
- Incubation for 1 hour at 37 °C
- 500 µl phenol/chloroform (1:1) were added, mixed properly
- Centrifugation for 5 minutes at full speed.
- The upper phase was transferred to a new tube
- The following step was performed twice:
  500 µl chloroform were added, mixed properly
  Centrifugation for 5 minutes at full speed
  The upper phase was transferred to a new tube
- At last 360 µl of the upper phase were transferred into the new tube
- 40 µl 3M sodium acetate and 800 µl ethanol absolute were added, mixed properly

- Incubation at -20 °C for 30 minutes
- Centrifugation for 5 minutes at full speed
- The supernatant was discarded
- The pellet was washed with 200 µl 70% ice cold ethanol, dried and dissolved in 10 µl 1x TE buffer overnight

Sodium acetate (3M, pH 5,2): for 1000 ml: 408,3 g; pH value adjusted to 5,2 using glacial acetic acid (100%); autoclaving;

1x TE buffer: for 1000 ml: 2 ml EDTA (0,5M, pH 8,0), 10 ml Tris-HCl (1M, pH 7,5), 988 ml A. dest.; autoclaving;

2.2.8.2 Extraction of genomic DNA via Generation Capture Column Kit from QIAGEN

Procedure and materials

- 1 ml sample of liquid culture was taken under sterile conditions
- Centrifugation for 1 minute at full speed
- Removal of 800 µl supernatant
- Suspension of the pellet in the residual fluid by pipetting up and down for 10 times
- 200 µl concentrated sample were transferred onto the centre of a Capture Column contained in a Waste Collection Tube
- Incubation for 1-60 minutes at room temperature
- 400 µl DNA Purification Solution 1 were added
- Incubation for 1 minute at room temperature
- Centrifugation for 10 seconds at 2000-12000 g
- The Capture Column was transferred to a new Waste Collection Tube
- 400 µl DNA Purification Solution 1 were added
- Incubation for 1 minute at room temperature
- Centrifugation for 10 seconds at 2000-12000 g
- 200 µl DNA Elution Solution 2 were added

- Centrifugation for 10 seconds at 2000-12000 g
- The Capture Column was transferred to a DNA Collection Tube
- 100 µl DNA Elution Solution 2 were added
- Incubation at 99 °C for 10 minutes
- Centrifugation for 20 seconds at 2000-12000 g

2.2.8.3 DNA extraction from VLPs using GES reagent (protocol 1)

The following protocol is derived from a protocol for extraction of genomic DNA from bacteria (Pitcher et al 1989) and includes cell lysis by GES reagent, which also inhibits nucleases, and precipitation of DNA with ammonium acetate and isopropanol – a combination that is especially applicable to precipitate small volumes of DNA. Ammonium acetate stabilizes the DNA while isopropanol precipitates it.

VLP pellets had been resuspended in TBT buffer when this protocol or a modified version of it was used for DNA extraction.

Procedure and materials

- 100 µl of VLP suspension were mixed with 500 µl GES reagent (freshly prepared!)
- Incubation for 5 minutes at room temperature and for 5 minutes on ice
- 250 µl of ice cold ammonium acetate (7,5M) were added and mixed immediately
- Incubation for 10 minutes on ice
- 0,54 volume of ice cold isopropanol were added and mixed properly
- Incubation for 10 minutes on ice
- Centrifugation for 5 minutes at full speed
- The supernatant was discarded and the pellet washed once with 200 µl ice cold 70% ethanol and once with 100 µl ethanol absolute
- The pellet was dissolved in 10µl 1x TE buffer for 48 hours at room temperature

7,5M ammonium acetate: for 100 ml: 57,81 g; sterile filtration;

GES reagent: for 100 ml: 60 g guanidium thiocynate, dissolved in 20 ml EDTA (0,5M) and 20 ml A. dest. at 65 °C; when cooled down to room temperature 5 ml 10% sarcosyl were added; filled up to 100 ml with A. dest.; sterile filtration;

2.2.8.4 DNA extraction from VLPs using the Gentra® Puregene® Yeast/Bact. Kit supplied by QIAGEN (protocol 2)

VLPs had been resuspended in Cell Suspension Solution (provided with the kit) when this protocol or a modified version of it was used.

Procedure and materials

- 300-400 µl particle suspension were centrifuged for 10 or 30 minutes at full speed
- The supernatant was discarded, the pellet resuspended in 300 µl Cell Lysis Solution
- Incubation for 5 minutes at 80 °C to lyse cells
- 100 µl of protein precipitation solution added and vortexed for 20 seconds at high speed
- Centrifugation for 3 minutes at 13000-16000 g
- The supernatant was transferred to a tube containing 300 µl isopropanol and mixed gently by inverting 50 times
- Centrifugation for 1 or 4 minutes at 13000-16000 g
- The supernatant was discarded, the pellet was washed with 300 µl 70% ethanol and dried
- 10-20 µl 1x TE buffer were added and vortexed for 5 seconds at medium speed
- Incubation at room temperature over night

2.2.8.5 Mini preps using the QIAprep Spin Miniprep Kit from QIAGEN

Mini preparation is a rapid method to extract and purify plasmids from bacteria.

Procedure and materials

- 1,5 ml LB amp medium or LB kan medium were inoculated with a single colony
- Incubation over night at 37 °C and 250 rpm
- Centrifugation for 3 minutes at 16000 g
- Complete resuspension of the pellet in 250 µl Buffer P1 by pipetting up and down
- 250 µl Buffer P2 were added, tube was inverted 4-6 times
- 350 µl Buffer N3 were added, tube was inverted 4-6 times immediately
- Centrifugation for 10 minutes at full speed
- The supernatant was transferred to a QIAprep spin column
- Centrifugation for 30-60 seconds at full speed
- 500 µl Buffer PB added
- Centrifugation for 30-60 seconds at full speed
- The flow through was discarded
- 750 µl Buffer PE were added
- Centrifugation for 30-60 seconds at full speed
- The flow through was discarded
- Centrifugation for 1 minute at full speed
- The QIAprep spin column was placed into a new 1,5 ml tube
- 50 µl A. dest. were pipetted onto the centre of the QIAprep spin column
- Centrifugation for 1 minute at full speed

LB amp medium: for 1000 ml LB medium: when cooled down 1 ml ampicillin solution (50 mg/ml) added;

LB kan medium: for 1000 ml LB medium: when cooled down 3 ml kanamycin solution (10 mg/ml) added;

2.2.9 Protein precipitation using PCI and CI

Some DNA samples had to be processed for protein precipitation because their high protein content did not allow proper size fractionation via agarose gel electrophoresis (VLP DNA extracted using GES reagent) or because they contained substances from upstream applications that were inhibitory to downstream applications (genomic DNA extracted using the Generation Capture Column Kit).

PCI was added because the phenol precipitates proteins. CI was added to remove the remaining phenol.

Procedure and materials

- The DNA solution was filled up to 270 µl with A. dest.
- 200 µl PCI (25:24:1) were added, mixed properly
- Centrifugation for 5 minutes at full speed
- The upper phase was transferred into a new tube
- 200 µl CI (24:1) were added and mixed properly
- Centrifugation for 5 minutes at full speed
- To precipitate the DNA 30 µl sodium acetate (3M) and 600 µl ethanol absolute were added, mixed properly and left at -20 °C for 30 minutes
- Centrifugation for 5 minutes at full speed
- The supernatant was discarded and the pellet washed with 200 µl 70% ethanol, dried and dissolved in 10 µl 1x TE for 48 hours at room temperature

PCI: for 100 ml: 50 ml phenol (equilibrated), 48 ml chloroform and 2 ml isoamyl alcohol were mixed.

CI: for 100 ml: 48 ml chloroform and 2 ml isoamyl alcohol were mixed

2.2.10 PCR

Polymerase Chain Reaction is a method to amplify DNA fragments exponentially. Flanking, synthetically produced primers allow the amplification of a specific region of the genome but therefore the flanking sequences of the desired region have to be known.

In this work genomic DNA of *A. kielensis* was used as template DNA for amplification of a part of the 16S rDNA via PCR. This region is highly conserved in prokaryotes and its sequence in *A. kielensis* is already known.

All PCRs were performed using a Biometra T3000 Thermocycler from Whatman and including a negative control using A. dest. instead of genomic DNA. The GoTaq® Green Master Mix from Promega and a 10x primer mix containing the following degenerated primers were used:

- aga gtt tga tcm tgg ctc ag-3'
- tac ggy tac ctt gtt acg act t-3'

The primer sequences were taken from the literature (Du et al 2010) and were synthesized by VBC Biotech (Austria).

Procedure and materials

For 25 µl reaction mixture:

- 10 µl genomic DNA
- 12,5 µl GoTaq® Green Master Mix, 2X
- 2,5 µl primer mix (10x concentrated)

Parameters chosen for PCR:

- Denaturation for 5 minutes at 95 °C
- 30 Cycles:
  Denaturation for 30 seconds at 95 °C
  Primer annealing for 30 seconds at 55 °C
  Polymerisation for 80 seconds at 72 °C
- Final polymerisation for 10 minutes at 72 °C

GoTaq® Green Master Mix, 2X (pH 8,5): 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP, and 3mM $MgCl_2$; in addition the buffer contains a compound that increases the density of the probe, and a yellow and a blue dye that function as loading dyes for agarose gel electrophoresis.

2.2.11 Amplification of high molecular weight DNA

As the amounts of DNA extracted from VLPs varied and were sometimes rather small some DNA samples had to be amplified for proper size determination via agarose gel electrophoresis or prior to shotgun library construction.

Therefore the REPLI-g Mini Kit from QIAGEN was used. It allows the amplification of large DNA molecules from small amounts of template DNA and ensures minimal sequence bias.

Procedure and materials

For 50 µl reaction:

- 5 µl DNA
- 5 µl Buffer D1 were added
- Vortexing and brief centrifugation
- Incubation for 3 minutes at room temperature
- 10 µl Buffer N1 were added
- Vortexing and brief centrifugation
- 30 µl master mix were added
- Incubation at 30 °C for 10-16 hours using a MWG Primus Thermocycler from MWG Biotech with lid heater set to 70 °C
- Incubation at 65 °C for 3 minutes to inactivate RPLI-g Mini DNA Polymerase with lid heater set to 70 °C

Buffer D1: for 7 reactions: 9 µl Reconstituted Buffer DLB, 32 µl nuclease free water; Reconstituted Buffer DLB: 500 µl added;

Buffer N1: for 7 reactions: 12 ml Stop Solution, 68 µl nuclease free water;

Master mix: for 1 reaction: 29 µl REPLI-g Mini Reaction Buffer, 1 µl REPLI-g Mini DNA Polymerase;

2.2.12 Agarose gel electrophoresis

Via agarose gel electrophoresis DNA and RNA fragments can be separated according to their size. The negatively charged nucleic acid molecules run in an electric field from the negative to the positive pole through an agarose gel. This gel functions as molecular sieve through which smaller DNA molecules move faster than larger ones. After dying the gel in an ethidium bromide solution the fragments are visible in UV light. The agarose content of the gel varies depending on the size of the fragments that should be separated. Agarose gel electrophoresis is also suitable to purify nucleic acids when subsequent elution from the gel is performed.

Ethidium bromide is a carcinogen substance that intercalates non-specifically into the DNA. Midori Green DNA Stain which is equally sensitive constitutes a safer alternative to colour nucleic acid fragments for observation in UV light. It is added to the gel prior to casting but as the colorant migrates from the negative to the positive pole most gels had to be dyed in ethidium bromide solution after they were run.

In this work agarose gel electrophoresis was used for detection and size fractionation and determination (VLP DNA, restricted recombinant plasmids) as well as for purification of DNA fragments prior to cloning (PCR products, VLP DNA fragments).

Procedure and materials

- For 100 ml 0,5-1% agarose gel 0,5-1 g agarose were dissolved in either 1x TBE or 1x TAE buffer by boiling using a microwave oven
- When cooled down (< 60 °C) 3 µl Midori Green DNA Stain (200 reactions/ml; NIPPON Genetics EUROPE GmbH) were added
- The gel was then poured into a gel rack into which a comb was placed
- When fully polymerised the gel was placed into a tank containing 1x TAE buffer

- The gel was loaded with samples containing Loading Dye (6X; Fermentas) and size markers and run at 30-70 V/cm

1x TAE buffer: for 1000 ml: 4,84 g Tris, 1,14 ml acetic acid, 2 ml 0,5M EDTA;

1x TBE buffer: for 1000 ml: 10,8 g Tris, 5,5 g Boric Acid, 2 ml 0,5M EDTA;

Ethidium bromide solution: for 100 ml buffer: 10 µl ethidium bromid solution (10mg/ml; Carl Roth);

2.2.13 End repair of insert DNA for blunt end cloning

To generate blunt ended DNA fragments for blunt end ligation an end repair step had to be performed. This was done using the DNATerminator® End Repair Kit from Lucigen® Corporation.

Procedure and materials

For 50 µl reaction:

- 38 µl insert DNA
- 10 µl 5X DNATerminator® End Repair Buffer
- 2 µl DNATerminator® End Repair Enzymes

The reaction was incubated for 30 minutes at room temperature and stopped by subsequent incubation at 70 °C for 15 minutes.

2.2.14 Recovery of DNA fragments from agarose gel

In order to clone DNA fragments they have to be purified and size fractionated first. This was done by agarose gel electrophoresis and subsequent elution from the gel. The desired DNA fraction was cut out from the gel under exposure to UV light and recovered from the gel piece using the illustra GFX™ PCR DNA and Gel Band Purification Kit from GE Healthcare.

Procedure and materials

- The gel piece was weighed
- For each 10 mg 10 µl Capture buffer type 3 were added (300 µl minimum)
- Tube was inverted several times
- Incubation at 60 °C for 15-30 minutes until complete dissolution of the gel piece; tube was inverted every 3 minutes
- A GFX MicroSpin column was placed into a Collection tube
- Up to 800 µl of the buffer sample mix were transferred onto the GFX MicroSpin column contained in the Collection tube
- Incubation for 1 minute at room temperature
- Centrifugation for 30 seconds at 16000 g
- The flow through was discarded and the GFX MicroSpin column was placed into the Collection tube again
- With samples > 800 µl the last 4 steps were repeated until all of the sample was loaded
- 500 µl Wash buffer type 1 were added
- Centrifugation for 30 seconds at 16000 g
- The Collection tube was discarded, the GFX MicroSpin column was placed into a DNAse free 1,5 ml tube
- 10 µl A. dest. were transferred to the centre of the GFX MicroSpin column
- Incubation for 1 minute at room temperature
- Centrifugation for 1 minute at 16000 g

2.2.15 Cloning

2.2.15.1 Cloning of PCR products

For further analyzes the PCR products were ligated into a plasmid and the recombinant plasmids transformed into the appropriate bacterial strain.

To clone PCR products the pGEM®-T Easy vector system from Promega was chosen. The vector is 3015 bp in length and is provided in linear form (cut at position 60 with EcoRV) with a single thymidine-overhang at its 3'-terminus. This overhang allows a

sticky end ligation with the PCR products that carry a single adenosine-overhang at its 5`-terminus. These 5'-overhangs are produced by the Taq polymerase during PCR. 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.

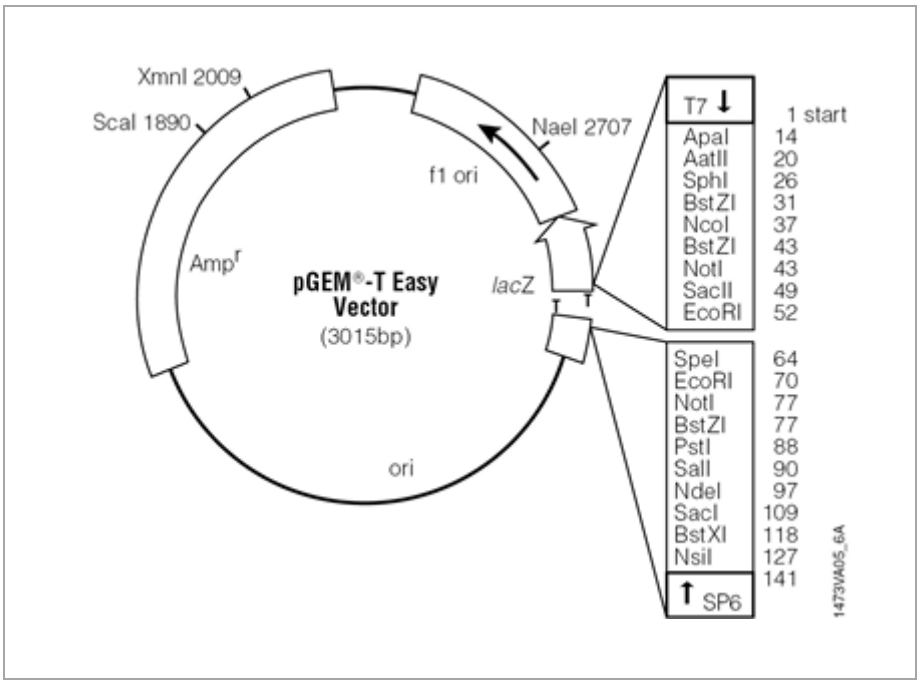

Fig 1 pGEM®-T Easy vector (adapted from www.promega.com)

Colonies containing a recombinant plasmid were selected by ampicillin and blue white selection using IPTG and X-Gal. Only bacteria which have taken up the vector containing the ampicillin resistance gene are able to grow on LB amp plates. Bacteria that have taken up a recombinant plasmid are recognized by their white colour as the insert disrupts the β-galactosidase encoding region. If the vector does not contain an insert β-galactosidase will be expressed and turn the colourless X-Gal into an indoxyl derivative which anodises to 5,5'-dibromo-4,4'-dichloro-indigo. This compound renders colonies of clones containing a plasmid without insert blue.

2.2.15.1.1 Preparation of E.coli strain JM109 competent cells

Competent cells are characterised by their ability to take up foreign DNA fragments. Cells can be made permeable to DNA by electroporation or chemically. Here it was done chemically by exposing the cells to TSS buffer.

Procedure and materials

- A cryo culture of *E.coli* strain JM109 (Promega) was stroked out on an LB plate and incubated at 37 °C over night
- Clones of a single colony were picked to inoculate 5 ml LB medium
- Incubation over night at 37 °C and 250 rpm
- 500 µl of this preculture were used for inoculation of 50 ml LB medium
- Incubation at 37 °C and 250 rpm until an OD of 0,3-0,4 was reached
- The flask was placed on ice and its content was dispensed into 4 sterile capo-test tubes
- Centrifugation at 3000 rpm for 10 minutes
- The supernatant was discarded, each pellet was placed on ice
- Resuspension in 1 ml ice cold 1x TSS buffer
- 1,5 ml tubes were cooled and filled with 100 µl of the suspension
- The tubes were frozen immediately in liquid nitrogen and stored at -80 °C

1x TSS buffer: for 100 ml: 10 g PEG (polyethylene glycol) 3350 dissolved in 25 ml A. dest., 10 ml 0,5M $MgCl_2$, 5 ml 10% DMSO (dimethyl sulfoxide) and 45 ml LB medium added; pH value adjusted to 6,5; filled up to 100 ml with LB medium; sterile filtration;

2.2.15.1.2 Ligation into pGEM®-T Easy vector

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

Procedure and materials

For 10 µl ligation reaction:

- 3 µl DNA

- 5 µl 2X Rapid Ligation Buffer
- 1 µl pGEM®-T Easy vector (50 ng/µl)
- 1 µl T4 DNA Ligase (3 u/µl)

The ligation reaction was incubated for 1 hour at room temperature.

2X Rapid Ligation Buffer: 60mM Tris-HCl (pH 7.8), 20mM MgCl2, 20mM DTT, 2mM ATP, 10% polyethylene glycol;

2.2.15.1.3 Transformation

Transformation means the uptake of free, non-viral DNA by competent cells.

Here the pGEM®-T Easy vector containing the PCR product had to be brought into competent cells of the *E.coli* strain JM109.

Procedure and materials

- 100 µl competent cells were thawed on ice (but not entirely!)
- 2 µl ligation reaction were added
- 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
- 950 µl SOC medium added
- Incubation for 1,5 hours at 37 °C and 150 rpm
- 2-3 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
- 100-330 µl of the transformation reaction were stroked out on each plate and incubated at 37 °C overnight

SOC medium (= SOB medium + 20mM glucose): for 1000ml: 20 g peptone, 5 g yeast extract, 5 g 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;

0,1M IPTG (isopropyl β-D-1-thiogalactopyranosidase): for 10 ml: 238 mg dissolved in A. dest.; sterile filtration;

2% X-Gal (w/v; 5-bromo-4-chloro-3-indolyl- β-D-galactosidase): for 5 ml: 100 mg dissolved in 5 ml N,N'-dimethylformamide;

2.2.15.2 Cloning of VLP DNA fragments

In order to construct a shotgun library of the VLP DNA the CLONESMART® HCKan Chemically Competent Blunt Cloning Kit (SOLOs) from Lucigen® Corporation was chosen for cloning of blunt ended DNA fragments. The kit was provided with the pSMART-HCKan-vector, which is 1788 bp in length and *E. cloni* 10G chemically competent cells.

The design of the transcription free high copy vector with kanamycin selection guarantees (according the producer) successful cloning of any DNA, no cloning bias, no sequence gaps and no false negative clones. With greater than 99% recombinants no screening is needed.

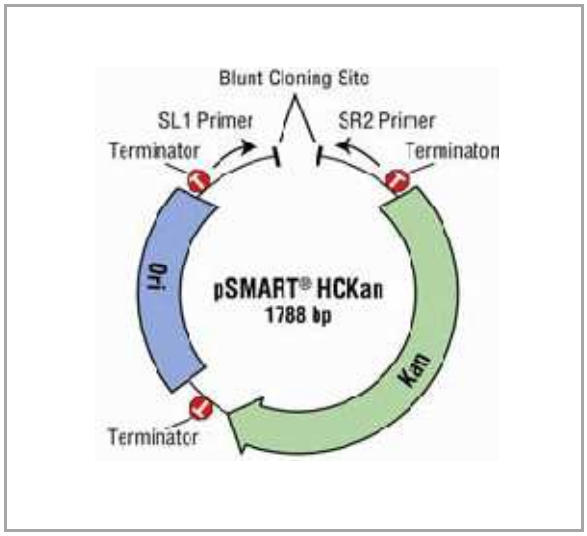

Fig 2 pSMART- HCKan-vector (adapted from www.lucigen.com)

2.2.15.2.1 Ligation

Procedure and materials

For 10 µl reaction:

- 6,5 µl DNA
- 2,5 µl 4X CloneSmart Vector Premix (pSMART vector, ATP, buffer)
- 1 µl CloneSmart DNA Ligase (2 u/µl)

The reaction was incubated for 2 hours at room temperature and 15 minutes at 70 °C to stop the reaction.

2.2.15.2.2 Transformation

Procedure and materials

- 40 µl *E. cloni* 10G chemically competent cells where thawed completely on wet ice
- 1 µl ligation reaction was added
- Incubation on ice for 30 minutes
- Heat shock at 42 °C for 45 seconds using a water bath
- Incubation on ice for 2 minutes
- 960 µl Recovery Medium were added
- Incubation for 1 hour at 37 °C and 250 rpm in the dark
- 100 µl transformed cells were plated on LB kan plates
- Incubation over night at 37 °C in the dark

2.2.16 Amplification of the shotgun library and preparation for long term storage

Amplification of the shotgun library was done using a modified version of a method described by Weis (2001).

Procedure and materials

- Each LB kan plate was flooded with 2 ml of LB medium
- The colonies were rubbed off the agar using a sterile rubber policeman

- The suspension of all plates was pooled into a sterile 50 ml tube resulting in a total volume of 3,8 ml
- The suspension was mixed properly with 670 µl sterile glycerol so that a final glycerol concentration of 15% was reached
- 500 µl of the suspension were transferred into 1 ml tubes and stored at -80 °C

2.2.17 Restriction analysis

As 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. Subsequent agarose gel electrophoresis offered information about the presence and the size of the insert.

Procedure and materials

For 10 µl restriction reaction:

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

The restriction reaction was incubated at 37 °C for 2 hours using an Eppendorf ThermoStatplus thermoblock.

10x SuRE/Cut Buffer H: 0,5M Tris-HCl, 1M NaCl, 100mM $MgCl_2$, 10mM DTE; pH 7,5 (at 37 °C);

2.2.18 Sequencing

Sequencing was done by 4base-lab (Germany).

Primers used for sequencing:

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

2.2.19 List of suppliers of the used materials

A	
Acetic acid	Carl Roth GmbH + Co. KG
Acridinorange	MERCK GesmbH
Agar-Agar	MERCK GesmbH
Agarose	Promega GmbH
Ammonium acetate	MERCK GesmbH
Ampicillin solution (50 ng/ml)	Carl Roth GmbH + Co. KG
Antimicrobial susceptibility test discs	Carl Roth GmbH + Co. KG

C	
Chloroform	Carl Roth GmbH + Co. KG
Citifluor	Christine Gröpl Elektronenmikroskopie
CLONESMART® HCKan Chemically Competent Blunt Cloning Kit (SOLOs)	Lucigen Corporation
Conical centrifuge tubes, 50 ml	SPL Labware

D	
DMSO (dimethyl sulfoxide; 10%)	Sigma-Aldrich Handels GmbH
DNA Terminator® End Repair Kit	Lucigen Corporation

E	
E.coli strain JM109	Promega GmbH
EcoRI (10 u/µl;)	Roche Diagnostics GmbH
EDTA	Sigma-Aldrich Handels GmbH
Ethanol	MERCK GesmbH
Ethidium bromid solution (10mg/ml)	Carl Roth GmbH + Co. KG

F

Filter, 0,02 µm pore size	Whatman GmbH
Filter, 0,05 µm pore size	Millipore GmbH
Filter, 0,1 µm pore size	Costar
Filter, 0,2 µm pore size	Whatman GmbH
Formaldehyde	Carl Roth GmbH + Co. KG

G

Generation Capture Column Kit	QIAGEN GmbH
Gentra® Puregene® Yeast/Bact. Kit	QIAGEN GmbH
GFX™ PCR DNA and Gel Band Purification Kit	GE Healthcare
Glacial acetic acid	Carl Roth GmbH + Co. KG
Glucose	Fluka Chemie AG
Glycerol	Carl Roth GmbH + Co. KG
GoTaq® Green Master Mix (2X)	Promega GmbH
Guanidium thiocynate	Sigma-Aldrich Handels GmbH

I

IPTG (isopropyl β-D-1-thiogalactopyranosidase)	Carl Roth GmbH + Co. KG
Isoamyl alcohol	Carl Roth GmbH + Co. KG
Isopropanol	Fluka Chemie AG

K

Kanamycin solution (30 µg/ml)	Carl Roth GmbH + Co. KG
KCl	Carl Roth GmbH + Co. KG

M

Medium 514	Carl Roth GmbH + Co. KG

$MgCl_2$	Carl Roth GmbH + Co. KG

N

N,N'-dimethylformamide	Fisher Scientific
NaCl	Carl Roth GmbH + Co. KG

P

Paraffin oil	Kwizda Pharma GmbH
PEG (polyethylene glycol) 3350	Carl Roth GmbH + Co. KG
Pepton	SERVA Electrophoresis GmbH
pGEM®-T Easy vector (50 ng/µl)	Promega GmbH
Phenol	Carl Roth GmbH + Co. KG
Primer aga gtt tga tcm tgg ctc ag-3'	VBC Biotech (Austria)
Primer tac ggy tac ctt gtt acg act t-3'	VBC Biotech (Austria)
Proteinase K (20 mg/ml 1x TE)	Sigma-Aldrich Handels GmbH

Q

QIAprep Spin Miniprep Kit	QIAGEN
Quick seal tubes	Beckman Coulter GmbH

R

Rapid Ligation Buffer (2X)	Promega GmbH
REPLI-g Mini Kit	QIAGEN
Rotilabo® syringe filters 0,45 µm	Carl Roth GmbH + Co. KG
Rotilabo® syringe filters 0,22 µm sterile	Carl Roth GmbH + Co. KG
Rotilabo® syringe filters 0,22 µm	Carl Roth GmbH + Co. KG

S

Sarcosyl (10%)	Sigma-Aldrich Handels GmbH
SDS (sodium lauryl sulfate)	Carl Roth GmbH + Co. KG
Sodium acetate	MERCK GesmbH

SuRE/Cut Buffer H (10x)	Roche Diagnostics GmbH
SYBRE®GOLD nucleic acid gel stain (10000x in DMSO)	Invitrogen GmbH

T

T4 DNA Ligase (3 u/µl)	Promega GmbH
Tris	Carl Roth GmbH + Co. KG

X

X-Gal	Carl Roth GmbH + Co. KG

Y

Yeast extract	Carl Roth GmbH + Co. KG

2.3 In silico work

Sequence analysis was performed using the programs nucleotide blast and blastx (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3 Results

3.1 Growth Curve

In order to gather some information about the growth characteristics of *A. kielensis* the optical density of culture GS1.15.C was observed over 4 weeks. The growth curve is shown in Fig 3. The typical growth phases (lag phase, exponential phase, stationary phase, death phase) were not observed as ideally expected with a pure culture. Nevertheless the culture seemed to have entered the stationary phase after ~100 hours and not reached the death phase when the period of observation ended.

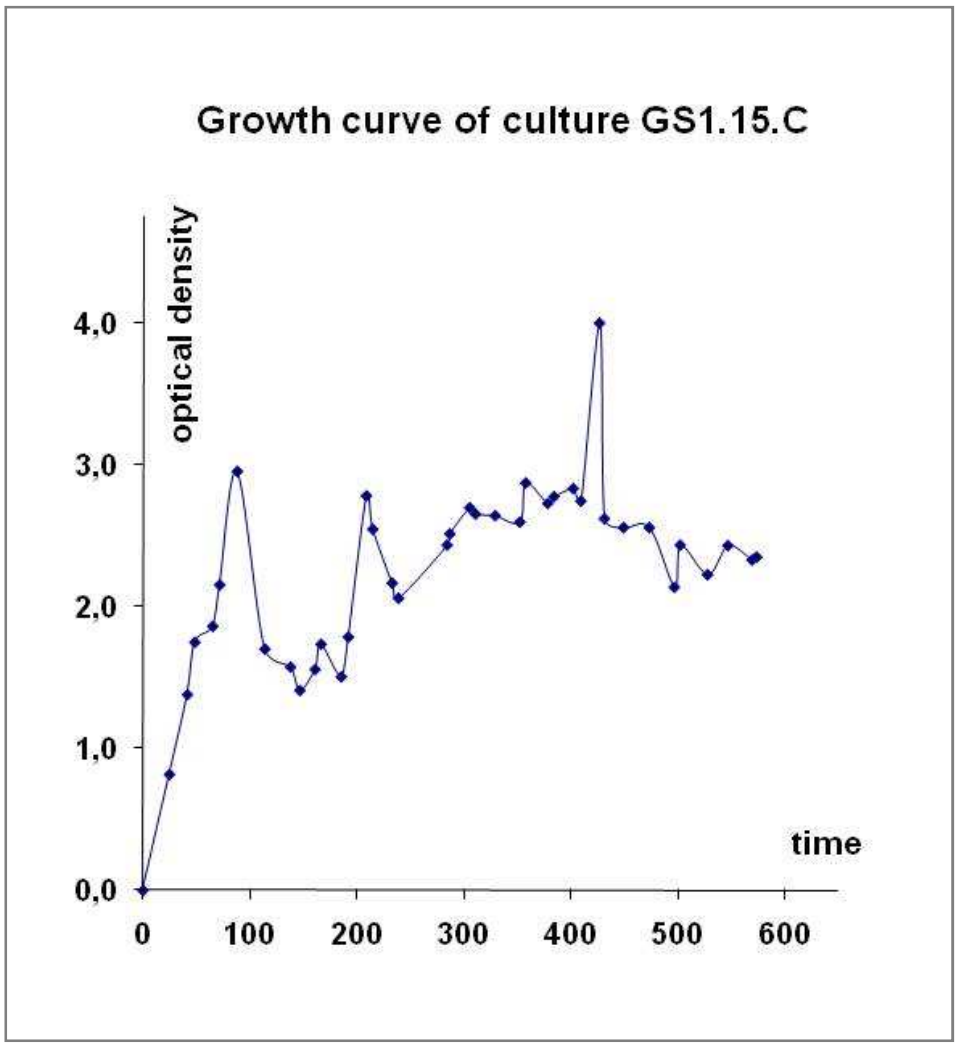

Fig 3 growth curve of culture GS1.15.C; time given in hours;

3.2 Size and shape of *A. kielensis* and its VLPs

Samples of liquid cultures and aliquots of VLP suspensions were prepared for examination under an electron microscope. Bacterial cells of culture GS1.2.B are shown in Fig 4 A-D. Almost round electron dense inclusion bodies which are supposed to be precursors of VLPs are indicated by arrows. Up to three of these bodies (data not shown) were found per bacterial cell and were about 80-125 nm in diameter. The bacterial cells varied in shape (see Fig 4 A vs. Fig 4 B-D) and were about 550-1400 x 450-650 nm in size.

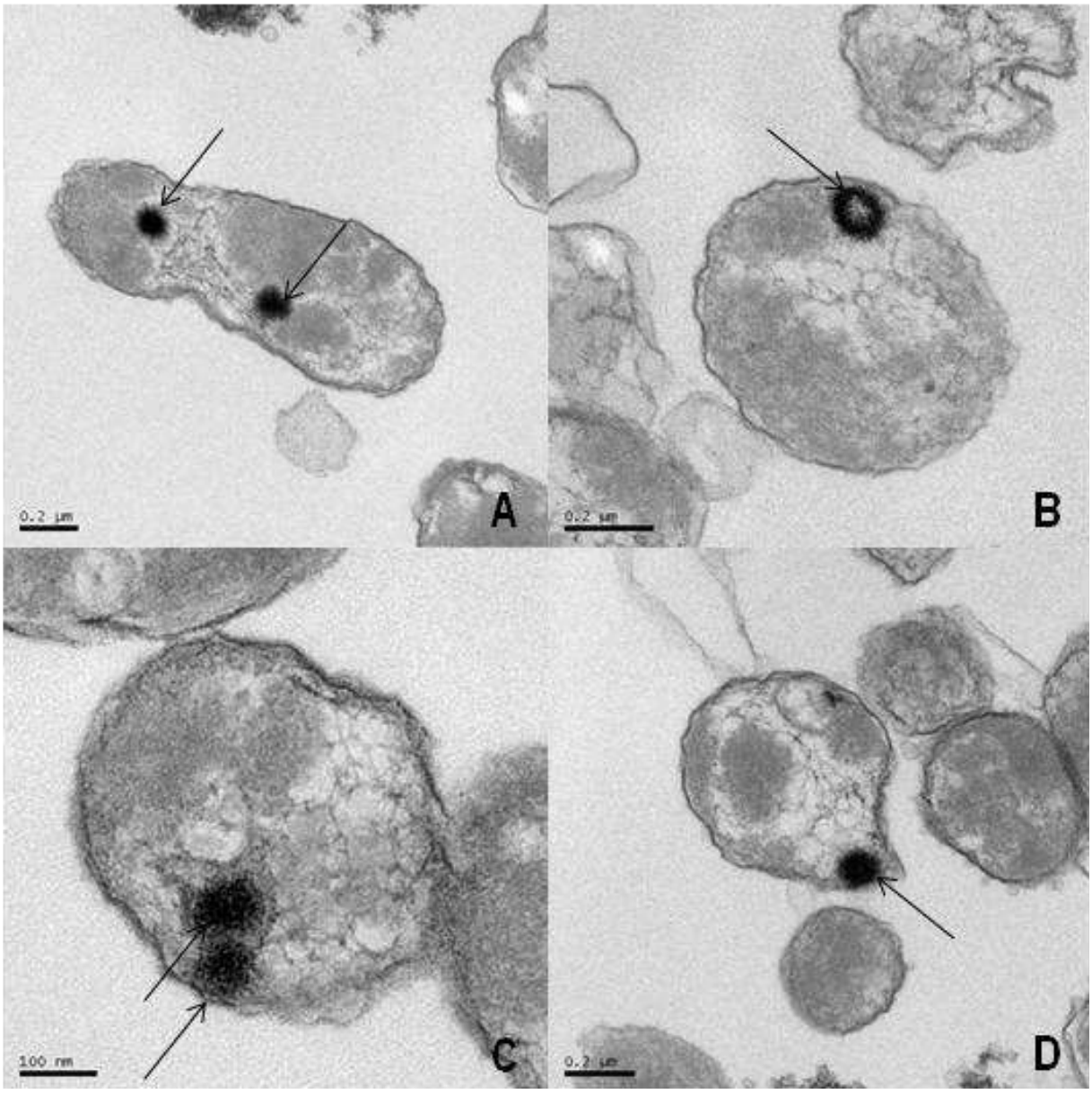

Fig 4 A-D: thin section of culture GS1.2.B (289 h); electron dense bodies are indicated by arrows;

VLPs of culture GS1.1.A (280 h) are shown in Fig 5 A-D. The particles are about 80-155 x 100-200 nm in size.

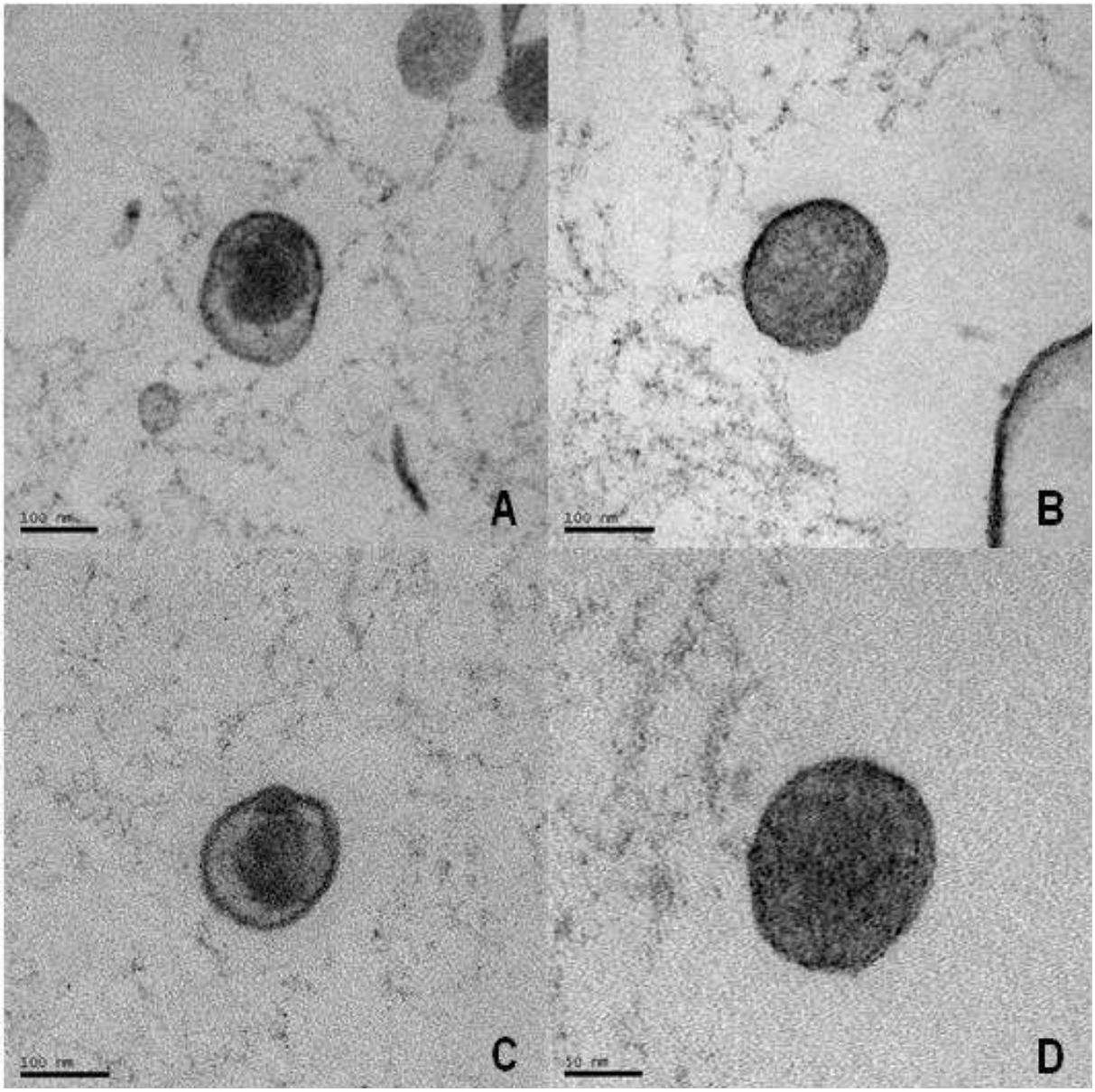

Fig 5 A-C: thin sections of VLP suspension from culture GS1.1.A (280 h);

3.3 Dispensation of bacterial aggregates

Samples were taken for epifluorescence microscopy from each culture immediately before the harvest of VLPs was started. It was confirmed that *A. kielensis* is an aggregate forming bacterium. These aggregates were not observed with all samples, varied in size and often seemed to be coated by a structure that could not be identified yet (see Fig 6).

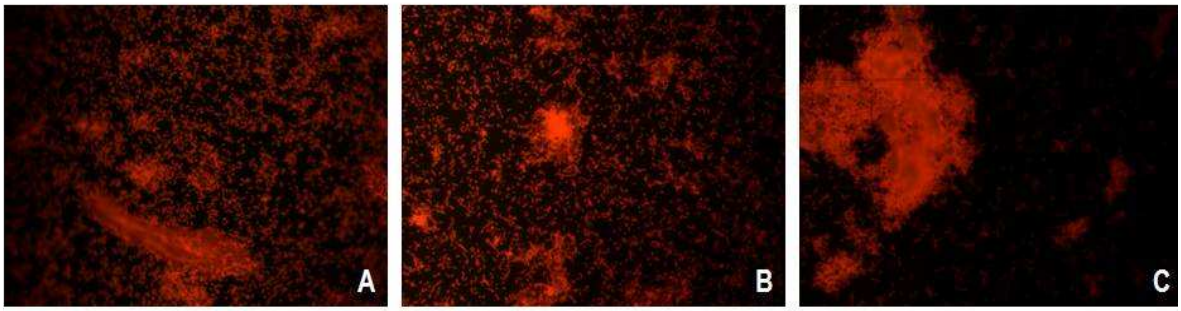

Fig 6 epifluorescence micrographs of 10 µl samples of *A. kielensis* cultures pulled through 0,1 µm pore sized filters and stained with acridinorange; **A**: GS1.9.D (425 h); **B:** GS1.10.C (449 h); **C:** GS1.8.C (474 h);

We hypothesised that these aggregates would consist of bacterial cells and VLPs and that dispensation would lead to higher amounts of VLPs during particle harvest and subsequently to higher amounts of VLP DNA. This was done by sonication as described in chapter 2.2.5.1. The parameters chosen for sonication were suitable to dissolve the observed aggregates (see Fig 7).

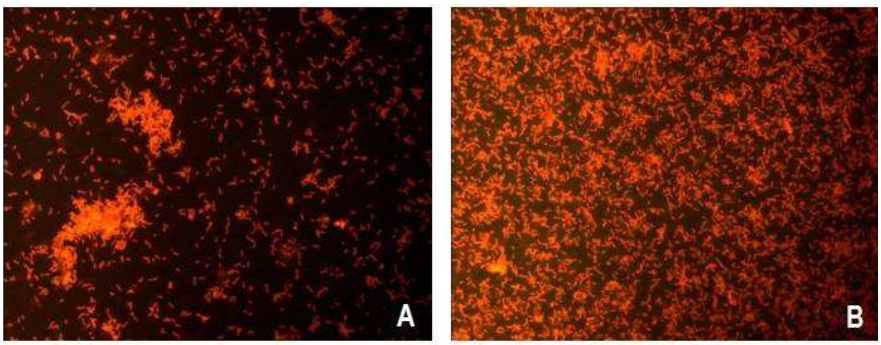

Fig 7 epifluorescence microcraphs of 10 µl samples of *A. kielensis* culture GS1.13.D (474 h); **A:** untreated; **B:** sonicated twice for 30 sec at 100 W with 1 minute break;

3.4 Observations during VLP harvest

3.4.1 Bacteria pellet

The bacterial pellet varied in size, shape, stability and colour depending on the age of the culture and whether the culture had been sonicated or not. Pellets from pooled cultures GS1.7.C/D (2014 h), GS1.15.A/D (379 h) and GS1.16.B/C (376 h) are shown in Fig 8. One half of each pooled culture was sonicated the other half not. Pellets from sonicated cultures tended to be more stable while pellets from cultures that were not sonicated tended to be slightly larger. But the size of the pellet as well as the colour composition varied most with age.

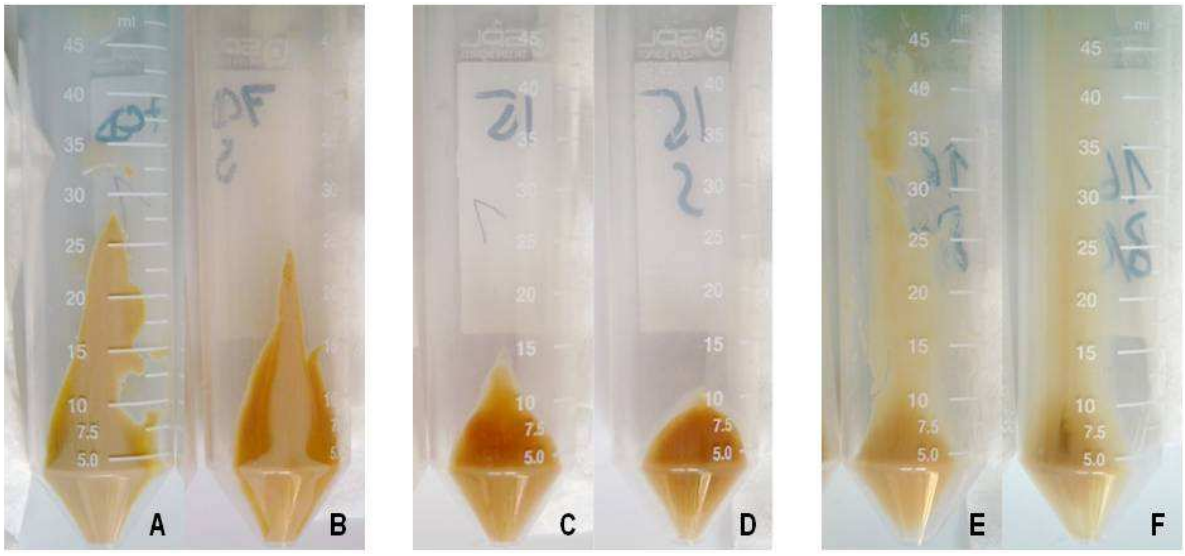

Fig 8 bacterial pellets; **A:** culture GS1.7.C/D (2014 h); **B:** sonicated culture GS1.7.C/D (2014 h); **C:** culture GS1.15.A/D (379 h); **D:** sonicated culture GS1.15.A/D (379 h); **E:** culture GS1.16.B/C (376 h); **F:** sonicated culture GS1.16.B/C (376 h);

3.4.2 VLP pellet

The VLP pellets varied as well in size and colour depending on the age and treatment of the culture prior to particle harvest (data not shown). Sonication prior to particle harvest resulted in pellets that were larger and more intense in colour.

All test cultures showed a colourless pellet while all pellets from cultures derived from the glycerol stock were at least slightly green pigmented. All test cultures and cultures derived from the glycerol stock were grown in the dark, but the preculture used for the glycerol stock was grown in the light.

3.5 Test for antimicrobial activity of *A. kielensis* and its VLPs

A. kielensis and its VLPs were tested for antagonistic activity against *Staphylococcus aureus, Listeria monocytogenes, Bacillus subtilis, Bacillus cereus, Vibrio cholerae* and *Vibrio alginolyticus* following the disc diffusion method as described in chapter 2.2.7. The test was carried out several times using bacteria and products of cultures of different ages. The cultures had not been sonicated. The desired effect (clear areas in the bacterial lawn around the charged filter discs) was observed on *Staphylococcus aureus* and *Listeria monocytogenes* but varied depending on the used *A. kielensis* culture. No effect was shown on *Bacillus subtilis, Bacillus cereus, Vibrio cholerae* and *Vibrio alginolyticus*.

3.5.1 Antagonism against *L. monocytogenes and S. aureus*

Overnight cultures of *L. monocytogenes* and *S. aureus* were grown in 5 ml LB medium each and diluted until an OD of approximately 0,006 and 0,040 was reached respectively. 100 µl of each diluted culture were plated on 514+Y+P plates.

The following pooled cultures were used for the test:

- GS1.6.C/D (2251 h)
- GS1.7.C/D (2014 h)
- GS1.15.A/D (379 h)
- GS1.16.B/C (376 h)

The filters were charged with the following suspensions and solutions:

- Filter 1: bacterial pellet resuspended in A. dest.
- Filter 2: sterile filtrate

- Filter 3: VLP pellet resuspended in 1x TBT buffer
- Filter 4: supernatant obtained from the ultracentrifugation
- Filter 5: A. dest. (control for filter 1)
- Filter 6: 514+Y+P (control for filters 2 and 4)
- Filter 7: 1x TBT buffer (control for filter 3)

No effect was shown on any of the bacteria when bacteria and products of culture GS1.16.B/C (376 h) were used (data not shown).

Pictures of some plates are shown in and Fig 9 and Fig 10. Unfortunately the images did not always reflect the full degree of the observed effect.

An antimicrobial activity of *A. kielensis* (filter 1) and the sterile filtrate (filter 2) was observed against *L. monocytogenes* when culture GS1.6.C/D (2251 h) was used (see Fig 9 A). When culture GS1.7.C/D (2014 h) was used changes in the lawn were only observed around filter 1. Thereby the filter was surrounded by bacteria (see Fig 9 B). These bacteria did neither look like *A. kielensis* nor like *L. monocytogenes*.

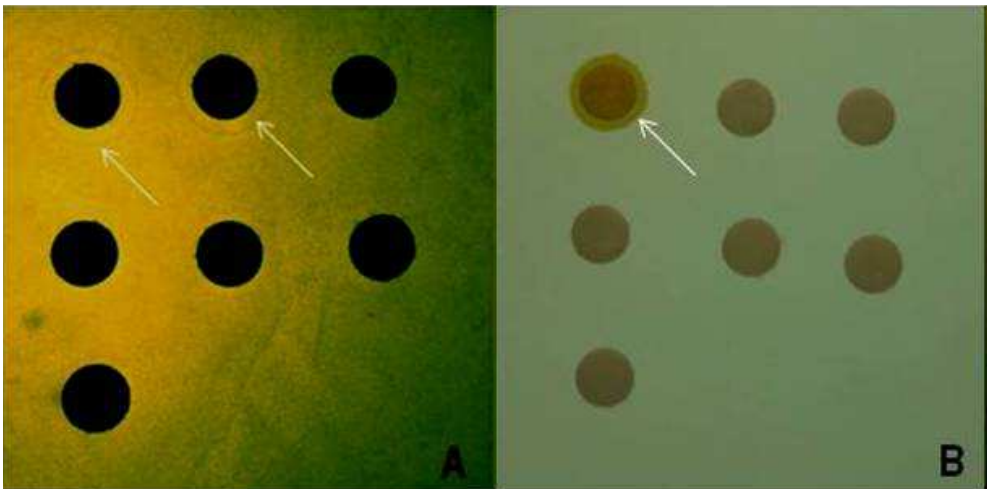

Fig 9 antagonistic activity against *L. monocytogenes*; changes in the lawn of *L. monocytogenes* are indicated by arrows; **A:** bacteria and products of culture GS1.6.C/D (2251 h); **B:** bacteria and products of culture GS1.7.C/D (2014 h);

The desired effect was observed on *S. aureus* around filter 1 and 2 when culture GS1.6.C/D (2251 h) was used (see Fig 10 A). When culture GS1.15A/D (379 h) was used an antibacterial effect was observed only around filters 1 and 2 (see Fig 10 B) and with

culture GS1.7.C/D (2014 h) no effect was observed around any of the filters (data not shown).

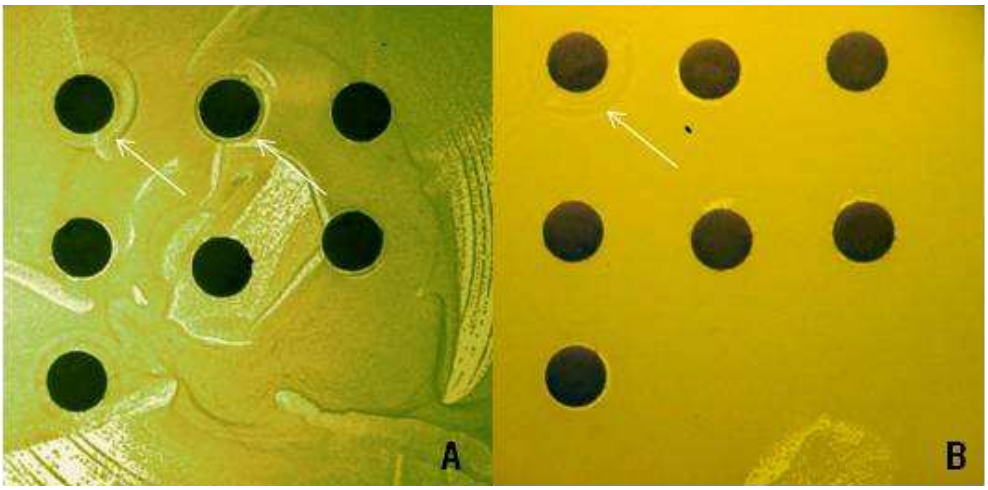

Fig 10 antagonistic activity against *S. aureus*; changes in the lawn of *S. aureus* are indicated by arrows; **A:** bacteria and products of culture GS1.6.C/D (2251h); **B:** bacteria and products of culture GS1.15.A/D (379 h);

It must be said that also around some of the control filters 5 (A. dest.), 6 (514+Y+P medium) and 7 (TBT buffer) slight changes in the lawn were observed. These filters tended to weep when they were placed on the plates which was not observed with filters 1-4.

3.6 Test for cell-freeness of the VLP suspensions

To ensure that the obtained VLP suspensions were free of bacterial cells, aliquots were plated on 514+Y+P plates and prepared for epifluorescence microscopy as well (data not shown). Rare contaminations with bacterial cells could always be correlated with contaminations of the staining apparatus, the A. dest. used during the staining procedure or the solution chosen for resuspension of the VLP pellet. Microscopy further revealed that the amount of VLPs varied depending on the processed culture (data not shown).

3.7 Verification of *A. kielensis* via PCR amplification and sequencing of the 16S rDNA

To ensure that the cultivated bacteria belonged to the species *A. kielensis*, genomic DNA was extracted from some cultures and used as template for amplification of the 16S rRNA encoding region via PCR. The PCR product was purified by agarose gel electrophoresis and cloned using the pGEM®-T Easy vector system from Promega. Sequencing was done by 4base-lab Germany. By blastn search it was verified that the cultivated bacterium was *A. kielensis* (see chapter 3.10.1).

3.7.1 PCR results

PCR was performed using primers and parameters as described in chapter 2.2.10 to amplify the 16S rDNA of *A. kielensis*. As expected the obtained fragments were about 1450 bp in length. The DNA bands were eluted from the gel and used for cloning.

PCR results from cultures GS1.2.A and GS1.2.C are shown in Fig 11 A. Genomic DNA from both cultures was extracted following the protocol described in chapter 2.2.8.1 and was pooled. PCR results from cultures GS1.6.A, GS1.6.B, GS1.7.A and GS1.7.B are shown in Fig 11 B. From these cultures genomic DNA was extracted using a kit from QIAGEN (see chapter 2.2.8.2). PCR was not successfully performed until the genomic template DNA was processed for protein precipitation which indicates that a compound of the used elution buffer inhibited PCR. PCR products are marked by arrows.

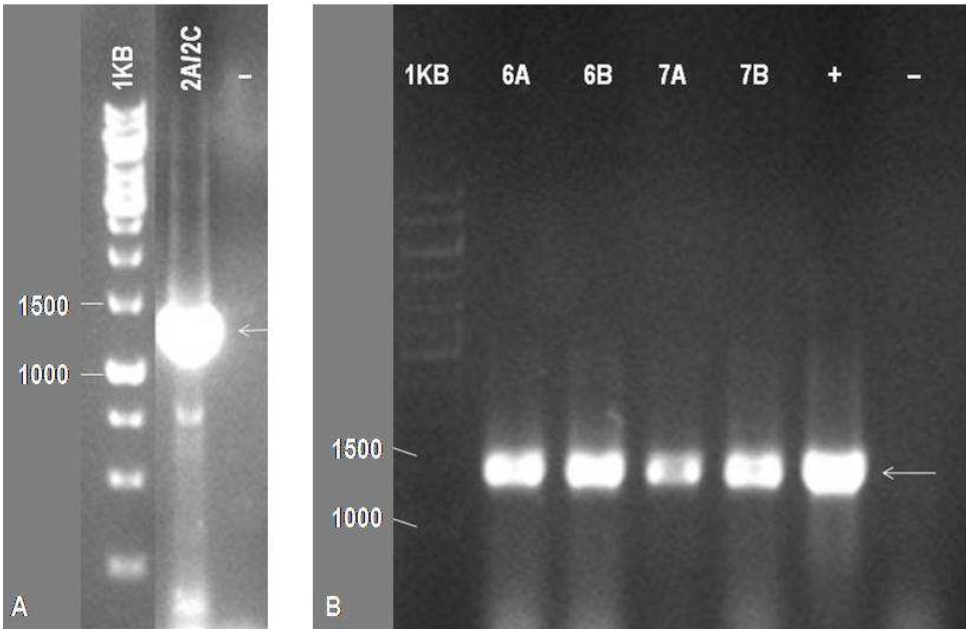

Fig 11 PCR amplified 16S rDNA of *A. kielensis*; 1% agarose gel in 1x TAE buffer; 1KB: size marker in bp; arrows mark bands of PCR products and primers; **A:** PCR products 2A/2C; pooled, genomic DNA extracted from culture GS1.2.A and GS1.2.C used as template; **B:** PCR products 6A, 6B, 7A, 7B; genomic DNA extracted from cultures GS1.6.A, GS1.6.B, GS1.7.A, GS1.7.B used as template;

3.7.2 Digestion of recombinant plasmids with EcoRI

As the insert site of the pGEM®-T Easy vector is flanked by two EcoRI restriction sites digestion with this enzyme resulted in the separation of vector and insert DNA. Three DNA bands could be distinguished by agarose gel electrophoresis indicating that the 16S rDNA of *A. kielensis* contains an EcoRI restriction site as well (see Fig 12). The ~3000 bp long fragment represents the vector while the ~630 and the ~850 bp long fragments represent the two parts of the restricted insert.

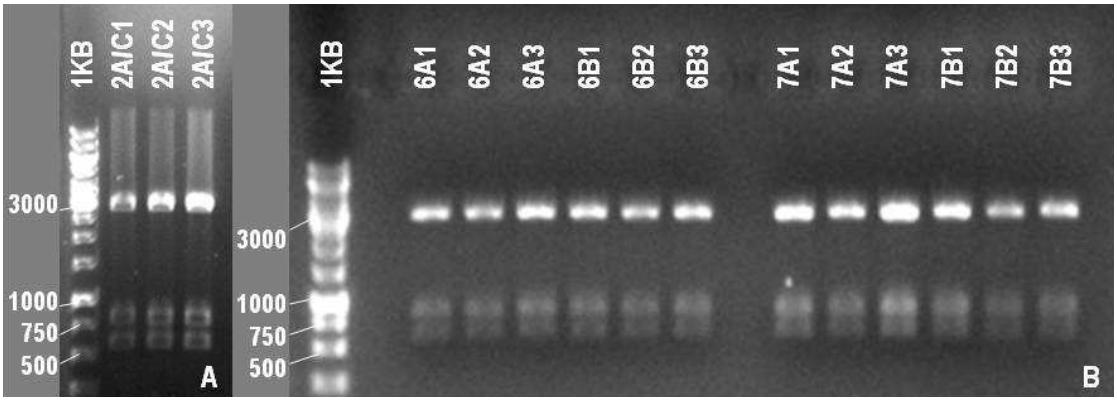

Fig 12 digestion of recombinant plasmids with EcoRI; 1% agarose gel in 1x TAE buffer; 1KB: size marker in bp; **A:** digested plasmids from clones 2A/C1-3; **B:** digested plasmids from clones 6A1-3, 6B1-3, 7A1-3 and 7B1-3;

3.8 Establishment of a method for DNA extraction from VLPs

For DNA extraction several modified versions of the protocols described in chapter 2.2.8.3 and 2.2.8.4 had been tested. Some were more efficient than others and some were entirely inapplicable.

When using the Gentra® Puregene® Yeast/Bact. Kit from QIAGEN no VLP DNA was obtained when the original protocol for DNA extraction from gram negative bacteria was followed. The original protocol demanded resuspension of the VLP pellet in Cell Lysis Solution, but only if the VLPs were suspended in Cell Suspension Solution first, DNA could be extracted from some of the cultures. According to the handbook supplied with kit Cell Suspension Solution was only required for isolation of DNA from gram positive bacteria or yeast.

Agarose gel electrophoresis revealed that the obtained VLP DNA was about 40 kbp in length but varied in size as well as in the amount of DNA (see Fig 13 and Fig 14) depending on the processed culture.

Fig 13 shows VLP DNA of test cultures A2 (474 h) and A3 (331 h). The cultures had not been sonicated. The amount of VLP DNA from the older culture A2 was significantly higher than that from culture A3. Apparently also the length of the extracted DNA differed between the two cultures and was estimated to be about 40-80 kbp.

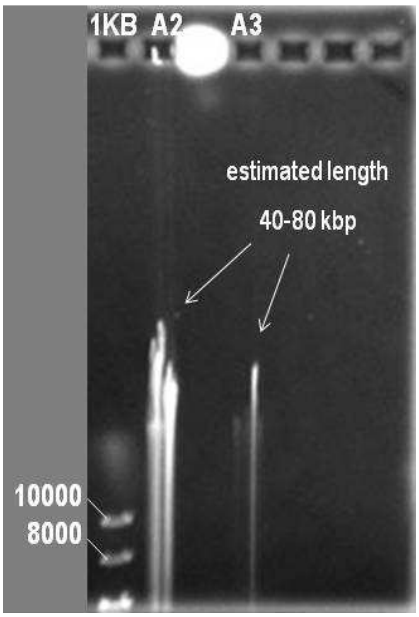

Fig 13 size fractionation of VLP DNA from test cultures A2 and A3 using 0,5% agarose gel in 1x TBE buffer; VLP DNA was extracted following protocol 1 without subsequent protein precipitation; 1KB: size marker in bp; A2: VLP DNA extracted from test culture A2 (474 h); A3: VLP DNA extracted from test culture A3 (331 h);

VLP DNA from cultures GS1.4.C (639 h) and GS1.5.B (304 h) was extracted following protocol 2 and was about the same size (~40 kbp), whereby DNA from culture GS1.5.B seemed to be slightly larger. The signal in lane 5B (DNA from VLPs of the younger culture) was stronger than that in lane 4C. Additionally a strong signal at 2-4 kb was observed with lane 5B and a weak one with lane 4C, as well as in the slots of both lanes (see Fig 14).

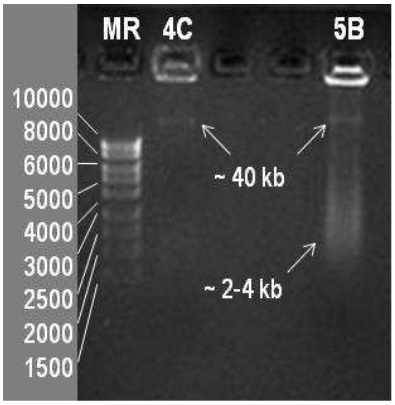

Fig 14 size fractionation of VLP DNA from cultures GS1.4.C and GS1.5.B using 1% agarose gel in 1x TAE buffer; VLP DNA was extracted following protocol 2; MR: size marker in bp; 4C: VLP DNA from culture GS1.4.C (639 h); 5B: VLP DNA from culture GS1.5.B (304 h);

In general DNA extraction was more often successful and yielded in higher amounts of DNA when the cultures had been sonicated before the harvest of VLPs was started (see Fig 15). Cultures GS1.7.C and GS1.7.D had been pooled and one half was sonicated and one not before the harvest of particles was started. The same was done with cultures GS1.15.A and GS1.15.D and with cultures GS1.16.B and GS1.16.C. From VLPs of the pooled cultures GS1.7.C/D and GS1.15.A/D DNA could only be extracted when the cultures had been sonicated. From VLPs of the pooled culture GS1.16.B/C DNA was extracted no matter whether it was sonicated or not.

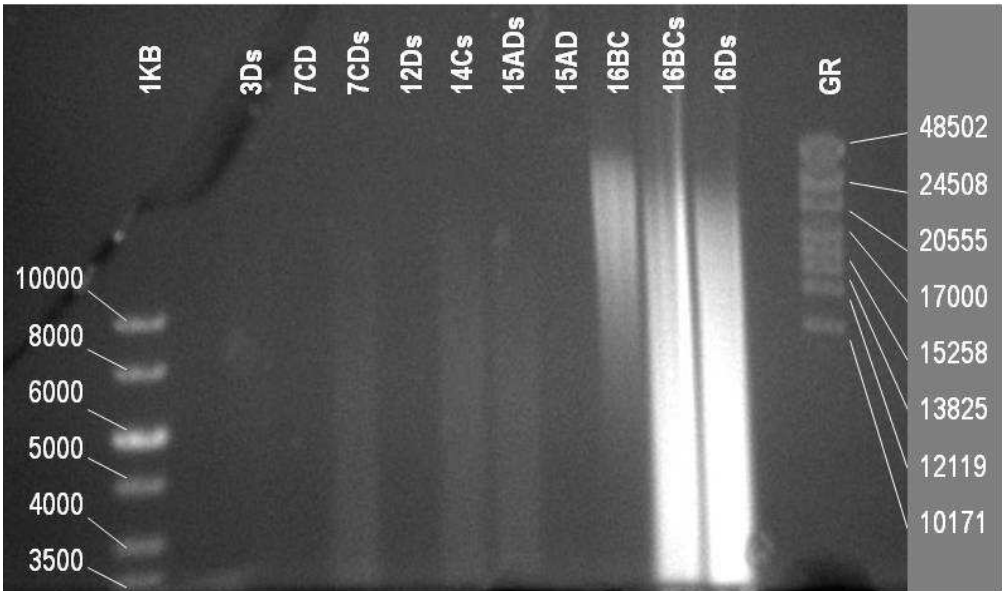

Fig 15 size fractionation of VLP DNA from different cultures using 0,5% agarose gel in 1x TAE buffer; VLP DNA was extracted following protocol 2; 1KB, GR: size markers in bp; s: cultures had been sonicated; 3Ds: VLP DNA from culture GS1.3.D (2854 h); 7CD and 7CDs: VLP DNA from the pooled culture GS1.7.C/D (2014 h); 12Ds: VLP DNA from culture GS1.12.D (983 h); 14Cs: VLP DNA from culture GS1.14.C (718 h), 15AD and 15ADs: VLP DNA from culture GS1.15.A/D (379 h); 16BC and 16BCs: VLP DNA from culture GS1.16B/C (376 h); 16Ds: VLP DNA from culture GS1.16.D (381 h);

All cultures of which VLPs were either processed following protocol 1 with subsequent protein precipitation or following protocol 2 are enlisted in table 2. Cultures are assorted according to their age. It is indicated in blue when the DNA extraction was successful. It is indicated by +/- whether the cultures had been sonicated or not.

Culture	Age	Sonication	Protocol
GS1.5.B	304	-	2
GS1.9.A	352	-	1
GS1.9.B	352	+	1
GS1.16.B/C	376	+	1
GS1.16.B/C	376	-	1
GS1.15.A/D	379	+	1
GS1.15.A/D	379	-	1
GS1.16.D	381	+	1
GS1.9.C	425	-	1
GS1.9.D	425	+	1
GS1.10.A	449	-	1
GS1.10.C	449	+	1
GS1.12.A	450	-	1
GS1.12.B	450	+	1
GS1.13.A	450	-	1
GS1.13.B	450	+	1
GS1.2.C	473	-	1

Culture	Age	Sonication	Protocol
GS1.2.C	473	-	2
GS1.10.B	473	-	1
GS1.10.D	473	+	1
GS1.8.C	474	-	2
GS1.8.D	474	+	2
GS1.13.C	474	-	1
GS1.13.D	474	+	1
GS1.4.C	639	-	2
GS1.5.C	663	-	2
GS1.5.D	663	-	2
GS1.14.C	718	+	1
GS1.7.C/D	2014	-	1
GS1.7.C/D	2014	+	1
GS1.6.C/D	2251	-	1
GS1.6.C/D	2251	+	1
GS1.3.D	2854	+	1

Table 2 DNA extraction from VLPs; 200 ml of each but one culture were processed either following protocol 1 with subsequent protein precipitation or following protocol 2; with culture GS1.2.C 100 ml were processed following protocol 1 and 100 ml following protocol 2; it is indicated in blue when VLP DNA extraction was successful; it is indicated by +/- whether the culture had been sonicated or not;

3.9 Shotgun library construction

As the DNA amount was limited, DNA extracted from VLPs of a 473 h culture (GS1.2.C) was amplified via the REPLI-g Mini Kit from QIAGEN (see Fig 16) and used for the construction of a shotgun library. The shotgun library was prepared for long term storage as described in chapter 2.2.16.

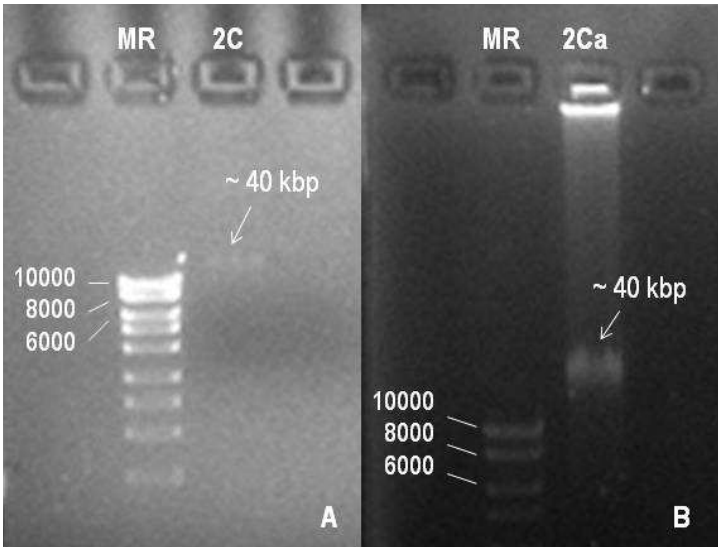

Fig 16 size fractionation of amplified VLP DNA using 1% agarose gel in 1x TAE buffer; MR: size marker in bp; **A:** VLP DNA extracted from culture GS1.2.C (473 h; lane 2C) following protocol 2; **B:** amplified VLP DNA from culture GS1.2.C (lane 2Ca);

The amplified VLP DNA was sheared randomly by sonication (see chapter 2.2.5.2) and prepared for blunt end cloning as described in chapter 2.2.13. Afterwards the DNA was purified and size fractionated via agarose gel electrophoresis (see Fig 17). Fragments between ~1000 and ~5000 bp were eluted from the gel and cloned using the CLONESMART® HCKan Chemically Competent Blunt Cloning Kit (SOLOs) from Lucigen® Corporation.

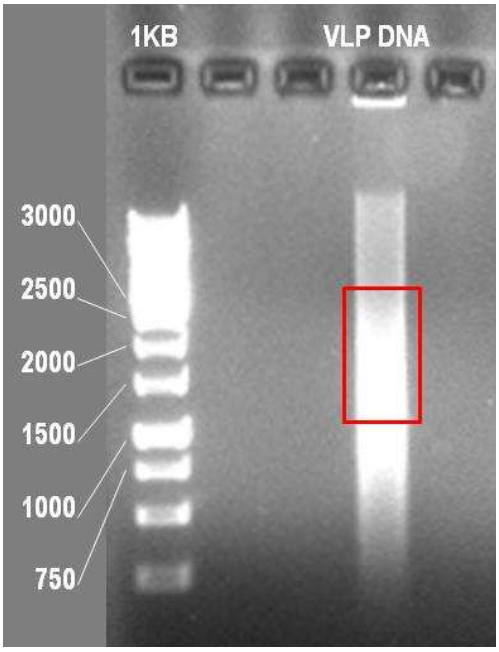

Fig 17 size fractionation of sheared, end repaired VLP DNA via agarose gel electrophoresis using 1% agarose gel in 1x TAE buffer; 1KB: size marker in bp; red rectangle marks DNA band that was eluted from the gel and used as insert DNA for cloning;

3.9.1 The shotgun library

The first attempt to construct a shotgun library yielded in an insufficient amount of clones (46 clones). Hence, the remaining end repaired and purified DNA (23,5 µl), which had been stored at -20 °C, was concentrated via heating (65 °C) until a volume of 6,5 µl was reached. Then the ligation into the pSMART-HCKan-vector was repeated as described in chapter 2.2.15.2.1. Transformation into chemically competent *E.cloni* cells was performed twice. Once as described in chapter 2.2.15.2.2 using 1 µl of the fresh ligation reaction and once using 3 µl. Each transformation reaction was plated on three LB kan plates using 320 µl transformed cells for one plate. The plates were incubated overnight at 37 °C in the dark.

The transformation using 3 µl ligation reaction yielded in significantly more clones than the one using 1 µl. Therefore the plates from the "3 µl transformation" were processed for amplification and long term storage (see chapter 2.2.16). Each of these three plates contained ~1295 colonies, consequently the construction of the shotgun

library had yielded in a total of ~3890 clones. Assuming that the average insert size was about 2000 bp all clones of the shotgun library contain a total of 7780000 bp.

3.9.2 Digestion of recombinant plasmids from clones of the shotgun library

Recombinant plasmids of 43 clones of the shotgun library were digested with EcoRI. Digestion resulted in separation of the vector DNA from the insert DNA. Results are shown in Fig 18. The used vector (pSMART-HCKan-vector) was 1788 bp in length. The average insert DNA fragment varied in size from approximately 1500 to 5000 bp. A few smaller inserts were also obtained.

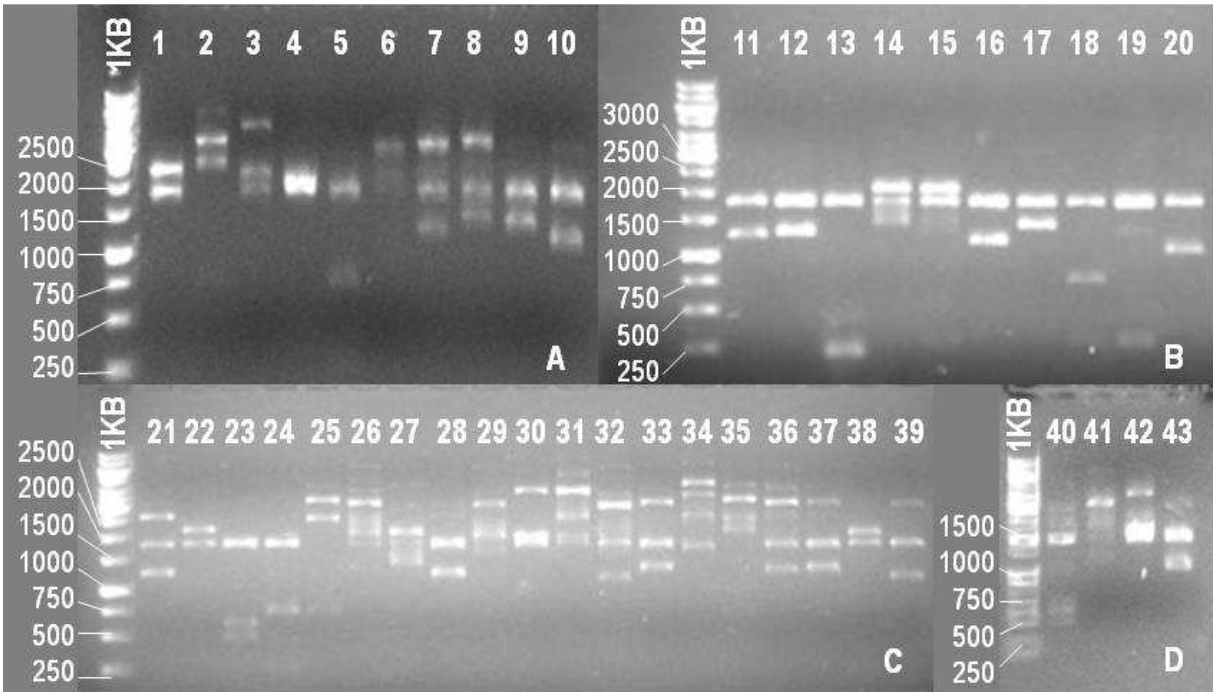

Fig 18 digestion of recombinant plasmids from 43 clones of the VLP DNA library; 1% agarose gel in 1x TAE buffer; 1KB: size marker in bp;

3.10 Sequence Analysis

Sequencing was done by 4base-lab Germany.

3.10.1 16S rDNA

Five clones (2AC3, 6A3, 6B1, 7A3, 7B1) were processed for sequencing and the obtained sequences analysed using the program blastn. All sequences were positively identified as belonging to the species *A. kielensis* as all of them made a top match with *A. kielensis* clone *SE79* (AY771772.1) with an e value of 0,0.

The e value (expect value) resembles the number of hits which can be expected to be obtained by chance when searching a database of a particular size. It describes the random background noise. The more significant the match, the lower the e value is.

Below the alignment of the consensus sequence of all 5 clones with the sequence of *A. kielensis* clone SE79 is shown:

```
Consensus sequence of clones 2AC3, 6A3, 6B1, 7A3 and 7B1
Sequence of clone SE79

1    AACGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAACGGTCTCTTCGGAGGCAGTGGCAGACGGG 68
1    AACGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAACGGTCTCTTCGGAGGCAGTGGCAGACGGG 68

69   TGAGTAACGCGTGGGAATATACCTATCAGTACGGAACAACAGTTGGAAACGACTGCTAATACCGTATA 136
69   TGAGTAACGCGTGGGAATATACCTATCAGTACGGAACAACAGTTGGAAACGACTGCTAATACCGTATA 136

137  CGCCCTTCGGGGGAAAGATTTATTGCTGATAGATTAGCCCGCGTTAGATTAGCTAGTTGGTGGGGTAA 204
137  CGCCCTTCGGGGGAAAGATTTATTGCTGATAGATTAGCCCGCGTTAGATTAGCTAGTTGGTGGGGTAA 204

205  AGGCCTACCAAGGCGACGATCTATAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACG 272
205  AGGCCTACCAAGGCGACGATCTATAGCTGGTTTGAGAGGATGATCAGCCACACTGGGACTGAGACACG 272

273  GCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATG 340
273  GCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATG 340

341  CCGCGTGTGTGATGAAGGCCTTAGGGTTGTAAAGCACTTTCAACGGTGAAGATAATGACGGTAACCGT 408
341  CCGCGTGTGTGATGAAGGCCTTAGGGTTGTAAAGCACTTTCAACGGTGAAGATAATGACGGTAACCGT 408

409  AGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGAA 476
409  AGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGAA 476

477  TTACTGGGCGTAAAGCGCACGTAGGCGGATTGATCAGTTAGAGGTGAAATCCCAGGGCTCAACCCTGG 544
477  TTACTGGGCGTAAAGCGCACGTAGGCGGATTGATCAGTTAGAGGTGAAATCCCAGGGCTCAACCCTGG 544

545  AACTGCCTTTAATACTGTCAGTCTAGAGATCGAGAGAGGTGAGTGGAATTCCGAGTGTAGAGGTGAAA 612
545  AACTGCCTTTAATACTGTCAGTCTAGAGATCGAGAGAGGTGAGTGGAATTCCGAGTGTAGAGGTGAAA 612

613  TTCGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGCTCGATACTGACGCTGAGGTGC 680
613  TTCGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGCTCGATACTGACGCTGAGGTGC 680

681  GAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGGAAGCTAGCCG 748
681  GAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGGAAGCTAGCCG 748

749  TCGGGCAGTATACTGTTCGGTGGCGCAGTTAACGCATTAAGCTTCCCGCCTGGGGAGTACGGTCGCAA 816
749  TCGGGCAGTATACTGTTCGGTGGCGCAGTTAACGCATTAAGCTTCCCGCCTGGGGAGTACGGTCGCAA 816

817  GATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAA 884
817  GATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAA 884
```

```
885  CGCGCAGAACCTTACCAGCCCTTGACATACCGATCGCGGTATCTGGAGACAGATACTTTCAGTTAGGC 952
885  CGCGCAGAACCTTACCAGCCCTTGACATACCGATCGCGGTATCTGGAGACAGATACCTTCAGTTAGGC 952

953  TGGATCGGATACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA 1020
953  TGGATCGGATACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA 1020

1021 ACGAGCGCAACCCTCGCCTTTAGTTGCCAGCATTAAGTTGGGCACTCTAGAGGGACTGCCGGTGATAA 1088
1021 ACGAGCGCAACCCTCGCCTTTAGTTGCCAGCATTAAGTTGGGCACTCTAGAGGGACTGCCGGTGATAA 1088

1089 GCCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTACGGGCTGGGCTACACACGTGCTACA 1156
1089 GCCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTACGGGCTGGGCTACACACGTGCTACA 1156

1157 ATGGTGGTGACAGTGGGCAGCGAGACGGCAACGTCGAGCTAATCTCCAAAAACCATCTCAGTTCGGAT 1224
1157 ATGGTGGTGACAGTGGGCAGCGAGACGGCAACGTCGAGCTAATCTCCAAAAACCATCTCAGTTCGGAT 1224

1225 TGGGGTCTGCAACTCGACCCCATGAAGTTGGAATCGCTAGTAATCGTGGATCAGCATGCCACGGTGAA 1292
1225 TGGGGTCTGCAACTCGACCCCATGAAGTTGGAATCGCTAGTAATCGTGGATCAGCATGCCACGGTGAA 1292

1293 TACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGTTCTACCCGAAGGTGCTGTG 1360
1293 TACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGTTCTACCCGAAGGTGCTGTG 1360

1361 CTAACCGCAAGGAGGCAGGCAACCACGGTAGGGTCAGCGACTGGGGTG 1408
1361 CTAACCGCAAGGAGGCAGGCAACCACGGTAGGGTCAGCGACTGGGGTG 1408
```

3.10.2 VLP DNA

Some of the clones of the shotgun library were processed for sequencing in order to become a first insight into the nature of the VLP DNA, especially to determine whether the origin is bacterial or viral. Sequences and blastx results of insert DNA from these clones are enlisted. The best result according to its e value is given below the sequence as well as the e value itself.

Clone 1

```
ATGAATCAGCTTGATTCGTAACTTCGAATTTAGTAAACCTTCAATTTTAGTTAACGTAAAGTAAATTTTTAA
AAAATGTTCAATATACGAGTAAAAAGGGCTGTTTTATTGAAGAGTGGAGTAAAATTTATCACTGCGCTTACT
TTCTTCTTGGGGGGCGAGCTTTCCCCAGTTCAAGCGCAAACACCGACATATTATGACCCCGAATGGCGCATC
ACTGCACCTTCATTAGCGGGGTTGCCACGTCTTCGGTTTTTAACAAGCCTTGATTTCCCACCGTTCAATTTT
GCTGACGCCAATAAAAAACCAACCGGCTTTAATGTGGATTTAGCTCGCGCGATTTGCACTGAACTCAAGATA
TCAGCGAAATGTGAAATTCAAGCATTGCCTTGGGCAGACCTTGAACCCGCGCTTGAAGCGCGACGCGGTGAG
GCGATTATTGCGGGCACAGCAATTACAGCCGAAAAACGTGCTACTTACAATTTGAGCCATAGCTATTTTAAG
TTTCCGGCACGCTTTATTGCCAATAAAAGCGCCGAAGAATTATCGGNNNNNNNNNNNNCGCAGGAAAAAAG
AATGCGCCAAAAATATCGATGAAAATAATTTACCATGGGAGAGATTGATTAGCCAGCGCCGTGATTTAAGCG
CGCCCGTCATTTCTGCGCGCTCCAGCTTTGGCGACCATAATTCATAAATCGCCATTGCGGTGAAGATGCTCA
AAAAAGCTGCAAGTCTAATGGTCGCTTCTGACACGGCTTTATTCCTTATTTTTCAAACCCTTTTCCGATATT
TCACCTACTGATTGCAAATCACAAGGTGTTGAATATATGGCGCCGTGTTTGTGCTAGGCATGTGTTTGGCGG
CAATCTCAATGCTTTTGAGGCGTTCGCACGCGCCATTGTTACGCAGTTCTGATATTGGCCCTTCTCATTGCG
GCAACGATGGGGTAGGAAATCGCGCAGTTTCAAACCTAGGAATTTTAACATGAGTGCCAATCCGGCTTTGCC
TATTCTTGATATAACACCAGCAGCTATTGAAGCCGCCAAGGTGAGCAAGGCTTGGCCGTTTGAAGAGGCGCG
GAAGATAACAAAGCGTTTTGAGAAAACAGGTTTTCCAGAAACTGTTCTTTTCGAAACAGGCTATGGCCCATC
CGGCTTGCCGCACATTGGAACATTCGGAGAAGTCGCGCGCACCACAATGGTACGCACAGCGTTCCGTCTTTT
GACTGAAGATAAAGTCGCGACAAAACTCATCTGCTTTTCTGATGATATACGAATCTCTGA
```

NNNNNNN: not determined region of about 1250 bp (according to the results of the restriction digestion)

Left region: extracellular solute-binding protein family 3; 1e-25;

Right region: Lysil t-RNA Synthetase; 7e-33;

Clone 3

AATGATTCAGCTTGATTCGTTCGCCAATTGCTCTACCTGCTAGTTGTTCCGATGCAGCAGCAAGGCCGTAAA
AAAATATGAGGATACGAAAAAGAAATTTAGCAATATTGCATTCGCTGCCAGCGTTACCGTTCCCATATTGGC
ACCAAGCCGCGCAAACAGTGAGAATGCGATCAAAAGAGCGAAAGAACGGATCATGATGTCGCGATTGACATT
GAGCATTTTGAGCAAAGACGATCGGTCCAGAACTTCCTTCCATGTTGGTTTTTGGCTTTGATCAAACCCACG
CCATAGCCAGATAAAAGAGCCGAGCGCCACCGTCGCTTCTGCGATCACTGTGCCCCAAGCAACGCCCACAAT
GTTCCATTCAAGCACAAGGCCAAGCACGACTGATAAAGCGATGTTTAAGCCATTAACGACAGACTGGACACC
TAAGCTTGCCATTGCTCTGTTTTGGCCCAGCAGCGTTCCAAGAATTGCATAATTTATCAGACTAAAGGGCGC
CCCTAAAAAACGAATTGCCATATAGGTCGTTGCAGCGTCACGCACACCGTTTTCAGGACTAATCCAATATAG
TCCCAGTTCGCGAATGAAAGGCGAAAGCGGCAGAACCAGCACACCAATCATTTGCAGCGGTAATAATAGCGC
GCCCAGAACCAAGCTTTGTTGCTCACGCTCATCGCCCCCGCCCCAAGGGCCTGTGCGACAAACCCNNNNNNN
NNNNNNATGATCCGTTCTTTCGCTCTTTGATCGGCAATTCTCACTGTTTGCGCGGCTTGGTGCCAATATGGG
AACGGTAAACGCTGGCAGCGAATGCAATATTGCTAA**AATTTCTTTTTCGTATCCTCATATTTTTTAGACGGC**
CTTGCTGCTGCATCGGAACAACTAGCAGGTAGAGCAATTGGCGTGGGCTCGCGTTCTGCCCTGTGGCAAGCG
ATCAAAAAATCATGCGGCTTTGGTTTTGCATTATCCTTTTTCGCAGCGGTGCTTTTGTTGGTTTTTGGAGAT
CAATTTATTGATATGATGACGACAGCTGAAGATGTGCGGATCATTGCGAAAACATATTTACCTTGGGCGGCG
GCGTATTGCATGATCTGGGTGTCTATCCGATTTCACTGGCAAGGTATTTGCTTGGTGAGCTGGAATTGGTTT
CAAGTGAGTGGAAAGCTGCCCCCAATGGCGTCAACAAGTCAGCGACACTCCATTTAAAATCCGGCGCTATGC
CCATTACGATCAATGTGGGCTTTGGGCCAGAACAGGGTAACACATTTGACATATATGGCGAAAAAGGAGCGC
TGCGGATTGATCGGCATTTCTTGCGCACGGATTCAGCAGCCATCTGGCATGGCGCTCAAAGCCATTTACCCC
CAGCAAGCGGCGGCTTCATAAATCGTCTTCGAAATAAATTTTCTTTGCAAGGCGGAACACGCAAAACATTTG
CGCGGCAAAGTCACGGGCTTAATTTTCAAGCTGCAGCATTTCAGGCTGCGCTCGGGCAAAAATTACCCGAAC
ATGCAGTGATGCCACTTGATGAAAGCGCAGAGGTTTTACACATCATCGAGACGAATCTCCAGATT

NNNNNNN: not determined region of about 1000 bp (according to the results of the restriction digestion);

Left region, right region: Part 1 (Frame -3, 1e-46) and part 2 (Frame+1, 4e-5):

Sodium:dicarboxylate symporter:Multi antimicrobial extrusion protein MatE;

When the right region was blasted separately additionally part 3 was found:

oxidoreductase domain-containing protein; 2e-16;

Clone 5

GTTCAGCTTGATTCGTGCCGTGATCAACAGGCTTCGCGCTGTCAAAAAATTGCTAATTCGTTTGAAGCCAAG
CGTAGGCCAGTGGCTGAGGCGCTGGCTTTGTTTTACCCCAAACAATTATCCGCAAACTTCTGCGCTTTACAG
CGGCGGCGGCTTTCGCAAATTCGCCGCTTTTAACGGCCGCGCACCGCCATGTCTTGGTGCTGCGCATTATAC
ATTTGACCGATATAAATCGCACGGCCCGTCGCGATAATTTTATCGTAGGGCCAATAAAAAGATGGGCACTAT
TCCGGTATGATTAAATCATACTTTCTGATAAAATAAGTTCTGAGAGGATGAATCGATGGATAAGAGAACAAT
CAGCCTACCCGATGAGCATGCGGCCTATATTGACCAGAAAGTGAGTTCTGGCGATTACGCTTCTGCGAGCGA
GGTTGTGCGTGCAGGACTTCGCGCATTGCAAGAACGTGACCGCGCCGTTGAAAACTGGCTACACCAACAGGT
TGCCCCTGCCTATGACGCGATGATGCAAGACCCATCGCGTGGGCTTTCTGCCAAATCTGTGTTTGATGAAAT
CCGCGCCCATCACAATGAAAAAACCAAAGGCCCAGCGTGAAACAACGCACCGTAGAATTTGCGCCAGAAGCA
AGCGGCGATCTATTCGCGCTCTATGATTGGATTAGCGTGAAAGCCAGCCCTGCTGTTGCAATGGGATATATC
GAGCGCATTGAGACCTATTGCAATGAAATCGACTTTGCATCGGAGCGCGGCCAAATGCGCAATGATATCCGT

TCAGTCTGCGCATTGTCGGGTTTGACAGAGAATTCCCCTCACTCAGTTGCGGCTAACGCGCAAATGCGCGAA
GCCTCACATTTCTCCTCCGCAGCGGCAGAGAAA

Part 341-580 (of 897): putative addiction module antidote protein, CopG/Arc/MetJ family; 1e-20;

Clone 6

TGTGAATCAGCTTGATTCGTGCCGTGATCAACAGGCTTCGCGCTGTCAAAAAATTGCTAATTCGTTTGAAGC
GGGGGTAGGCCAGTGGCTGAGGCGCTGGCTTTGTTTTACCATTAAAAATATCCGCAAACTTCTGCGCTTTAC
AGCGGCGGCGGCTTTTCAAATTCCTCTTTTAACTTTATTTCTCTCTTTNNNNNNNNNNNNNGGGTGGGCTTT
CTGCCAAATCTGTGTTTGATGAAATCCGCGCCCATCACAATGAAAAAACCAAAGGCCCAGCGTGAAACAACG
CACCGTAGAATTTGCGCCAGAAGCAAGCGGCGATCTATTCGCGCTCTATGATTGGATTAGCGTGAAAGCCAG
CCCTGCTGTTGCAATGGGATATATCGAGCGCATTGAGACCTATTGCAATGAATTCGACTTTGCATCGGAGCG
CGGCCAAATGCGCAATGATATCCGTTCAGGTCTGCGCATTGTCGGGTTTGACAGAGAATTCCCCTCACTCAG
TTGCGGCTAACGCGCAAATGCGCGAAGCCTCACATTCCTCTCCCGCAAGCGGCAGAGGAAAATCAGCGCTCC
CTATCTCTCCCCTTGTGGGAGAGAAAGCGAAAACTTGGGCTTGGCTTGCCAAGTCCTTAGTTTTGGCAAGAG
AGGGGATATAACGCCGCTCTCCGTCATCCTATGGCTTGACCATAGGATCCATTCCGTGAAGTTCACTTGGGT
GTTTTTTCTTGAAGAATGATACAGGACGAATCTCTGAT

NNNNNNN: not determined region of about 1800 bp (according to the results of the restriction digestion);

Right region: plasmid stabilization system; 7e-11;

Clone 7

GATCTAGAGATTCGTCGATGATGCCTTGTTGCAAAAGGCACGAATTTTTGTGGACAGCTTTGAAACGACAGT
CGAGCATATTGGTGAATTGATAATACCGCTTGCGTCGGGCGCTATTGAGCGCAGTGCCGTGCTCGGTGATTT
CTATGATCTGGTAACAAGCAGCGTCGGGCGGCAATCTAAAGATGAGATCACCGTTTTCAAAAATGGCGGCGG
CGCGCATCTCGATCTGATGGTCGCAGACCGAATCTGGAAAGCGATGAATTAAGCTGGTGCTGACTACATTTG
CGCAGCGAAAATGCGACAGTGTCGGAATTGAAATTATCGCAAAAGCTAGGTGTTGTCATCGACAGTCATGCT
CTGTCGGTATTCCGCGCATAACTTGGCGGTAGTCTAATCAATAGATAAGGGCAGGCTTTTTATTCAAAACAA
GCCCGCCCTTAATTTTTAAATGTCGTTAAATTACGCTTTTGCACCGATGATCATGACTTCAACCATATAATC
CGATGTTACCAGGCGCGATTCGATACACGCGCGTGCTGGCGGGTTTTTCGNNNNNNNNNNNNGATCCTCCC
CCTTTTTGTACCCCCCCCGGAATCGTCCACACTCCCGCCCAGCCCCCCCTTCCCGCGCGTCTACCAATTCCA
CCAGAGGGGCACATAGATCAACGAATCAAGCTGAACACA

NNNNNNN: not determined region of about 4300 bp (according to the results of the restriction digestion);

Left region: ornithine cyclodeaminase/mu-crystallin; 3e-21;

Clone 8

CGTCTAGAGATTCGTCCGCGCATACCACCGAGTTGAGATGTGCTATGAGGCAGGGCCAACAGGTTATGGACT
GTATCGGCAGATTACGGCGTTTGGGTTTTCCTGTTGCGTGGTTGCCCCGTCCCTGATCCCGATGCGTTCAGG
TGAACGGATAAAAACTGACAGGCGTGATGCGATGCGTCTGGCGCGCCTTCTAAGGGCGGGGGAATTAACACC
AGTTTGGGTACCCGATGAGACCCACGAGGCCATGCGAGATTTGGTGCGGGCACGCGAAAGTGCCGCTGAAGA
TCAGCGCCACAAACGCCAGTTGATTTCGGCCTTTCTCCTGCGCCATGGTCGAATTTATCACCGACCCAAGAC
ATGGACAATGCGTTATCGCCGGTGGCTACAACAACAGTCCTTTGATCACCCTGCGCAACAGATCGCTTTACA
GGAAATGATTATGGCCGAGCGTCACGCTGTCGAACGTGTTGCCCGACTAACCGACGGTATCGAACAGTTAAT
TCCAAATTGGCAATTGGGACCGGTTGTTGATGCATTACAAGCACTGCGCGGTGTTGCGCTGATCAGTGCTGT
TACATTTATGGCTGAGATTGGCGATGTTCGCCGATTTGAAAATCCGCGCAAGTTGATGGCCTATTTGGGCTT

GGTACCCAGTGAGTATTCCACGGGTCAGACGACCAAACGTGGCGGGATCACCAAAGCTGGCAATTCTAGGGT
CCGGCGCACGCTGGTGGAGGGAGCTTGGACATATCGGTTTCCAGCGCGAGTCGGCGAAAGGAAGCTCTACGT
CCTGCAAAAACTACCGCCTGAGATACAGGACATCGCTTGGAAGGCACAATCAAGGCTGACAGCGCGATATCG
GCGGCTTAGCCAGCGCGGTAAGAAGAAGACAGTGATAACAACGGCAATCGCCCGAGAAATGTCAGCATTCAT
GTGGGATATTGCCCGAAGGACAATGCCAGTGCCTTAGCCGGTCCAACCTTTCACGCCTATTGGCGGGAACGA
GATCACGGCAGGGGAATATCCGACATCGCTTTGTGGCCAACATAAGTTGACGCCCGATGTAAGACAGGAATA
GCCCCGAGACGCACATGCGGTCATGCGGTATCCAATCCGCGTATCAGAGTTTGTTAACCGACGTCTTCAGAT
CTCGTTCCCACCAATGTGCGCCACAGTAACGTTGAAACCCGCCAGCGGGTGCTTTGACGCGCATAAATCATT
GACAAAAGACATCAGAGCGATGCGGCGATCACACGGTCTCGGCATAATTCCACGGCAATAACGCGTCTACAT
TAGATTGCTTATGACCGTTCACAATGGCTGTGAGCGTGGCAGTTAGGTAAGCTTGAGGATCGATTGCATTGA
GACGAATCAAGCTGATTCACA

Part 32-964 (of 1389): transposase IS116/IS110/IS902; 3e-136;

Clone 9

TTCTGAGATTCGTCCCCCCCATGAAGAGCGCGCCAATCGACACGCCGGACACGACACCATACAGCACCAAAG
GAATGGATGGCGGAATGATCGGCCCAATCACACCTGATGCGGCTACGACTGCGGCAGAAAATGATGCGTCGT
ATCCACGGCGCTTCATCATAGGCCCCCCCATGAAGAGCGCGCCAATCGACACGCCGGACACGACACCATACA
GCACCAAAGGAATGGATGGCGGAATGATCGGCCCAATCACACCTGATGCGGCTACGACTGCGGCAGAAAATG
ATGCGTCGTATCCACGGCGCTTCATCATAGGCCCCCCCCATGAAGAGCGCGCCAATCGACACGCCGGACACG
ACACCATACAGCACCAAAGGAATGGATGGCGGAATGATCGGCCCAATCACACCTGATGCGGCTACGACTGCG
GCAGAAAATGATGCGTCGTATCCACGGCGCTTCATCATAGGCCCCCCCATGAAGAGCGCGCCAATCGACACG
CCGGACACGACACCATACAGCACCAAAGGAATGGATGGCGGAATGATCGGCCCAATCACACCTGATGCGGCT
ACGACTGCGGCAGAAAATGATGCGTCGTATCCACGGCGCTTCATCATAGGCCCCCCCATGAAGAGCGCGCCA
ATCGACACGCCGGACACGACACCATACAGCACCAAAGGAATGGATGGCGGAATGATCGGCCCAATCACACCT
GATGCGGCTACGACTGCGGCAGAAAATGATGCGTCGTATCCACGGCGCTTCATCATAGGCCCCCCCATGAAG
AGCGCGCCAATCGACACGCCGGACACGACACCATACAGCACCAAAGGAATGGATGGCGGAATGATCGGCCCA
ATCACACCTGATGCGGCTACGACTGCGGCAGAAAATGATGCGTCGTATCCACGGCGCTTCATCATAGGCCCC
CCCATGAAGAGCGCGCCAATCGACACGCCGGACACGACACCATACAGCACCAAAGGAATGGATGGCGGAATG
ATCGGCCCAATCACACCTGATGCGGCTACGACTGCGGCAGAAAATGATGCGTCGTATCCACGGCGCTTCATC
ATAGGCCCCCCCCATGAAGAGCGCGCCAATCGACACGCCGGACACGACACCATACAGCACCAAAGGAATGGA
TGGCGGAATGATCGGCCCAATCACACCTGATGCGGCTACGACTGCGGCAGAAAATGATGCGTCGTATCCACG
GCGCTTCATCATAGGCCCCCCCATGAAGAGCGCGCCAATCGACACGCCGGACACGACACCATACAGCACCAA
AGGAATGGATGGCGGAATGATCGGCCCAATCACACCTGATGCGGCTACGACTGCGGCAGAAAATGATGCGTC
GTATCCACGGCGCTTCATCATAGGCCCCCCCATGAAGAGCGCGCCAATCGACACGCCGGACACGACACCATA
CAGCACCAAAGGAATGGATGGAACGAATCAAGCTGAATTC

Part 12-561, part 352-1080 and part 1118-1447 (of 1480): GK25652 [Drosophila willistoni]; 3e-22;

Clone 10

TCCGCTGACATTAAAGCTGATCAGTCTTTTGACAAAAATGAAAGGCTTGCGCGGAACGCCCTTCGACCCATT
TGGCCGGAGTGAAGAGCGTAAAGCGGAACGTGCTTTAATTACGCAATATGAGCAGGATATTGAACTGGTTTT
GAATAAGCTTGATTCTGCGCCTGCACACATAGCGCTGGCTCTTTTATCATCGGTTGATCAGGTGCGGGGCTT
TGGTCCTGTCAAAGATGAAGCGCGGGCAGCTCATGATAAGAGACGGCAAGACCTCTTGAACCAGATCAATAA
GCCTGAGATCAGCAAGATTGCGGCTGAATGATCTAGCCTCCACATTTTGCGTTCTGTGACCGCTTGTTTTTT
TGAGTGCATCAACATCGATTAATTCGGTGTTGATGCCTTTGTTTGTGGCTGAAACGAATCAAGCTGATTCAT
CAGG

Part 2-292 (of 436): indolepyruvate ferredoxin oxidoreductase; 1e-18;

Clone 11

ATCAAGAGAATTCGTTGATGCAGCCATTCGCACCGTGCTTAGTCAGGCATTGTCACGTGCTGGGTATGATGT
TCGCTTAACGTCGAATGCCTCAACTTTATGGCGTTGGGTGGCTGAAGGTGAAGGCGACTGCGTGATCAGTGA
TGTGGTTATGCCGGATGAAAATCTATTTGATCTTCTGCCGCGCATGAAAAAAATGCGCCCAGATCTGCCGGT
TATTGTCATGAGTGCGCAAAACACATTTATGACGGCGATCAGAGCTTCTGAAAACGGCGCCTATGAATATCT
TCCAAAGCCGTTTGACCTTGTAGAGCTCACTGCGATTATTGAGCGTGCGCTACAGGAAGCGCGGCGCAAACC
GACTGATAGCAAGAGTGCTGTGGCAGAAAAAGATGAAGCCATGCCGCTTGTTGGCCGCTCGCCAGCGATGCA
GGAGATATACCGTGTTTTGGCGCGCATGATGCAGTCTGATCTCACGGTTTTGATCAATGGCGAATCAGGAAC
CGGCAAAGAGTTGGTTGCGCGTGCGCTGCATGAATATGGCAAGCGCAAAAAGGGCCCGTTTGTTGCGATCAA
TATGGCAGCTATCCCGCGCGATTTGATTGAGTCTGAGCTTTTCGGTCATGAAAAAGGGGCATTTACTGGCGC
CCATAGCCGTGCCACAGGCCGGTTTGAACAAGCGGAAAATGGCACTTTGTTTCTTGATGAAATCGGTGACAT
GCCTATGGATGCGCAAACGCGTTTGTTGCGTGTGCTTCAGCAGGGCGAATATACGACGGTTGGCGGACGCAC
ACCGATCAAAACAAATGTGCGTATTGTGACCGCGACGAATAAAGAGCTGCGTAGCCTTATCAATCAAGGGCT
GTTTCGCGAAGACTTATATTATCGCTTGAATGTTGTTCCGCTGCGCATACCGCCTTTGCGTGAACGTGTTCA
GGATATTCCTGATCTGGTCAGCCATTTCTTGAAGAAAGTTCAAGAAGAAGGGCTTGAAGAAAAGATATTATT
GCCGGATGCGATTGAGGCACTTTTGCATCACTCTTGGCCGGGCAATGTGCGTGAACTTGAAAATCTGGTTCG
CAGATTGGCAGCGTTGTATCCGCAAGATGAAATAACAGCCGATATCATTTCATCTGAACTTCAAGACCCTGA
TAATCATAATTTGCCAGACATCGGTCACGTTGAAAACGGTAAAAATATCACCATCGCGCAAGCGGTTGAATT
AAATATGCAAAAATATTTCAATCATCAACGAATCAAGCTGAATCA

Part 17-1243 (of 1269): nitrogen regulation protein NtrC; 2e-170;

Clone 12

ATTCAGCTTGATTCGTTAAACGGTGCCTGTCTTAGCACCTTGATACTCATTTAAATCAAAACAGTAGGCCGA
TATGAAAACATTAACGCATCTTCAGCGACTGGAAGCTGAAAGCATACACATTATGCGCGAGGTCGTGGCTGA
GGTTGAAAATCCAGTCATGATGTATTCCATCGGCAAGGATTCGGCGGTGATGTTACATTTGGCGCTAAAAGC
CTTTTATCCATCACGCCCGCCATTTCCGCTACTGCATGTTGATACGACATGGAAGTTTCAGGAAATGTATAA
GTTCCGCGATAAAGTCGCTCAAAAGACAGGCATGGAGCTTTTAACGCACACGAACCCAGATGGGTTAGAGCA
GGGCGTTGGCCCGTTCTCGCATGGCTCGGCGGTCCATACCGACATTATGAAAACGCAGGCGCTCAAGCAGGC
TTTGGATCATTATAAGTTTGATGCGGCTTTTGGCGGTGCGCGGCGCGACGAAGAAAAGTCCCGTGCAAAAGA
ACGCATCTTTTCGTTCCGCACAGCAGACCACCGATGGTTCTTGGTCTGCGATTGAGTTTAGAAACTAGACTG
ATTTAGCTGCCTGCTTTTAATGGTTTTTGACAAGAGTAGAACGTAAAATAGCCCAAAGCCTTATCCTCAATT
ATGAGAACAGGCTTTGGGTTGAATGTCGTTTAGCCATCGAAAGGTGGGAGTTGTACTTTTACCATGTTGAAT
TGCGGTTATCTTTTTGCCGGCCAGTATTCGCAGTACAGCAAAGGAAAACGACAGAATCGCCAACATAAGAGC
GATCAGCTGAAAGATTCGACTTGAAGGCGTTCAATGTCCGTAATGCCATATGTGCGGTGAATATTGGCAGTG
ATAATTCAAGACCCTCATTGACGCTTTGCAGGTTTGAAATTGCACCAAATTTAGAAAAATTTCCTCCACTGT
GGCATCTTGAAAGCTGTTAAGTTCCTTGCAAGCAGCGGTTCAAGATTAAATTGTATGGGGTTGTATGTGAAA
ATAGTGAAAAATGTACTTGATATCGCTTGCTGGTTTGGATAGTCACAACTCCAGTTGGGGTGTAGCCAAGCG
GTAAGGCAACGCTTTTTGGTAGCGTGTACCGAAGGTTCGAATCCTTCCACCCCAGCCAAATTTATTTCTTCC
CCATTGCTCCTTAGTAGAAAAAACATATGGTCTCGCAAGAGACTTTAGAAAATGTGTTTTCATTTTTCTGGC
GCTGTAGACCTAGTAAAATAATTGCGATATCCGGCGGCTCTTCATTGACTTGAAGTGTCGATGGACGAATCT
CAGA

Part 83-541 (of 1300): sulphate adenylyltransferase subunit 2; 4e-68;

Clone 13

ATTCAGCTTGATTCGTCTTGTGGCCAAACAGACAGATTCAACCCACCTGCCCTTCTCCCGCTTGCGGGAGAA
GGTGGATACGAGTGGTAACGAGTAGACGGATGAGGGGGACAGAGAATTCCCCTCACCCAGTTGCGGCTAACG
CGCAAATGCGCGAAGCCTCACATCCCTCTCCCGCAGGCGGGAGAGGAAAAAGGATCGCCCGTTCGCGCAGGG
TTTATATTTGCCTTGTGGCCAAACAGACAGATTCAACCCACCTGCCCTTCTCCCGCTTGCGGGAGAAGGTGG
ATACGAGTGGTAACGAGTAGACGGATGAGGGGGACAGAGAATTCCCCTCACCCAGTTGCGGCTAACGCGCAA
ATGCGCGAAGCCTCACATCCCTCTCCCGCAGGCGGGAGAGGAAAAAGGATCGCCCGTTCGCGCAGGGTTTAT
ATTTGCCTTGTGGCCAAACAGACAGATTCAACCCACCTGCCCTTCTCCCGCTTGCGGGAGAAGGTGGATACG
AGTGGTAACGAGTAGACGGATGAGGGGGACAGAGAATTCCCCTCACCCAGTTGCGGCTAACGCGCAAATGCG
CGAAAGCCTCACNNNNNNNNNNNNNAAGTGGCCAAACAGACAGATTCAACCCACCTGCCCTTCTCCCGCTTG

CGGGAGAAGGTGGATACGAGTGGTAACGAGTAGACGGATGAGGGGGACAGAGAATTCCCCTCACCCAGTTGC
GGCTAACGCGCAAATGCGCGAAGCCTCACATCCCTCTCCCGCAGGCGGGAGAGGAAAAAGGATCGCCCGTTC
GCGCAGGGTTTATATTTGCCTTGTGGCCAAACAGACAGATTCAACCCACCTGCCCTTCTCCCGCTTGCGGGA
GAAGGTGGATACGAGTGGTAACGAGTAGACGGATGAGGGGGACAGAGAATTCCCCTCACCCAGTTGCGGCTA
ACGCGCAAATGCGCGAAGCCTCACTTCCCTCTCCCGCAGGCGGGAGAGGAAAAAGGATCGCCCGTTCGCGCA
GGGTTTATATTGGACGAATCTCAGAT

NNNNNNN: the length of this not determined region cannot be estimated according to the results of the restriction digestion with EcoRI because it contains repetitive section which contain a EcoRI site; digestion with another enzyme is needed;

Left region, right region: hypothetical protein LOC749915 [Pan troglodytes]; 9e-05;

Clone 16

ATTCTGGAGATTCGTCGACAGAGCCTCTGCTGCACCCAAACTTTTATTTTTTGCATCTGCTTCATTTTAAGC
CGAGTAATTTCTGCTTTTAGATCGCCGATGAGTTCTTTCAAATCGCCGACGGAAAGCTCATCAATCTTTTGG
CCCAGCTGATAAGGCTTTTTTCTCTTAGGTTCGTTTTCGTCGTCAAACATGATGTTATCCTCACTTTTAAAA
ACTGTCGTAAGATTAAACTCGCCACAATGCGGTGCCAAGTGAATAAGCATGTGACTAAAAGGAAGATGCGAT
GACCAATACAAAAAAGATGACGGCAGTAGAAATCACTGAGCCGGGTGGTCCAAAAGCGCTGAGTGTTTGCAA
ACGCGATAAACCGCAGCCTGCATCTCATGAAATATTGATTGAGGTGAGGGCCGCAGGCGTGAACCGGCCTGA
CGTGTTGCAACGTATGGGGGCTTATCCGCCGCCAAAGGGCGCATCAGACCTTCCGGGCCTAGAGGTCGCAGG
TATTGTTGAAGCGGTTGGCGAGGATGTTACGCGCTTTCGTGTGGGTGATTCGGTTTGCGCGCTCACTGCCGG
TGGCGGCTATGCTCAATATGTCAGCGTGGATGAGGGTAGCGTTTTGCCTGTGCCTGCGGGTTTAACAATGAC
GCAGGCCGCTGCTCTGCCTGAAACCTTCTTTACCGTTTGGCACAATGTTTTCGAGCGTGGCGGTTTGCGCAA
AGGCGAGGTCTTTCTTGTGCATGGTGGAACCTCTGGCATCGGCACGACCGCTATCCAACTGGCCAAAGCGAT
TGGCGCGATCGTCATCACGACTGTTGGCTCGGCTGAAAAAGCAGAAGCATGCAAAAAACTTGGTGCTGATCA
TGTCATTTCATACCGCGACGAAGATTTTGTGTCTGTCGTCAAACAGGTAACGGACGGGATTTGCGTTTTAAT
GTCTGTATTTTAACAGGAATAAACAGCGCAAAAAAAGAGAGCCGCAAAAGCGGCTCTTGAAGATATACAGGG
GAGGCGTCAAACAAAGCCAAAGCCTTGATTGAGTTCGAATAAATCCGAACAAAATGGTTAATATCTTATAAG
GGTTAATATGGCGTTAGCGCAGGCAATCATTTTTTTTTAGGCCCGTTCAGCTTTTGAACAGAGCCTCTGCTG
CACCCAAACTTTTATACGAATCAAGCTGAATCAT

Part 303-992 (of 1186): NAD(P)H quinone oxidoreductase, PIG3; 3e-79;

Clone 17

CGAGAGATTCGTCGCTCGGCTATCTTCGATGCGCTTTCAAAACATTTTGGCAATCATATGCCAGAGATAACA
TCGAGCCTGGCCGCGGTATGGTTGGCAATGCAGGCGTTATCAAATCAGAAGTTGTGAACCGATCCACTCCAC
AAGGATCACAATCTCGCTGCTTGTTCATCGTAAGGTGCATTCTTGCCTTCTTTTCAAGCGACATCGTTCTGA
CGCCGGAAAATCCGGTATTTGATTTTGAGGATTGGCCTAATGGCAACTGCACGTATTATCGACTTCTTCAAC
ACACGTAACCCTGCAACACCATGCTTGGTTGTTGACCTTGATGTTGTCGCCGACAA**CTTCACAAAATTCCGC**
CGCGCGCTTCCGCTATCTAAAATCTTCTATGGGGTTAAAGCAAACCCTGCGCCAGAAATCCTGCGCCTCCTT
GCTGATATGGGCTCTTCATTCGATTGCGCTTCTGTTGTTGAAATCGAAATGGCACTTGAAGCTGGTGCGACG
CCTGACCGTATTTCATACGGCAACACAATTAAAAAAGAGCGCGATATTGCTGCAGCTTTTGCGCATGGCGTT
TCCATGTTTGCCGTTGACTGCGAAGTAGAAGTTGAAAAAATTGCCCGCGCTGCACCTGGCTCACGTGTATTC
TGCCGCATTCTGACAGATGGCGAAGGCGCTGAATGGCCGCTTTCTCGCAAATTTGGCTGCGTGCCTGAAATG
GCAACAGATGTGCTTAAACACGCCGTTGCGCTTGGCCTCGTTGCACATGGCATTTCATTCCATGTCGGCTCT
CAATGACGACAGTTGACGCTTGGGACTTACCTCTTTCGCATGCACGCACGATTTTCGATGCGATGGCTAAAG
CTGGCGTACAGTTGAAAATGGTCAATATGGGCGGTGGTTTTCCAACGCGCTATTTGAAAGACATACCAACAG
CAGAAGCCTATGGCTCGGCTATCTTCGATGCGCTTTCAAAACATTTTGGCAATCATATGCCAGAGACAATCA
TCGAGCCTGGCCGCGGTATGGTTGGCAATGCAGGCGTTATCAAATCAGAAGTTGTGAACCGATCCACTCCAC
AAGGATCACAATCTCGCTGCTTGTTCATCGTAAGGTGCATTCTTGCCTTCTTTTCAAGCGACATCGTTCTGA
CGCCGGAAAATCCGGTATTTGATTTTGAGGATTGGCCTAATGGCAACTGCACGTATTATCGACTTCTTCAAC
ACACGTAACCCTGCAACACCATGCTTGGTTGTTGACCTTGATGTTGTCGCCGACAACTTCACAAAATTCCGC

CGCGCGCTTCCGCTATCTAAAATCTTCTATGCGGTTAAAACAAACCCTGCGCCAGAAATCCTGCGCCTCCTT
GCTGATATGGGCTCTTCATACGAATCAAGCTGATTCATTG

Part 17-217 (5e-19), **part 345-614** (7e-111), part 614-1153 (7e-111) and part 1281-1370 (0,58) of 1408: putative ornithine, DAP, or arginine decarboxylase protein;

Clone 18

CGCTGATGATTCAGCTTGATTCGTGCCGTGATCAACAGGCTTCGCGCTGTCAAAAAATTGCTAATTCGTTTG
AAGCCAAGCGTAGGCCAGTGGCTGAGGCGCTGGCTTTGTTTTACCCCAAACAATTATCCGCAAACTTCTGCG
CTTTACAGCGGCGGCGGCTTTCGCAAATTCGCCGCTTTTAACGGCCGCGCACCGCCATGTCTTGGTGCTGCG
CATTATACATTTGACCGATATAAATCGCACGGCCCGTCGCGATAATTTTATCGTAGGGCCAATAAAAAGATG
GGCACTATTCCGGTATGATTAAATCATACTTTCTGATAAAATAAGTTCTGAGAGGATGAATCGATGGATAAN
NNNNNNNNNNNNGGAAAATCAGCGCTCCCTATCTCTCCCCTTGTGGGAGAGAAAGCGAAAACTTGGGCTTGG
CTAGCCAAGTCCTTAGTTTTGGCAAGGGAGGGGATATAACGCCGCTCTCCGTCATCCTATGGCTTGACCATA
GGATCCATTCCGTGAAGTTCACTTGGGTGTTTTTTCTTGAAGAATGATTACAGGACGAATCTCTTGAATCGC

NNNNNNN: not determined region of about 250 bp (according to the results of the restriction digestion);

Left region, right region were blasted separately;

Left region: unnamed protein product; 4,1;

Right region: conserved hypothetical protein, HTH_MerR-SF super family; 3,2;

Clone 20

ATCAGCTTGATTCGTTTGGGAAGATAAGTACCGTTATGTCATCGAACTTGGCAAAGCCATGGAGAACTTGAT
GAAGCGCAGCGGACAGCAGAAAACAAAGTAAATGGCTGTGTTAGCCAAGTTTGGCTCGCGACATCCTGCACG
GATGAAGTCAAAAAAATCATTCATTTTGATGGCGCTTCTGATGCTCACATCGTGCGCGGCCTCGTTGCGATT
ATGATCGCCGCTTTGTCTGATCGCCAAGCAAAAGATATCGTAGATTTTGATGCTGAAGGCTTGATGAAAAAT
TTAGGGCTTGATAGCCACCTGTCGCCGCAACGTTCTAATGGTTTGCGTGCAATGATCGAGCGCATGAAGTCA
GACGCTAAAGCTGCGCTCGTTTAATACATTACATTTCTGGACGGTAATCTTCTTGTCCCCAATGACGCGGCT
TTGAAGATCGCGTTCTCACTTGGGGCGGGTTATAGTGGCGTGCTAACATAGACAGCGCAGTTTTAAGCATAA
GTTTTGCTGATCTTCGCGGCCATTGCCTCTCTCTTTCTATATCTTCCATTCCTTTTAAGAAACAACACACAT
CAAGTTGCTTGGGTGAAATATCAAGAAACAGCTTGATCATGAAAAATGAGCTTCGCTCGTGCTTGTAAAAGA
TCATTGACAGTTCTCTTTTAGAGTATGTGGCGATCTAAAAGCTATTCAATGTTCTTGATGGTTGGATAAGTT
CATCCTTGCCAAGCAGGTTTTCTTTAATTTTATAAAGAAAATCAGTATTTTTGCGCGTGAGGGAATAAATAC
CTATTAAATAGGGATGAAATTAATGTAGGAATATAGTCCTCACTACGGAGGACTGAATGCTTAAGACCAAAG
CAATAAAACGAAATAATTATCTGAATGATTTTTTGCCATCAGCAGCTTCTAAACAGCGTGCTGAAGATCAGA
TATATACCCCTTTGTGCGATTGTGCCGCCACTGTAAGTGCAGACTTACATATGGAGATTTGTGACGGTCTCA
TTGATATAATGGCCGCGCTTTTCAATGTTTCAGGGCGGCAATTGCGTCACCGACGAATCTCCAGA

Part 67-378 (of 1073): hypothetical protein mll7646, SufE super family; 8e-28; part 398-598 (of 1073): hypothetical protein; 2e-18;

Clone 21

TTCGGAAGAATCAGCTTGATTCGTTTCGGAGCGCGGCCAAATGCGCAATGATATCCGTTCAGGTCTGCGCAT
TGTCGGGTTTGACAGAGAATTCCCCTCACTCAGTTGCGGCTAACGCGCAAATGCGCGAAGCCTCACATTCCT
CTCCCGCAAGCGGCAGAGGAAAATCAGCGCTCCCTATCTCTCCCCTTGTGGGAGAGAAAGCGAAAACTTGGG
CTTGGCTTGCCAAGTCCTTAGTTTTGGCAAGAGAGGGGATATAACGCCGCTCTCCGTCATCCTATGGCTTGA
CCATAGGATCCATTCCGTGAAGTTCACTTGGGTGTTTTTTCTTGAAGAATGATTACAGTGTCAGCATCACGG

AATGGATCCCGCGACGTCGCAACGCTTGTCTTTGCTCGAAGGCCGAGGATGACGGCAGGATGGGCTGCCCCT
CCCTTCTCCCGCCTGCGGGAGAAGGGGGATACGAGTGGTAACGAGTAGACGGATGAGGGCGTTCCGCATCAA
ATTCCCCTCACCCAGTTGCGGCTAACGCGCAATGCGCGAAGCCTCACATCCCTCTCCCGCAAGCGGGAGAGG
AAAGCACCAATCTTATCCACCCACCACAAAACACATTCACACTGCGGTGATGAACCGTACCGCGAAAAACAT
TGCCATAGAGAAAAACCGCGCCGCGCTTTTGCGCCTCGTGCGGTGCTGGTTGATGATCGCCGCAATGTTAAG
CGCCAATCGCGTGCTGCCATGCCGGATTGGGTAATGGGTATCGCGCATCATTTTCAAAGTCGAAAAGGCGGC
CTATTATTTGCAGATTGCTTCCGGCTTCATGCAAGCAAAGGATTCCCTGCCCGTTGAGCCGCATGGACAAAT
AACAATCAAGGCCGTGATCAACAGGCTTCGCGCTGTCAAAAAATTGCTAATTCGTTTGAAGCCAAGCGTAGG
CCAGTGGCTGAGGCGCTGGCTTTGTTTTACCCCAAACAATTATCCGCAAACTTCTGCGCTTTACAGCGGCGG
CGGCTTTCGCAAATTCGCCGCTTTTAACGGCCGCGCACCGCCATGTCTTGGTGCTGCGCATTATACATTTGA
CCGATATAAATCGCACGGCCCGTCGCGATAATTTTATCGTAGGGCCAATAAAAAGATGGGCACTATTCCGGT
ATGATTAAATCATACTTTCTGATAAAATAAGTTCTGAGAGGATGAATCGATGGATAAGAGAACAATCAGCCT
ACCCGATGAGCATGCGGCCTATATTGACCAGAAAGTGAGTTCTGGCGATTACGCTTCTGCGAGCGAGGTTGT
GCGTGCAGGACTTCGCGCATGCAAGAGACGAATCTCAGATCGC

Part 1028-1327 (of 1339): putative addiction module antidote protein, CoqG/Arc/MetJ family; 2e-06;

Clone 23

GCGAATCTGAGATTCGTCTCTTTACATTTCTCGTATCTTTGATGATTTGGGTTGGATTTGAGAATTCAAATC
CCGGCTTCCAATTTGTTGAAGAAGCTGAATGGCTCGGTGGCGGGCTAACCTACCGTATGGGTGTGGACGGCA
TTTCAATGTTGTTTGTCATTTTGACAACCTTCTTGATGCCTTTCTGTATCCTTGCTGGCTGGGAGAGCGTTA
CCAAGCGTCTTAAAGCCTACATGATTGCTTTCCTTATTTTGGAAACACTCATGATCGGCGTGTTTACTGCGC
TTGATATCGTGCTGTTCTATGTCTTCTTTGAAGCGGGCCTTTGGAAACGCGGTGACGGCTGGCTTATTGATG
GGTTTGGTCCCAACAATATCGCAGCGCGTGTTTCTGGCCTCGCACAGCGTGCTGTGAAGGTTCAGACCGGAT
TTCTTTATCATTATGCGTTCGCCATGTTGATTGGTGTCGCTGCTCTCGTGACTTGGATTATGCTTGGAGGCA
TCCACTAATGGACGGTATGCCAATCCTCTCGCTCGTAACTTTCCTTCCTTTGGTTGGTGTTCTTGTTATTCT
GTTTATCAATGATGAAACAGAAGCGGCAAAACGCAACGTTCGGATGGTCGCGTTACTGACGACGATCTTTAC
ATTTCTCGTATCTTTGATGATTTGGGTTGGATTTGAGAATTCAAATCCCGGCTTCCAATTTGTTGAAGAAGC
TGAATGGCTCGGTGGCGGGCTAACCTACCGTATGGGTGTGGACGGCATTTCAATGTTGTTTGTCATTTTGAC
AACCTTCTTGATGCCTTTCTGTATCCTTGCTGGCTGGGAGAGCGTTACCAAGCGTCTTAAAGCCTACATGAT
TGCTTTCCTTATTTTGGAAACACTCATGATCGGCGTGTTTACTGCGCTTGATATCGTGCTGTTCTATGTCTT
CTTTGAAGCGGGCCTTTGGAAACGCGGTGACGGCTGGCTTATTGATGGGTTTGGTCCCAACAATATCGCAGC
GCGTGTTTCTGGCCTCGCACAGCGTGCTGTGAAGGTTCAGACCGGATTTCTTTATCATTATGCGTTCGCCAT
GTTGATTGGTGTCGCTGCTCTCGTGACTTGGATTATGCTTGGAGGCATCCACTAATGGACGGTATGCCAATC
CTCTCGCTCGTAACTTTCCTTCCTTGAACGAATCAAGCTGAATCAT

Part 21-147 (5e-41) and part 512-952 of 1198 (2e-59): proton-translocating NADH-quinone oxidoreductase, chain M;

Clone 24

TCGCATGATTCAGCTTGATTCGTGCCGTGATCAACAGGCTTCGCGCTGTCAAAAAATTGCTAATTCGTTTGA
AGCCAAGCGTAGGCCAGTGGCTGAGGCGCTGGCTTTGTTTTACCCCAAACAATTATCCGCAAACTTCTGCGC
TTTACAGCGGCGGCGGCTTTCGCAAATTCGCCGCTTTTAACGGCCGCGCACCGCCATGTCTTGGTGCTGCGC
ATTATACATTTGACCGATATAAATCGCACGGCCCGTCGCGATAATTTTATCGTAGGGCCAATAAAAAGATGG
GCACTATTCCGGTATGATTAAATCATACTTTCTGATAAAATAAGTTCTGAGAGGATGAATCGATGGATAAGA
GAACAATCAGCCTACCCGATGAGCATGCGGCCTATATTGACCAGAAAGTGAGTTCTGGCGATTACGCTTCTG
CGAGCGAGGTTGTGCGTGCAGGACTTCGCGCATTGCAAGAACGTGACCGCGCCGTTGAAAACTGGCTACACC
AACAGGTTGCCCCTGCCTATGACGCGATGATGCAAGACCCATCGCGTGGGCTTTCGCATGATTCAGCTTGAT
TCGTGCCGTGATCAACAGGCTTCGCGCTGTCAAAAAATTGCTAATTCGTTTGAAGCCAAGCGTAGGCCAGTG
GCTGAGGCGCTGGCTTTGTTTTACCCCAAACAATTATCCGCAAACTTCTGCGCTTTACAGCGGCGGCGGCTT
TCGCAAATTCGCCGCTTTTAACGGCCGCGCACCGCCATGTCTTGGTGCTGCGCATTATACATTTGACCGATA
TAAATCGCACGGCCCGTCGCGATAATTTTATCGTAGGGCCAATAAAAAGATGGGCACTATTCCGGTATGATT
AAATCATACTTTCTGATAAAATAAGTTCTGAGAGGATGAATCGATGGATAAGAGAACAATCAGCCTACCCGA

TGAGCATGCGGCCTATATTGACCAGAAAGTGAGTTCTGGCGATTACGCTTCTGCGAGCGAGGTTGTGCGTGC
AGGACTTCGCGCATTGCAAGAACGTGACCGCGCCGTTGAAAACTGGCTACACCAACAGGTTGCCCCTGCCTA
TGACGCGATGATGCAAGACCCATCGCGTGGGCTT

Part 348-572 (2e-19) and part 905-1114 (1e-18) of 1114: putative addiction module antidote protein, CopG/Arc/MetJ family;

Clone 26

ATCAGCTTGATTCGTTTGACTACCGCTTGCACAAGGTGTATCTTTCGATATCTAAACTCTATATGGGCTTGG
CTTTTAAAAGGCCAAGCCAGCTCAATCAGGAAAAACTGATGACTACCTTTTGTGCCTATATTATACGTCATG
GAATATGAGACATTGGACGAAGCAATCCATATGCAAAACGATGTACCACAAGGCTTGTCGTCCTGTATCTTT
ACGCTCAACATGCGTGAGGCGGAAACATTTTTATCAGCCACAGGGTCTGACTGCGGAATTGCAAACGTCAAT
ATTGGCCCATCGGGTGCAGAAATTGGCGGCGCATTTGGCGGTGAAAAAGAAACAGGCGGCGGGCGTGAAAGC
GGTTCAGACGCATGGAAAGGTTACATGCGGCGTCAGACCTCAACTGTCAATTATTCGGCTGAACTACCGCTT
GCACAAGGTGTATCTTTCGATATCTAAACTCTATATGGGCTTGGCTTTTAAAAGGCCRASSMATCTCAATCA
KGAAAAACTGATGACTACCTTTTGTGNNNNNNNNNNNNNCCTATATTATACGTCATGGAATATGAGACATTG
GACGAAGCAATCCATATGCAAAACGATGTACCACAAGGCTTGTCGTCCTGTATCTTTACGCTCAACATGCGT
GAGGCGGAAACATTTTTATCAGCCACAGGGTCTGACTGCGGAATTGCAAACGTCAATATTGGCCCATCGGGT
GCAGAAATTGGCGGCGCATTTGGCGGTGAAAAAGAAACAGGCGGCGGGCGTGAAAGCGGTTCAGACGCATGG
AAAGGTTACATGCGGCGTCAGACCTCAACTGTCAATTATTCGGCTGAACTACCGCTTGCACAAGGTGTATCT
TTCGATATCTAAACTCTATATGGGCTTGGCTTTAAAAGGCCGACGAATCTC

NNNNNNN: not determined region of about 900 bp (according to the results of the restriction digestion);

Left region (4e-39), right region (2e-28): aldehyde dehydrogenase family protein;

Clone 28

GCGGAATCTGAGATTCGTCCTGCATCCGTGCACCTTTGGGAAACGCTAGAAACTCTGCATTACATGCGTGAA
ATTGGCTATGATGGCTGGGTTGCGTATGATGTATTCACGCGTTCGGGTGATAATGTTGAGGCAATTGCGTCA
ACATTTGAGATCATGGAAGATCTCGATTCTCTTCTTGATAAAATTGGTAAGGACAAGATCCAGCAACTTATT
AAAGGTGGCCGTCCTCAGAACACTTATCGTGATTTGATCAAGGCGTTACTATGAAAATAGGACTTGGAACTT
ATTGCTTCCGTTGGTCAATCGGGCACAAAGATCGTGTGCCCGAAAAACCAATGACAGCGATGGATGTTTTAG
ATTTTGGTATTTCTGAAGGTTGCGCTGTTGTGCAATATGCGGACAATCTGCCGTTAGATAAACTATCCATTG
AAGAAATTGATGCGCTGGCTGAAAAGGCGCGTCAACACAATGTGATTTTGGAGCTGGGCACACAGTCTTTTG
ATGCGGAACAAGTTCGTGTGTATTTGGATATTGCCAAACGGATTAACGCTCCCATTTTACGCATAGCGCTGG
ACGGTGAAGATGCCGCGACTTCTATAGAGGATTTGGCGGCTGCATTTCGGCCACTGCTTGCTACTGCGCGGG
AAATTGGTTGCAAGATCGCTATTGAAAACCACTTTAACTATCCATCCCAACGGATGGTGACATTACTTGAAG
CGGTCAATGACGATTGCCTTGGGGTCTGCCTCGATGTTGCAAATTCGATTTGCGCAAAAGTTCTACATTCAC
TTTAATGACAATGATCGCGGTGCCGATTGGGATATGCTTCCTGCATCCGTGCACCTTTGGGAAACGCTAGAA
ACTCTGCATTACATGCGTGAAATTGGCTATGATGGCTGGGTTGCGTATGATGTATTCACGCGTTCGGGTGAT
AATGTTGAGGCAATTGCGTCAACATTTGAGATCATGGAAGATCTCGATTCTCTTCTTGATAAAATTGGTAAG
GACAAGATCCAGCAACTTATTAAAGGTGGCCGTCCTCAGAACACTTATCGTGATTTGATCAAGGCGTTACTA
TGAAAATAGGACTTGGAACTTATTGCTTCCGTTGGTCAATCGGGCACAAAGATCGTGTGCCCGAAAAACCAA
TGACAGCGATGGATGTTTTAGATTTTGGTATTTCTGAAGGTTGCGCTGTTGTGCAATATGCGGACAATCTGC
CGTTAGATAAACACGAATCAAGCTGAATCATAAAG

Part 267-776 (2e-52), part 1080-1229 (2e-12) of 1259: hypothetical protein OB2597_06015;

Clone 29

GAGATTCGTCCTGCATCCGTGCACCTTTGGGAAACGCTAGAAACTCTGCATTACATGCGTGAAATTGGCTAT
GATGGCTGGGTTGCGTATGATGTATTCACGCGTTCGGGTGATAATGTTGAGGCAATTGCGTCAACATTTGAG
ATCATGGAAGATCTCGATTCTCTTCTTGATAAAATTGGTAAGGACAAGATCCAGCAACTTATTAAAGGTGGC
CGTCCTCAGAACACTTATCGTGATTTGATCAAGGCGTTACTATGAAAATAGGNNNNNNNNNNNNNAAAATGC
ATCTTGCATCCGTGCACCTTTGGGAAACGCTAGAAACTCTGCATTACATGCGTGAAATTGGCTATGATGGCT
GGGTTGCGTATGATGTATTCACGCGTTCGGGTGATAATGTTGAGGCAATTGCGTCAACATTTGAGATCATGG
AAGATCTCGATTCTCTTCTTGATAAAATTGGTAAGGACAAGATCCAGCAACTTATTAAAGGTGGCCGTCCTC
AGAACACTTATCGTGATTTGATCAAGGCGTTACTATGAAAATAGGACTTGGAACTTATTGCTTCCGTTGGTC
AATCGGGCACAAAGATCGTGTGCCCGAAAAACCAATGACAGCGATGGATGTTTTAGATTTTGGTATTTCTGA
AGGTTGCGCTGTTGTGCAATATGCGGACAATCTGCCGTTAGATAAACACGAATCAAGCTGA

NNNNNNN: not determined region of about 2500 bp (according to the results of the restriction digestion);

Part 1 (7e-23), part 2 (9e-20) and part 3 (8e-13): putative xylose isomerase;

Clone 30

GCGGATCTGGAGATTCGTCCCGAGAGAATTTCTAAAAAGGAAAATCCATGACAAACTTTACTATTCACACGA
TTGAATCTGCACCTGAAGCAGGTAAGCCGCTTTTAGAAACATCACTTAAAAATAATGGTCGCATTCCAGGTT
TGCACGGCACCATGGCAGATGCACCGCCATTGCTCGCAGCTTATAATTTCGCGCATCAGCAGTTCATGGCAA
CCTCAATGACTGATGAGGAGAAAACTGTCGTTTGGCAAACAATCAATGTTGAAAACAATTGTCATTACTGTG
TGCCTGCGCATACTGGAATTGCGAAAATGATGAAGATCGATGATGCAATCACTGATGCTTTGCGCGATGAAA
CACCGCTTCCGACTGCCAAACTCGAAGCCTTGCGTACATTCACATTGGCTATGGTTCGTGACCGCGGTTTCG
TTGCTGAAGCCGATACTCAAGCCTTTTTAGATGCCGGTTTCACAGAAACGAACATCCTTGAAATCATTCTGG
GCGCTGCGCAAAAGCTGATGTCAAACTACACCAACCACTTTGCAAAAACGCCTGTTGACCAAGTGTTCCAAA
AGTTCGCTTGGGAAAAGAAAACAGCAGTCGCGGCTGAATAATTGAAGCGCCGGAGTGTGTGATCGCTTCGGC
ACTTACCCCGACCAAGTGACACCTATCTGTGTGGCGGGTGTTATGGTTTTTAGCCCTTCGCTTCTTGCGCTG
TTTTTCACCCGCTCTATGGTAATTGGTGCTTATGACACCTACCTCACTGTCCTATTTACAGCGAATGTGTTC
ACACTGTTGTTTGAAATCAATGATTTGCATTTATGGCTTTGCGTGATCGTCGTGTTATTAGACGCATCTGGA
GTGTATAATATGACCGACCCCTACGTATTGATATGTGNNNNNNNNNNNNNGAAAATACGGCAGCACGCCGAG
CAATCTGAGCAATCGCGCCAGCAGATGAAGGCACTGGGCGATAGCTTGGATTTGACTTTGCATGGACGGATG
ATTCCCGCATGCACAACACATTCAACACGCATCAATTGCTGCACTGGGCAGATACGCAAGGCCGTCGCCACG
ATCTTAAAATGGCGCTGTTCACAGCTCATTTCACGGATCAGCGCAACCTATCCGATGATAATGTGCTAGCTG
ATATAGCCGCTGAAATCGGCTTGAACCGCGAAGAGGCTTTGGCTGTTCTTGAAGATCAGCGCTATGCAAATG
ACGTGCGCGCCGCCGAAACTTTTTGGCAACAACAGGGTATTTCAGGTGTGCCGGCCGTCGTGTTTGATCGCA
AACACCTTGTCACAGGTGCACAAGGCGTGGAAAACTACACGAATATTCTGTCCCAAATCGCAAAATTGAAAA
GTTGACCCATGGCCCGCGCTGCAAATTATGATCGTGACGCCGCACTTGATGCGGCCATGGCTGTGTTTTGGC
GCAAAGGCTACCACGCAACGTCGCTAAAAGATTTGGAAGCGACACTTAGCATGAAGCCGGGCAGTATATATG
CTGCATTCGAGAGCAAAGAAAACCTCTACCTCTTGGCGATAGAACGTTATTTCGAAACGTCACGCCAAGGGT
TTAGAGCAAAGATGATGCAGTTTGAGTCACCACTGGAAGGGTTGGCCGCACAGTTTCAAAACTACGTTAGCC
TTGCGGATGCAGACCCGTCACGCCGATCTTGTATGTTGATGAAAACGCTTGTGGATACCAAATCCACAGATC
CCAAAATTGCTGAAGCTTCACAGAAATATTTAGATCGTATGTGCGCGGAGTTCGCAAAGACAAACGAATCAA
GCTGAATATCAGGA

NNNNNNN: not determined region of about 400 bp (according to the results of the restriction digestion);

Left region: hypothetical protein Pcryo_0971; 1e-62;

Right region: dsba oxidoreductase; 6e-46;

Clone 33

TCTGAGAATTCGTCCCCCCCATGAAGAGCGCGCCAATCGACACGCCGGACACGACACCATACAGCACCAAAG
GAATGGATGGCGGAATGATCGGCCCAATCACACCTGATGCGGCTACGACTGCGGCAGAAAATGATGCGTCGT
ATCCACGGCGCTTCATCATAGGCCCCCCCATGAAGAGCGCGCCAATCGACACGCCGGACACGACACCATACA
GCACCAAAGGAATGGATGGCGGAATGATCGGCCCAATCACACCTGATGCGGCTACGACTGCGGCAGAAAATG
ATGCGTCGTATCCACGGCGCTTCATCATAGGCCCCCCCCATGAAGAGCGCGCCAATCGACACGCCGGACACG
ACACCATACAGCACCAAAGGAATGGATGGCGGAATGATCGGCCCAATCACACCTGATGCGGCTACGACTGCG
GCAGAAAATGATGCGTCGTATCCACGGCGCTTCATCATAGGCCCCCCCATGAAGAGCGCGCCAATCGACACG
CCGGACACGACACCATACAGCACCAAAGGAATGGATGGCGGAATGATCGGCCCAATCACACCTGATGCGGCT
ACGACTGCGGCAGAAAATGATGCGTCGTATCCACGGCGCTTCATCATAGGCCCCCCCATGAAGAGCGCGCCA
ATCGACACGCCGGACACGACACCATACAGCACCAAAGGAATGGATGGCGGAATGATCGGCCCAATCACACCT
GATGCGGCTACGACTGCGGCAGAAAATGATGCGTCGTATCCACGGCGCTTCATCATAGGCCCCCCCATGAGA
GCGCGCCATCGACACGCCGGACACGACACCATACAGCATCAAAGATGGATGGCGGATGATNNNNNNNNNNNN
NCAATCGACACGCCGACACGACACCATACAGCACCAAAGGGAATGGATGGCGGAATGATCGGCCCAATCACA
CTTGATGCGGCTACGACTGCGGCAGAAAATGATGCGTCGTATCCACGGCGCTTCATCATAGGCCCCCCCATG
AAGAGCGCGCCAATCGACACGCCGGACACGACACCATACAGCACCAAAGGAATGGATGGCGGAATGATCGGC
CCAATCACACCTGATGCGGCTACGACTGCGGCAGAAAATGATGCGTCGTATCCACGGCGCTTCATCATAGGC
CCCCCCATGAAGAGCGCGCCAATCGACACGCCGGACACGACACCATACAGCACCAAAGGAATGGATGGCGGA
ATGATCGGCCCAATCACACCTGATGCGGCTACGACTGCGGCAGAAAATGATGCGTCGTATCCACGGCGCTTC
ATCATAGGCCCCCCCCATGAAGAGCGCGCCAATCGACACGCCGGACACGACACCATACAGCACCAAAGGAAT
GGATGGCGGAATGATCGGCCCAATCACACCTGATGCGGCTACGACTGCGGCAGAAAATGATGCGTCGTATCC
ACGGCGCTTCATCATAGGCCCCCCCATGAAGAGCGCGCCAATCGACACGCCGGACACGACACCATACAGCAC
CAAAGGAATGGATGGCGGAATGATCGGCCCAATCACACCTGATGCGGCTACGACTGCGGCAGAAAATGATGC
GTCGTATCCACGGCGCTTCATCATAGGCCCCCCCATGAAGAGCGCGCCAATCGACACGCCGGACACGACACC
ATACAGCACCAAAGGAATGGATGGAACGAATCAAGCTGAT

NNNNNNN: not determined region of about 3200 bp (according to the results of the restriction digestion);

Left region, right region: hypothetical protein [Strongylocentrus purpuratus]; 3e-18;

Clone 34

ATATTTCAGCTTGATTCGTGGATTG**GATGCTGAATTGGGCGGTCGAATCACAATTCAGGGCCCTCGTTTGGC**
GAAGGCAACGCCGCTACCCACACCATCCAGCCGGCCGAGCATTATCCAGTTAAATTTGACGATCAATGCACC
GTCTCAGATTTTTGTGCGCGGACGTGGATTGGATGCTGAATTGGGCGGTCGAATCACAATTCAGGGCCCTCG
TTTGGCGAAGGCAACGCCGCTACCCACACCATCCAGCCGGCCGAGCATTATCCAGTTAAATTTGACGATCAA
TGCACCGTCTCAGATTTTTGTGCGCGGACGTGGATTGGATGCTGAATTGGGCGGTCGAATCACAATTCAGGG
CCCTCGTTTGGCGAAGGCAACGCCGCTACCCACACCATCCAGCCGGCCGAGCATTATCCAGTTAAATTTGAC
GATCAATGCACCGTCTCAGATTTTTGTGCGCGGACGTGGATTGGATGCTGAATTGGGCGGTCGAATCACAAT
TCAGGGCCCTCGTTTGGCGAAGGCAACGCCGCTACCCACACCATCCAGCCGGCCGAGCATTATCCAGTTAAA
TTTGACGATCAATGCACCGTCTCAGATTTTTGTGCGCGGACGTGGATTTGGATGCTGAATTGGGCGGTCGAA
TCACAAATTCAGGGCCCTCGTTTTGGCGAAAGCAAACGCCCGCTACCCACACCATCCAGCCGGCCGAGCATT
AATCCAGTTAAATTTGACGATCAATGCACCGTCTTCAGATTTTTGTGCGCGGACGTGGAATTGGATGCTGAA
NNNNNNNNNNNNNGGTTGGAATCACAATTCAGGGCCCTCGTTTGGCGAAGGCAACGCCGCTACCCACACCAT
CCAGCCGGCCGAGCATTATCCAGTTA**AATTTGACGATCAATGCACCGTCTCAGATTTTTGTGCGCGGACGTG**
GATTGGATGCTGAATTGGGCGGTCGAATCACAATTCAGGGCCCTCGTTTGGCGAAGGCAACGCCGCTACCCA
CACCATCCAGCCGGCCGAGCATTATCCAGTTAAATTTGACGATCAATGCACCGTCTCAGATTTTTGTGCGCG
GACGTGGATTGGATGCTGAATTGGGCGGTCGAATCACAATTCAGGGCCCTCGTTTGGCGAAGGCAACGCCGC
TACCCACACCATCCAGCCGGCCGAGCATTATCCAGTTAAATTTGACGATCAATGCACCGTCTCAGATTTTTG
TGCGCGGACGTGGATTGGATGCTGAATTGGGCGGTCGAATCACAATTCAGGGCCCTCGTTTGGCGAAGGCAA
CGCCGCTACCCACACCATCCAGCCGGCCGAGCATTATCCAGTTAAATTTGACGATCAATGCACCGTCTCAGA
TTTTTGTGCGCGGACGTGGATTGGATGCTGAATTGGGCGGTCGAATCACAATCAGACGAATTCTC

Left region, right region: **part 1** (3e-12), part 2 (1e-13), **part 3** (1e-13) and part 4 (4e-13): gramicidin S biosynthesis GrsT protein [Ahrensia sp. R2A130]

Clone 36

CGATGATTCAGCTTGATTCGTTTGACTACCGCTTGCACAAGGTGTATCTTTCGATATCTAAACTCTATATGG
GCTTGGCTTTTAAAAGGCCAAGCCAGCTCAATCAGGAAAAACTGATGACTACCTTTTGTGCCTATATTATAC
GTCATGGAATATGAGACATTGGACGAAGCAATCCATATGCAAAACGATGTACCACAAGGCTTGTCGTCCTGT
ATCTTTACGCTCAACATGCGTGAGGCGGAAACATTTTTATCAGCCACAGGGTCTGACTGCGGAATTGCAAAC
GTCAATATTGGCCCATCGGGTGCAGAAATTGGCGGCGCATTTGGCGGTGAAAAAGAAACAGGCGGCGGGCGT
GAAAGCGGTTCAGACGCATGGAAAGGTTACATGCGGCGTCAGACCTCAACTGTCAATTATTCGGCTGAACTA
CCGCTTGCACAAGGTGTATCTTTCGATATCTAAACTCTATATGGGCTTGGCTTTTAAAAGGCCAAGCCAGCT
CAATCAGGAAAAACTGATGACTACCTTTTGTGCCTATATTATACGTCATGGAATATGAGACATTGGACGAAG
CAATCCATATGCAAAACGATGTACCACAAGGCTTGTCGTCCTGTATCTTTACGCTCAACATGCGTGAGGCGG
AAACATTTTTATCAGCCACAGGGTCTGACTGCGGAATTGCAAACGTCAATATTGGCCCATCGGGTGCAGAAA
TTGGCGGCGCATTTGGCGGTGAAAAAGAAACAGGCGGCGGGCGTGAAGCGGTTCAGACGCATGGAAAGGGTT
ACATGCGGCGTCAGACCTCAACTGTCAATTTATTCGGCTGACTACGCTTGCACATGGTGTATCTTCGATATC
TAAACTCTATATGGGCTTGGCTTTNNNNNNNNNNNNNCTAGTCAAGTATTCGTCTGACTACGCTGCACAAGT
GTATCTTCGATATCTAAACTCTATATGGGCTGCTTTAAAAGGCCAAGCCAGCTCAATCAGGAAAAACTGATG
ACTACCTTTTGTGCCTATATTATACGTCATGGAATATGAGACATTGGACGAAGCAATCCATATGCAAAACGA
TGTACCACAAGGCTTGTCGTCCTGTATCTTTACGCTCAACATGCGTGAGGCGGAAACATTTTTATCAGCCAC
AGGGTCTGACTGCGGAATTGCAAACGTCAATATTGGCCCATCGGGTGCAGAAATTGGCGGCGCATTTGGCGG
TGAAAAAGAAACAGGCGGCGGGCGTGAAAGCGGTTCAGACGCATGGAAAGGTTACATGCGGCGTCAGACCTC
AACTGTCAATTATTCGGCTGAACTACCGCTTGCACAAGGTGTATCTTTCGATATCTAAACTCTATATGGGCT
TGGCTTTTAAAAGGCCAAGCCAGCTCAATCAGGAAAAACTGATGACTACCTTTTGTGCCTATATTATACGTC
ATGGAATATGAGACATTGGACGAAGCAATCCATATGCAAAACGATGTACCACAAGGCTTGTCGTCCTGTATC
TTTACGCTCAACATGCGTGAGGCGGAAACATTTTTATCAGCCACAGGGTCTGACTGCGGAATTGCAAACGTC
AATATTGGCCCATCGGGTGCAGAAATTGGCGGCGCATTTGGCGGTGAAAAAGAAACAGGCGGCGGGCGTGAA
AGCGGTTCAGACGCATGGAAAGGTTACATGCGGCGTCAGACCTCAACTGTCAATTATTCGGCTGAACTACCG
CTTGCACAAGGTGTATCTTTCGATATCTAAACTCTATATGGGCTTGGCTTTAAAAGGCCGACGAATCTCAGA
TTCG

NNNNNNN: not determined region of about 3100 bp (according to the results of the restriction digestion);

Right region: aldehyde dehydrogenase family protein; 7e-39;

Clone 37

TCAGCTTGATTCGTTTGACCTGCGACCTTCAGGTTATGAGCCTGACGAGCTACCGGGCTGCTCCATCCCGCG
TCAATTGCTTACTTGAAGCGATTGACGCTACCCTGACAGGTAATTGCGAAGCAAATACCGAGAAGGGCCGCG
TCATTACATCACCAATAAAGAACTGATGATGGTGTTCATCTTTGTTTATCAAAGTTGAACATACAAAAAAGC
CGCTGACCTTACACCATAAGTGAGGTAAGCGGCCTTAAATGTAATGTTTATATTGTAATGAGAATACATATT
GTCCTTTGCAGACCTGGCAGCGACCTACTCTCCCGTGCCTTAAGACAAAGTACCATCGGCGCTGGGGCATTT
CACGGCCGTGTTCGGAATGGGAACGGGTGCAGCGACCCCGCCATAACCACCAGGTCAGCAAAGGACAATATG
TAAGAAGCTGGTAAAGCAAATTTGGCGCAGCCAAAATGCGTTTAAGTCGGCAGTCAATTGCGTAGCAAATGA
CGAGAGACTTACTTTAAGTGTTGTTTTCACAAACGTGAATACAAGCAATGAGAATGATGAAGTCGATCGAGC
TATTAGTAATGGTAAGCTTCATACCTTACGGTACTTCCACACCCCATCCTATCAACGTGGTGGTCTTCCACG
ACTCTCAGGGAATACTCGTTTTTAGGCTGGTTTCCCGCTTAGATGCCTTCAGCGGTTTATCCATTCCACACA
TAGCTACCCTGCAATGCTGCTGGCGCAACAACAGGTCCACCAGTGGTGTGTCCATCCCGGTCCTCTCGTACT
AGGGACAGATCCTATCAATATTCCTACACCCACGGCAGATAGGGACCGAACTGTCTCACGACGTTCTGAACC
CAACTCACGTACCGCTTTAAATGGCGAACAGCCATACCCTTGGGACCTGCTCCAGCCCCAGGATGCGATGAG
TCGACATCGAGGTGCCAAACAACCCCGTCGATATGGACTCTTGGGGGTCATCAGCCTGTTATCCCCGGCGTA
CCTTTTATCCGTTGAGCGATGGCCCTTCCACTCGGGACCACCGGATCACTATGACCGACTTTCGTCTCTGCT
CGACTTGTCAGTCTCGCAGTCAGGCGGGCTTATGCCATTGCACTCGACGAACGATTTCCGACCGTTCTGAGC
CCACCATCGCGCGCCTCCGTTACTCTTTAGGAGGCGTATTGGTTTGGGTATTGGTTTTGCCTCTGCGATCCC

GTTGCTAAGGCAGGCAATTGCGCAGCAAATGCCGAGAGCAGTTCAGGTTATGAGCTTAATGCAAAAAAGCCC
CTGATAAATCAGAGGCTTTAATGTACGAATCTC

Part 271-426 (of 1329): hypothetical protein; 2e-9; part 707-1147 (of 1329): cell wall-associated hydrolase; 6e-66;

Clone 39

ATCTGGAGATTCGTCCCCATGCTGCGTAGCGTCGAGCTTGAACCAATTGTCGATCTGGCTCAGATCAGCCAC
ATTTTGTGCGACACGCGGCTTATCGAAGAGGTTGCAGGCTTTGCGGCAGATAACAAACATGTAAAACATGTC
ATTGGCTTTGATGGCACATCAAACCATGAGGCAGAACTTGACCGACTTGCGCTTGAAAAGCCGGTTAAATTT
GATGCTGTGAAAACGGGCCGTGATGATGTTGCATTACTCGGCTTTACATCTGGCACGACAGGTAAGCCGAAA
GCCACCATGCATTTTCACCGTGATTTGCTGATGATTGCTGATGGATATGCAAAAGAGATTTTAGGTGTTGTG
CCAGACGATGTGTTTGTCGGCTCACCGCCTTTGGCTTTCACATTTGGCCTTGGCGGCCTTGCGATATTTCCG
CTGCGTTTTGGCGCAACCGCAACGCTGCTCGAAAATGCCAGCCCGCCCAATATGATTGAGATCATTGAGAAG
TATAAGGCTACGGTCTGTTTTACTGCTCCAACGGCTTACCGGGCCATGTTGGCGGCGATGGCAGAGGGCGCA
GATTTGTCCAGTTTGCGCGCGNNNNNNNNNNNNNNGCTGTTTCTGCAGGCGAAACTTTGCCTGCCCCTGTCTA
CGAGGAATGGATTGCTAAAACTGGCAAACCGATGCTTGACGGCATCGGGGCAACTGAGATGTTGCATATCTT
TATATCGAACCGCTTTGACGATCATCGCCCCGCATGCACAGGTAAACCGGTCACAGGCTATGAAGCAAAAGT
AGTCGATGATGAGGGTAATGAGGTGGCCGTCGGAACCGTAGGCCGCTTGGCGGTTCGGGGACCAACTGGATG
TCGCTATATGGCCGATGATAGGCAGAGCAATTATGTTCAGAACGGCTGGAATATCACCGGCGATAGTTTCAT
GATGGATGCTGAGGGATATTTGCATTTTGCCGCGCGCAATGACGATATGATTATTTCCAGCGGATACAATAT
TGCTGGACCAGAAGTTGAAGCAGCATTGCTAGGGCACCCTGCGGTGCGTGAATGTGCTGTTATTGGGGTCGC
AGATGAATCAAGAGGCTCGATAGTTCAAGCGCATATTGTTCTGAATGAAGGACAAAATGCAGATGATGCGCA
AATCAAACACCTACAAGAGCATGTAAAACAAACAATTGCGCCGAACGAATCAAGCTGA

NNNNNNN: not determined region of about 200 bp (according to the results of the restriction digestion);

Left region, right region: benzoate-coenzyme A ligase; 4e-177;

Clone 41

GAGATTCGTCCCCCCCATGAAGAGCGCGCCAATCGACACGCCGGACACGACACCATACAGCACCAAAGGAAT
GGATGGCGGAATGATCGGCCCAATCACACCTGATGCGGCTACGACTGCGGCAGAAAATGATGCGTCGTATCC
ACGGCGCTTCATCATAGGCCCCCCCCATGAAGAGCGCGCCAATCGACACGCCGGACACGACACCATACAGCAC
CAAAGGAATGGATGGSGGAATGATCGGCCCAATCACACCTGATGCGGCTACSACTGCGGCAGAAAATGATGC
GTCGTATCCACGGCGCTTCATCATAGGCCCCCCCCWKRAARAGSGSGCCAWTCRACMCSCCGRACMCRACMC
CWTACAGCACCAAAGGAAKGGATGGCGGAATGATCGGCCCAATCMCACCTGATGCGGCTACRACTGCGGCAR
AAAATGATGCGTCGTATCCACGGCGCTTCATCATAGGCCCCCCCCATGAARAGCGCGCCAATCGACACGCCGG
ACACGACACCATACAGCACCAAAGGAATGGATGGCGGAATGATCGGCCCAATCACACCTGATGCGGCTACGA
CTGCGGCAGAAAATGATGCGTCGTATCCACGGCGCTTCATCATAGGCCCCCCCCATGAAGAGCGCGCCAATCG
ACACGCCGGACACGACACCATACAGCACCAAAGGAATGGATGGCGGAAWTGATCGGCCCRATCACACCTGAT
GCGGCTMCGACTGSSGCAGAAAAATGATGCGTCGTATCCACGGCGCTTCAWCATAGGCCCCCCCATGAAGAG
SSCGCCATCNNNNNNNNNNNNNNCCCCCTACCGCCCCAAAGGGAAGGGAGGGGGAATGATTCGCCCAATCCCC
CCTGATGGGGGTTCGAATGCGGCAGAAAAAGATGGGTTGTATTCCCGGGGGTTTATTATTGGCCCCCCCCAT
GAAGAGCGCGCCAATCGACACGCCGGACACGACACCATACAGCACCAAAGGAATGGATGGCGGAATGATCGG
CCCAATCACACCTGATGCGGCTACGACTGCGGCAGAAAATGATGCGTCGTATCCACGGCGCTTCATCATAGG
CCCCCCCATGAAGAGCGCGCCAATCGACACGCCGGACACGACACCATACAGCACCAAAGGAATGGATGGCGG
AATGATCGGCCCAATCACACCTGATGCGGCTACGACTGCGGCAGAAAATGATGCGTCGTATCCACGGCGCTT
CATCATAGGCCCCCCCCATGAAGAGCGCGCCAATCGACACGCCGGACACGACACCATACAGCACCAAAGGAAT
GGATGGAACGAATCAAGCTGA

NNNNNNN: not determined region of about 200 bp (according to the results of the restriction digestion);

Left region, right region: part 1 (0,016), part 2 (0,11), part 3 (5e-9): nascent polypeptide-associated complex subunit alpha isoform a; [Homo sapiens];

Clone 42

TCAGCTTGATTCGTTTGTCTTTGCGAACTCCGCGCACATACGATCTAAATATTTCTGTGAAGCTTCAGCAAT
TTTGGGATCTGTGGATTTGGTATCCACAAGCGTTTTCATCAACATACAAGATCGGCGTGACGGGTCTGCATC
CGCAAGGCTAACGTAGTTTTGAAACTGTGCGGCCAACCCTTCCAGTGGTGACTCAAACTGCATCATCTTTGC
TCTAAACCCTTGGCGTGACGTTTCGAAATAACGTTCTATCGCCAAGAGGTAGAGGTTTTCTTTGCTCTCGAA
TGCAGCATATATACTGCCCGGCTTCATGCTAAGTGTCGCTTCCAAATCTTTTAGCGACGTTGCGTGGTAGCC
TTTGCGCCAAAACACAGCCATGGCCGCATCAAGTGCGGCGTCACGATCATAATTTGCAGCGCGGGCCATGGG
TCAACTTTTCAATTTTGCGATTTGGGACAGAATATTCGTGTAGTTTTCCACGCCTTGTGCACCTGTGACAAG
GTGTTTGCGATCAAACACGACGGCCGGCACACCTGAAATACCCTGTTGTTGCCAAAAAGTTTCGGCGGCGCG
CACGTCATTTGCATAGCGCTGATCTTCAAGAACAGCCAAAGCCTCTTTCGCGGTTCAAGCCGATTTCAGCGG
CTATATCAGCTAGCACATTATCATCGGATAGGTTGCGCTGATCCGTGAAATGAGCTGTGAAACAGCGCCATT
TTTAAGATCGTGGCGACGGCCTTGCGTATCTGCCCAGTGCAGCAATTGATGCGTGTTGAATGTGTTGTGCAT
GCGGGAATTCATCCCGTCCATGCAAAGTCAAATCAAAGCTATCGCCCAGNNNNNNNNNNNNNATGCAAATCA
TTGATTTCAAACAACAGTGTGAACACATTCGCTGTAAATAGGACAGTGAGGTAGGTGTCATAAGCACCAATT
ACCATAGAGCGGGTGAAAAACAGCGCAAGAAGCGAAGGGCTAAAAACCATAACACCCGCCACACAGATAGGT
GTCACTTGGTCGGGGTAAGTGCCGAAGCGATCACACACTCCGGCGCTTCAATTATTCAGCCGCGACTGCTGT
TTTCTTTTCCCAAGCGAACTTTTGGAACACTTGGTCAACAGGCGTTTTTGCAAAGTGGTTGGTGTAGTTTGA
CATCAGCTTTTGCGCAGCGCCCAGAATGATTTCAAGGATGTTCGTTTCTGTGAAACCGGCATCTAAAAAGGC
TTGAGTATCGGCTTCAGCAACGAAACCGCGGTCACGAACCATAGCCAATGTGAATGTACGCAAGGCTTCGAG
TTTGGCAGTCGGAAGCGGTGTTTCATCGCGCAAAGCATCAGTGATTGCATCATCGATCTTCATCATTTTCGC
AATTCCAGTATGCGCAGGCACACAGTAATGACAATTGTTTTCAACATTGATTGTTTGCCAAACGACAGTTTT
CTCCTCATCAGTCATTGAGGTTGCCATGAACTGCTGATGCGCGAAATTATAAGCTGCGAGCAATGGCGGTGC
ATCTGCCATGGTGCCGTGCAAACCTGGAATGCGACCATTATTTTTAAGTGATGTTTCTAAAAGCGGCTTACC
TGCTTCAGGTGCAGATTCAATCGTGTGAATAGTAAAGTTTGTCATGGATTTTCCTTTTTAGAAATCTTCTCG
GGACGAATC

NNNNNNN: not determined region of about 100 bp (according to the results of the restriction digestion);

Left region: hypothetical protein Pcryo_0971; 1e-62;

Right region: part 1: dsba oxidoreductase; 4e-24; part 2: putative TetR-family transcriptional regulator; 5e-31;

Clone 43

TCAGCTTGATTCGTTTGATTCCGCAATGCGGCTAAGTTTTTGGAATTAGAATCAAAATGACTAAATCAGTTT
TTATTGACGGCGAGCACGGAACCACAGGCTTGCAAATTGCAGAACGGCTTGCTGCACGCGATGACATCAATC
TTTTGTCGCTTGCGATGGAAGATCGGCGCGACAATGAAAAGCGAACAACAATGCTGCGCGATGCTGATATTG
CGATTCTATGTTTACCCGATGATGCTGCGCGCGAAGCTGTTGCACTGGCTGAAGGTTCAGGCACACGGTTTA
TTGATGCGTCAACTGCGCACCGTATTGCAGATGGCTGGGTCTATGGTTTTGCAGAAAGCGAGCCGGGCCAGC
GCGAAAAAATCATCCACGCGCAAAATGTTACTAACCCGGGCTGTTATTCAACAGGCGCGATTGCCCTGCTGC
GTCCCCTCACCTTAGCGGGTCTATTACCCAGCACTTATCCGGTGACGATAAATGCGGTTTCTGGCTATTCAG
GCGGCGGCAAGCAGATGATTGCGCAGATGGAAGATGCTAAGCGTGATGACCCGATAAAGGCCCCTTATTTTG
CCTATGCGCTNNNNNNNNNNNNNGGCAGGCAAATCAGCCTTATTGCTTCCTTGGACAATTTGGGCAAAGGCG
CATCAGGCGCAGCTGTTCAAAACCTCAACCTGATGCTTCAAGCCTAATTTAAGTGAGGCCGTGCGTGTGTGC
ACGGCCATCTTTTAGCGGTAGCGGCCCAACAAGCCCGCGCCAATGCGCAACCGCAAATGCTAGAACTATAAA
CGCCAAGCCTTGGTGCACGAGTGCCCAATCCAGCTGAACCTCCGTTAGCAGCGTTGCAATACCAACAATCGC
CTGACCTATCACCAAGGACGAATCTCT

NNNNNNN: not determined region of about 2300 bp (according to the results of the restriction digestion);
Left region, right region: N-acetyl-gamma-glutamyl-phosphate reductase [Ahrensia sp. R2A130]; 3e-52;

Most of the sequences determined so far were of bacterial origin. Three sequences - namely the inserts of clone 9, clone 33 and clone 41 - show homologies exclusively to a eucaryotic protein.

A total of 35876 bases was sequenced.

4 Discussion

4.1 Growth characteristics of *A. kielensis*

4.1.1 Atypical growth curve

Continuous OD measurements were done over 4 weeks to construct a growth curve of *A. kielensis*. The obtained curve (see Fig 3) did not show the typical growth phases – lag phase, exponential phase, stationary phase and death phase – as ideally expected with a bacterial pure culture. Instead discontinuous peaking and declining of the curve was observed.

Schleheck et al (2009) observed the growth of *Pseudomonas aeruginosa* PAO1 and found that this strain grows in aggregates and disperses upon starvation leading to an increase in the optical density during starvation phase when the bacterial cell number is already declining. Therefore OD measurements would not allow distinguishing between growth and starvation phase. Furthermore it was noted by Martens-Habbena and Sass (2006) that OD measurements do not reflect the actual cell number of a bacterial culture if the observed bacteria formed aggregates or if particles were present in the culture.

With *A. kielensis* cultures both the formation of aggregates and the production of VLPs was observed by epifluorescence microscopy which might explain the unusual shape of the obtained growth curve. Nevertheless the *A. kielensis* culture seemed to have entered the stationary phase at about 100 h and not to have reached the death phase when the period of observation ended.

4.1.2 Variations in the size, shape, stability and colour composition of bacterial pellets

The bacteria pellets of *A. kielensis* obtained during the process of VLP harvest varied in size, shape, stability and colour composition (see Fig 8). These variations depended both on the time of incubation of the processed bacterial culture and the

circumstance whether the culture was sonicated before the harvest of VLPs was started or not.

First of all it has to be mentioned that the 514+Y+P medium used for cultivation of *A. kielensis* tended to flocculate and might contribute to the composition of the pellet as well. That the size of the pellets varied with the age of the processed culture was not surprising as the cell number contained in the processed culture is expected to vary as well.

No information was found on the influence of sonication on the stability of bacterial pellets. The presence of aggregates might lead to more instable pellets as they varied in size and shape and might promote the formation of micro cavities inside the pellet which increase its instability.

Varieties in the colour composition of the bacterial pellets might be due to metabolic changes during long term incubation of *A. kielensis.*

4.1.3 Pigmentation of VLP pellets

Although no pigmentation of the liquid cultures was observed, VLP pellets from *A. kielensis* cultures derived from the glycerol stock were found to be at least slightly green pigmented. Interestingly no pigmentation was found with pellets from the four test cultures. In this work all cultures were grown in the dark except of the preculture which was used for the glycerol stock. This leads to the assumption that the exposure to light might activate the expression of these pigments.

To date it has not been reported that the species *A. kielensis* produces pigments. But Allgaier et al (2003) reported that four strains (DFL-13, DFL-42, DFL- 43, DFL-44) isolated from dinoflagellates and possessing the PufLM genes were related to the species *A. kielensis*. The PufLM genes encode for the photosynthetic reaction center subunits L and M. Allgaier et al (2003) suggested that, although bacteriochlorophyll a production was not observed with the strains mentioned above, it might be induced in most bacterial strains possessing the PufLM genes, if the suitable conditions were found for incubation.

Today the strains DFL-13, DFL-42 and DFL-44 are classified to the genus *Ahrensia* while strain DFL-43 is classified as *Hoeflea phototrophica* DFL-43. Interestingly in a later publication (Pradella et al 2004), which was based on results of the former mentioned one (Allgaier et al 2003), the strain DFL-43 was described as producing bacteriochlorophyll a. Analysis of the genome of this strain revealed that it possesses a linear (~36 kbp) as well as a covalently closed circular plasmid (107 kbp) which both could only be detected weakly by PFGE. The PfuLM genes were located on the bacterial chromosome and not on extrachromosomal elements.

Accounting for the relationship between the strains mentioned above and *A. kielensis* it might be that the pigmentation found with VLPs of our bacterium is as well based on the presence of bacteriochlorophyll a. Further research is needed to determine for sure which pigment is causing the green coloured VLP-pellet of *A. kielensis*.

4.2 Electron microscopy

Electron microscopy revealed that VLPs of *A. kielensis* were about 80-155 x 100-200 nm in size. No capsid like structures were observed. As for both bacteriophages and GTAs the presence of a capsid is characteristic (see chapters 1.2.1 and 1.2.2), we assumed that it is very unlikely that VLPs of *A. kielensis* do represent bacteriophages or GTAs.

It is more likely that our VLPs either represent outer membrane vesicles (MVs; see chapter 1.2.3) or constitute an entirely new phenomenon which has not been described yet.

4.3 Test for antimicrobial activity of *A. kielensis* and its VLPs

The test for antimicrobial activity of *A. kielensis* and its VLPs was carried out against *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus subtilis*, *Bacillus cereus*, *Vibrio cholerae* and *Vibrio alginolyticus* several times using bacteria and products from *A. kielensis* cultures of various ages (379 h, 2014 h, 2251 h) following the disc diffusion method (see chapter 2.2.7). The desired effect (clear areas in the bacterial lawn around the charged filter discs) was only observed on *S. aureus* and *L. monocytogenes* which are

both gram positive bacteria, but not on *B. cereus* and *B. subtilis* (gram positive) and the gram negative bacteria *V. cholera* and *V. alginolyticus*. In general the observed effect was rather mild and there remains a slight chance that (in some cases) the changes in the lawn represent artefacts.

The observed antibacterial effect depended on the processed culture and the product used, indicating that the antimicrobial compounds were not expressed continuously during the growth of *A. kielensis*. Bruhn et al (2005) found that various factors influenced the production of an antimicrobial compound by a bacterium of the *Roseobacter* clade. The antibacterial compound was found only with high cell densities – indicating that quorum sensing might play a role in its production – and its presence was always accompanied by a brownish pigment. Temperature, aeration and salinity of the bacterial cultures were crucial for the detection of the antimicrobial compound. Therefore altering the growth conditions of *A. kielensis* might have positive implications for further tests.

The antimicrobial activity of *A. kielensis* and its VLPs was observed with the bacterium itself and with the sterile filtrate but not with the VLP-free supernatant, obtained from the ultracentrifugation and not with the concentrated VLP fraction. VLPs of different bacteria – including *A. kielensis* – have already been shown to exhibit a lethal effect on the recipient *E. coli* strain AB1157. According to Chiura (1997) the exposure of the recipient bacterium to VLPs of *A. kielensis* resulted in an efficiency of plating (EOP) of ~17%. Therefore it was quite surprising that no effect was observed around filters charged with the concentrated VLP fraction. This might have several reasons.

First of all we had no possibility to determine the concentration of the used VLP suspension. Chiura (1997) achieved a MOI (multiplicity of infection; the average number of infectious agents per cell) of 0,22 when incubating the recipient bacterium (*E. coli* AB1157) with VLPs of *A. kielensis*. It might be that the applied VLP concentration was too low. Especially regarding the fact that the *A. kielensis* cultures used for the test had not been sonicated. As mentioned before sonication was performed to disperse bacterial aggregates of *A. kielensis*, which we believe, to consist of bacterial cells and VLPs. Therefore it might well be that a significant portion of the VLPs was captured in the bacterial pellet and not contained in the used VLP suspension. Sonication was not

performed with cultures used for the antibacterial test to ensure the integrity of the used VLPs.

Furthermore Matsuyama et al (1986) found that the production of membrane vesicles with *Serratia marcescens* depended on the growth conditions of the bacterium. They observed the presence of extracellular vesicles only with cultures that were grown at 30 °C but not with cultures that were grown at 37 °C. Unfortunately due to various circumstances in our labratory it was not possible to keep the temperature and the oxidative conditions of the *A. kielensis* cultures stable. They varied between 26-31 °C and 120-200 rpm. This might as well have impact on the VLP production of *A. kielensis*, the success of VLP harvest and subsequently on the antibacterial test. The growth parameters chosen in our laboratory for the cultivation of *A. kielensis* also differed from those chosen by Chiura (1997: PPES II medium, incubation at 120 rpm and 25 °C for 100 h) and might contribute to the different results according to the lethal effect of VLPs of *A. kielenesis*. Again altering the growth conditions might positively affect the VLP production and subsequently the success of the antibacterial test.

Another possibility is that the absence of the antimicrobial effect of the VLP fraction is due to the pathogenic bacteria selected for this test. Beveridge (1999) noted that the lytic potency of outer membrane vesicles on other bacteria depended on the peptidoglycan layer type of the recipient bacteria. Although we do not know for sure of which type the envelope of our VLPs is, based on electron microscopy, it seems quite plausible that it is derived from the outer membrane of the producing gram negative bacteria. Li et al (1998) isolated membrane vesicles from 15 different gram negative bacterial strains and tested their lytic activity against 17 gram negative and gram positive bacteria with different cell wall composition. They confirmed the importance of the peptidoglycan chemotype and furthermore found that the capability of MVs to lyse other bacteria is given, as long as the recipient bacterium suffers from insufficient nutrition and is growing poorly. As mentioned above no antibacterial effect was observed on the two gram negative strains *V. cholera* and *V. alginolyticus*. But considering the findings of Beveridge (1999) and Li et al (1998) it might well be that *A. kielensis* and its products would exhibit the desired effect on other gram negative bacteria which have not been tested yet - particularly as a lethal effect has already been shown on the gram negative bacterium *E. coli* AB1157 (Chiura 1997).

In general the observed antimicrobial effect was rather mild and varied depending on processed *A. kielensis* culture and its products. Regarding the fact that a significant lethal effect of VLPs of *A. kielensis* against *E. coli* strain AB1157 has already been shown (EOP of ~17%, Chiura 1997) it seems rather implausible that the observed changes in the lawns of the tested pathogenic bacteria represent only artefacts. Instead it seems more conclusive that the protocol for the antibacterial test requires optimization. The conducted tests should be regarded as pre tests and their findings should be considered when optimizing the protocol for further tests. Concluding, the growth conditions of *A. kielensis* cultures should be altered in order to increase the VLP production and the production of antibacterial compound. The concentration of the VLP fraction should be determined – for example by electron microscopy – and if needed increased. Also the set of pathogenic bacteria might be altered and standardization of their cultivation by preparation of a glycerol stock might be useful.

Further studies are needed to determine the optimal age of the *A. kielensis* culture, at which it reaches its full antibacterial potential.

4.4 VLP DNA

4.4.1 Extraction of VLP DNA

DNA extracted from VLPs of *A. kielensis* was on average about 40 kbp in length although variations were observed (see Fig 13 and Fig 14). Therefore it exceeds by far the average length of DNA encapsulated in so far described gene transfer agents (GTAs; see chapter 1.2.2) which is typically < 14 kbp in length (Lang et Beatty 2007).

For DNA extracted from membrane vesicles (MVs; see chapter 1.2.3) of gram negative bacteria in some cases no precise length is given. MVs of *N. gonorrhoeae* were reported to harbour linear DNA molecules of unidentified length and circular plasmids which were 4,2 kbp, 7,1 kbp and 36 kbp long (Dorward et al 1989). Even longer, heterogenous chromosomal DNA was also found to be associated with MVs but unfortunately their length was not given in the publication by Dorward et al (1989).

Yaron et al (2000) analysed the DNA content of DNAse treated MVs of *E. coli* O157:H7 via electron microscopy and found linear DNA, circular plasmid DNA as well as large rosette like structures. The length of the rosette like structures had not been estimated by Yaron et al (2000) but based on the electron micrographs shown they should be several 100 kbp long.

With some but not all samples (see Fig 14) an additional DNA fraction of about 2-4 kbp was observed. DNA fractions of various sizes were found within membrane vesicles of *E. coli* O157:H7 (Yaron et al 2000) and *N. gonorrhoeae* (Dorward et al 1989). But in both cases the smaller fractions resulted in distinct bands of a specific size during gel electrophoreses and were believed to represent small circular plasmids. The additional DNA fraction of about 2-4 kbp might be due to the uptake of degraded, extracellular DNA which might be derived from lysed cells or simply due to DNA degraded by the experimental conditions. Renelli et al (2004) reported that MVs of *P. aeruginosa* PAO1 were able to take up exogenous DNA. The same was reported with MVs of *H. influenzae* and *H. parainfluenzae* (Kahn et al 1982).

In general VLP DNA extraction was more often successful when the processed culture was sonicated before the harvest of VLPs was started. As mentioned before *A. kielensis* is an aggregate forming bacterium. We hypothesised that these aggregates consisted of bacterial cells as well as of VLPs and that a significant portion of the VLPs was not accessible for the particle harvest and subsequent DNA extraction, until these aggregates were dispersed by sonication. The obtained results confirmed this hypothesis.

Nevertheless DNA extraction from VLPs of some cultures was not successful although they had been sonicated. One possible reason might be that the VLP production varied during the growth of *A. kielensis*. As mentioned before it was shown for membrane vesicles of *S. marcescens* that they were only produced under certain growth conditions, namely, when the bacterium was grown at a certain temperature (Matsuyama et al 1986). A glycerol stock was prepared, aliquoted and used for inoculation especially to ensure standardized conditions of the grown *A. kielensis* cultures. But unfortunately due to various incidences in our laboratory it was neither possible to keep the temperature nor the oxygenic conditions always constant. The cultures were grown at 26-31 °C and 120-200 rpm. Both parameters are crucial for bacterial growth. This might explain why with

VLPs of some cultures DNA extraction was not successful although the cultures had been sonicated. And it might also explain why with VLPs of different cultures which were of the same age and treated identically (sonicated or not sonicated) DNA extraction was once successful and once not.

Another explanation for this phenomenon would be that the VLPs were produced constantly but that the content of the VLPs varied. Kolling and Matthews (1999) investigated membrane vesicles of *E. coli* O157:H7 and noted that the DNA content within the vesicles varied, but they didn`t further explain or discuss their observation.

4.4.2 Origin of VLP DNA

To determine the origin of the DNA encapsulated with VLPs of *A. kielensis* some clones of the VLP shot gun library (see chapter 3.10.2) were sequenced. Analyses of the obtained sequences using the program blastx revealed that the VLP DNA was of bacterial origin, as no viral sequences were identified so far. Three sequences, namely that of clone 9, clone 33 and clone 41 could be identified as eukaryotic ones, which makes future research even more promising.

The fact that no viral DNA could be detected until now once more underscores that our VLPs do not represent phages. It has already been shown by electron microscopy that no capsid like structures were associated with VLPs of *A. kielensis*. But some dsDNA phages, for example those belonging to the Plasmaviridae family (Ackermann 2003), do not possess a capsid. Nevertheless our sequencing results exclude the possibility that the examined VLPs represent such phages.

It is known, that the DNA of gene transfer agents (GTAs) is also of bacterial origin. As described in chapter 1.2.2 GTAs are virus like particles that only carry random pieces of the bacterial donor genome. Their genome is relatively small (< 14 kb; Lang et Beatty 2007) in contrast to the genome of *A.kielensis* VLPs (about 40 kbp). Furthermore the described GTAs resemble small, tailed phages - a structure which was not observed with our VLPs.

Membrane vesicles (MVs) of some gram negative bacteria, including *N. gonorrhoeae*, *P. aeruginosa* PAO1 and *E. coli* O157:H7, were reported to contain DNA beside of proteins (Dorward et al 1989, Renelli et al 2004, Kolling and Matthews 1999, Yaron et al 2000).

The DNA from membrane vesicles of *E. coli* O157:H7 turned out to be very heterogenous. Yaron et al (2000) demonstrated via electron microscopy that they contain linear DNA fragments, circular plasmids and large rosette like structures and analyzed by PCR that this vesicle DNA was of chromosomal, plasmid and even viral origin.

4.4.3 Conclusions

We found that the VLPs of *A. kielensis* contain mostly large dsDNA molecules (about 40 kbp) of bacterial and not of viral origin. Three eucaryotic sequences could be identified.

It is possible that the VLPs of *A. kielensis* represent a form of DNA containing outer membrane vesicles but it is also possible that VLPs constitute an entirely novel phenomenon.

The VLPs of *A. kielensis* could be correlated with an antimicrobial activity – which components are responsable for this will show the future research work.

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Abstract

The virus like particles (VLPs) of our interest are – for the most part – released by marine bacteria by budding, They contain large dsDNA (~20-500 kbp) molecules of unknown origin and are capable to mediate gene transfer to the recipient *E.coli* AB1157. Furthermore VLPs of different bacteria – including those of *A. kielensis* – exhibited a significant lethal effect on the recipient bacterium.

To gain a deeper insight into this so far only purely described and understood phenomenon, we cultivated the marine bacterium *Ahrensia kielensis*. We used a cell free fraction of its VLPs to determine the origin of the DNA encapsulated within these particles and to test whether VLPs showed an antibacterial effect on different pathogenic bacteria.

A method for DNA extraction from VLPs was established and the obtained DNA used to construct a shotgun library. Partially sequencing revealed that the main fraction of the VLP DNA of *A. kielensis* was of bacterial and not of viral. These findings will have crucial implications on the design of further research projects, especially on those which will focus on elucidating the mechanisms underlying the capability of VLPs to mediate gene transfer or to induce an antibacterial activity.

The test for antibacterial activity was carried out several times against *Staphylococcus aureus, Listeria monocytogenes, Bacillus subtilis, Bacillus cereus, Vibrio cholera* and *Vibrio alginolyticus. A. kielensis* cultures of different ages and their products were used. Antagonistic activities were detected against *S. aureus* and *L. monocytogenes* but not against the other four pathogenic bacteria. In general the observed effect was only mild and varied depending on the *A. kielensi*s culture used. Considering the fact that a significant lethal effect has already been observed against *E. coli* AB1157, we do believe that a comparably strong effect will be shown against other pathogenic bacteria which have not been tested yet.

Accounting for the rapid increase and spread of antibiotic resistant bacteria, VLPs might be of high medical and pharmaceutical significance, although their antibacterial effect against the so far tested pathogenic bacteria was only mild. Further research will be needed to find out under which parameters and against which bacteria VLPs of *A. kielensis* can exhibit the full extent of their antibacterial potency.

Zusammenfassung

Die in dieser Arbeit untersuchten "virus like paricles" (VLPs) werden zum Großteil von marinen Bakterien freigesetzt, ohne dabei die Zelllyse des produzierenden Bakteriums zu verursachen. Sie beinhalten lange doppelsträngige DNA-Moleküle (~20-500 kbp) und sind in der Lage, genetische Information auf *Escherichia coli* AB1157 zu übertragen. Weiters können diese VLPs einen milden letalen Effekt auf das Rezipientenbakterium ausüben.

Um einen tieferen Einblick in dieses bisher kaum beschriebene und verstandene Phänomen zu erlangen, haben wir das marine Bakterium *A. kielensis*, von dem wir wussten, dass es VLPs produziert, kultiviert. Wir haben eine zellfreie Fraktion von VLPs verwendet, um die Herkunft der in den Partikeln eingeschlossenen DNA zu bestimmen und zu testen, ob sie eine antibakterielle Wirkung auf verschiedene pathogene Bakterien ausüben.

Eine Methode zur DNA-Extraktion aus VLPs wurde etabliert und die gewonnene DNA eingesetzt, um eine "Shotgun Library" zu erstellen. Die partielle Sequenzierung zeigte, dass die Partikel-DNA bakteriellen und nicht viralen Ursprungs ist. Dieses Ergebnis wird entscheidenden Einfluss auf das Design weiterer Forschungsprojekte haben, vor allem auf jene, die es zum Ziel haben werden, die Mechanismen aufzuklären, auf denen die Fähigkeit von VLPs zum Gentransfer und ihrer möglichen antimikrobiellen Aktivität beruht.

Der Test auf antibakterielle Wirkung wurde mehrere Male durchgeführt. Dabei wurden *A. kielensis* Kulturen verschiedenen Alters und ihre Produkte verwendet und gegen *Staphylococcus aureus, Listeria monocytogenes, Bacillus subtilis, Bacillus cereus, Vibrio cholera* und *Vibrio alginolyticus* getestet. Die antagonistische Aktivität wurde nur gegen *S. aureus* und *L. monocytogenes* beobachtet, aber nicht gegen die anderen vier pathogenen Bakterien. Im Allgemeinen war der beobachtete Effekt nur schwach ausgeprägt und variierte abhängig davon, welche *A. kielensis* Kultur verwendet wurde. Unter Berücksichtigung der Tatsache, dass ein letaler Effekt bereits gegen *E. coli* AB1157 beobachtet wurde, glauben wir, dass sich ein vergleichbar starker Effekt auch bei anderen, bisher noch nicht getesteten pathogenen Bakterien zeigen wird.

Berücksichtigt man die schnellen Zunahme und Verbreitung von antibiotika-resistenten Bakterien, dürften VLPs von hoher medizinischer und pharmazeutischer Bedeutung sein, obwohl ihr antibakterieller Effekt gegen die bisher getesteten Bakterien nur schwach ausgeprägt war. Weitere Forschung ist notwendig, um herauszufinden unter welchen Bedingungen und gegen welche Bakterien VLPs von *A. kielensis* ihre volle antibakterielle Wirksamkeit entfalten können.

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