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Charakterisierung potentiell pathogener *Vibrio* spp. mittels *rpoB*-DHPLC (Denaturierender Hochdruck Flüssigkeitschromatographie)

von

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angefertigt im Lehrstuhl für Evolutionsökologie und Biodiversität der Tiere in Kooperation mit dem Alfred-Wegener-Institut, Helmholtz-Zentrum für Polar- und Meeresforschung

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Characterization of potentially pathogenic *Vibrio* spp. by *rpoB*-DHPLC (Denaturing High Performance Liquid Chromatography)

by

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Abstract

Rising sea water temperature due to global warming enhances the conditions for *Vibrio* spp. to grow and disperse even in temperate waters of the North and Baltic Sea. Because of the increased incidence of Vibrio infections in the last years, a rapid and accurate method is required to analyze and identify complex *Vibrio* spp. populations, specifically potential pathogenic *Vibrio* species, in environmental samples. A PCR-DHPLC (Denaturing High Performance Liquid Chromatography) has been developed based on the *rpoB* gene of the genus Vibrio, which is a promising method to not only identify but also separate *Vibrio* spp. in mixed samples due to their different running characteristics of amplified PCR products.

To facilitate the identification of potential human-pathogenic species we designed Vibrio specific primers based on *rpoB* sequences of *Vibrio* spp. strains isolated at Helgoland Roads (North Sea). These primers were combined to amplify fragments of 100-400 bp of this *rpoB* gene. Using the PCR products of four different *Vibrio* species, we systematically improved the DHPLC conditions, including column temperature and acetonitrile gradient. Finally, we compared the PCR-fragment separation with and without a 40-bp clamp attached to the amplification primers.

We developed primer-sets for *rpoB*-DHPLC and verified the primer-sets by successful amplification of 20-23 different *Vibrio* species from a total number of 31. We showed that for optimal identification and separation of the amplified fragments by DHPLC adaptation of column temperature, acetonitrile gradient as well as attachment of GC-clamp to the respective primer was essential. Furthermore, we proved that the *rpoB*-DHPLC assay is a sensitive tool to differentiate between *Vibrio* species, but is limited for Vibrio isolates with a similar GC-content. We investigated that not only the amount of variability in the *rpoB*-gene is the discriminative factor for separation in the DHPLC-system, also the GC-content, amplicon-length and melting-domains play are determining for optimal differentiation of *Vibrio* species**.** In conclusion, *rpoB*-DHPLC is a promising perspective to identify and separate *Vibrio* spp. in mixed samples, considering primer-sets meeting the findings described above.

1. Introduction

1.1. The genus Vibrio and its pathogenicity

The name Vibrio derives from the Italian physician Filippo Pacini who discovered *Vibrio cholerae* (*V. colerae*) in 1854 (Thompson, Iida et al. 2004). Bacteria of the genus Vibrio belong to the *Gammaproteobacteria*, are gram-negative, primarily facultative anaerobe curved rods, found in aquatic habitants and in association with eukaryotes (Thompson, Iida et al. 2004; Farmer, Janda et al. 2005). The genus Vibrio is a highly diverse group with about 63 species, of which 13 are described as human pathogen species (Eiler and Bertilsson 2006; Eiler, Johansson et al. 2006) including the species *V. cholerae*, the causative agent for the cholera disease. Infections caused by the pathogen *V. cholerae* are of major importance in developing countries with a low standard of sanitary conditions. The recent annual report of "The World Health Organization" (2011) noticed at least 589.854 worldwide cholera infections including 7816 deaths in a total of 58 countries (http://www.who.int/wer). The two most important human pathogenic *Vibrio* spp. besides *V. cholera* are *V. parahaemolyticus* and *V. vulnificus*. *V. parahaemolyticus*, for instance, is worldwide the causative agent for foodborne gastroenteritis outbreaks (Yeung and Boor 2004) caused by ingestion of raw or undercooked seafood as well as contact with contaminated sea water. Infections with *V. vulnificus* are much rarer but can cause wound infections or primary septicemia most notably in immunocompromised persons (Thompson, Iida et al. 2004; Drake, DePaola et al. 2007). The Centers for Disease Control and Prevention reported that bloodstream infections induced by *V. vulnificus* are fatal about 50 % of the cases (Prevention 1996).

In addition to human illnesses, Vibrio can also affect marine organisms, e.g. bivalves, particularly oyster, fish, shrimp or corals. Coral bleaching have increased dramatically since the last few decades and leads to coral reef degradation worldwide. However, it has been discovered that of eight pathogens associated with coral diseases, four belong to the family of *Vibrionaceae* (Kushmaro, Banin et al. 2001; Ben-Haim, Thompson et al. 2003).

Rising Vibrio infections in humans and massive infections of bivalve-populations have already been observed in the past in connection with an increasing growth rate during the summer months (Hsieh, Fries et al. 2007; Paz, Bisharat et al. 2007; Blackwell and Oliver 2008). Due to the worldwide trade, shipping lanes and fishery industry and in terms of global warming a higher rate of Vibrio infections are predictable.

1.2. How climate change correlates with the abundance of *Vibrio* species

Warming of the climate system is unambiguous showing records of increased global air and ocean temperatures, widespread melting of glaciers and ice caps, and rising average sea level. Global average temperatures have increased by nearly 0.8 °C and the mean sea surface temperatures (SST) by approximately 0.7 °C since the late nineteenth century (Trenberth 2007). Also a rapid warming of European Seas has been observed, with the Baltic, North, and Black Seas showing the greatest increasing temperatures (Fig. 1). A long term series, carried out by the Alfred-Wegener Institute for Polar and Marine research, even showed significantly risen sea surface temperatures by a mean value of 1.67 °C since 1962 for Helgoland Roads (Wiltshire, Malzahn et al. 2008). In addition to the rising temperatures a reduction in salinity in estuaries and coastal wetlands is expected (Hakkinen 2002). Furthermore, significant changes in the German Bight for light and nutrient conditions have been detected over the last 45 years (Wiltshire, Kraberg et al. 2010).

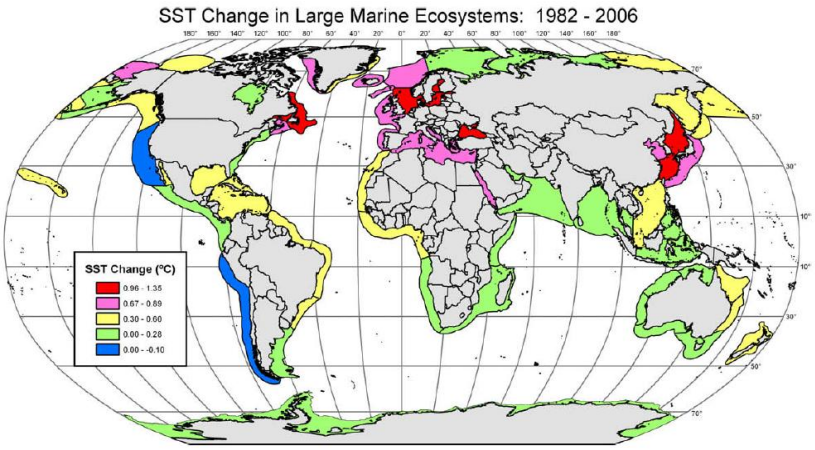

Fig. 1: Net Sea Surface Temperature change (°C) in Large Marine Ecosystems, 1982-2006. Rapid warming (red and pink) is among other areas observed in the European Seas (Belkin 2009).

These global changes will definitely influence the marine ecological system (Harley, Randall Hughes et al. 2006). For example, increasing sea water temperatures are in important factor in the proliferation of many microbial mediated infectious diseases (Baker-Austin, Stockley et al. 2010) and changes in bacterial community structures also might influence the population dynamics of *Vibrio* species (Vibrio spp.). Typically, the abundance of pathogenic *Vibrio* spp. is reported for warmer regions, but in consequence to the rising sea temperatures potentially pathogenic *Vibrio* spp. could become significant even in temperate zones, like the North and Baltic Sea (Colwell 1996; Paz, Bisharat et al. 2007; Baker-Austin, Stockley et al. 2010).

1.3. Distribution and spread of potentially pathogenic *Vibrio* spp.

Pathogenic *Vibrio* spp. are widely distributed in estuarine and marine environments, commonly reported in the USA and in many Asien countries, but also become significant in European waters (Baker-Austin, Stockley et al. 2010). The highest concentration of pathogenic Vibrio is generally stated in the summer months when water temperatures are rising (Hsieh, Fries et al. 2007; Paz, Bisharat et al. 2007; Blackwell and Oliver 2008). A previous long-term study of Oberbeckmann and coworkers (2011) correspondingly demonstrates a positive correlation of the occurrence of *Vibrio* spp. with the rising seasonal sea surface temperatures for the German Bight.

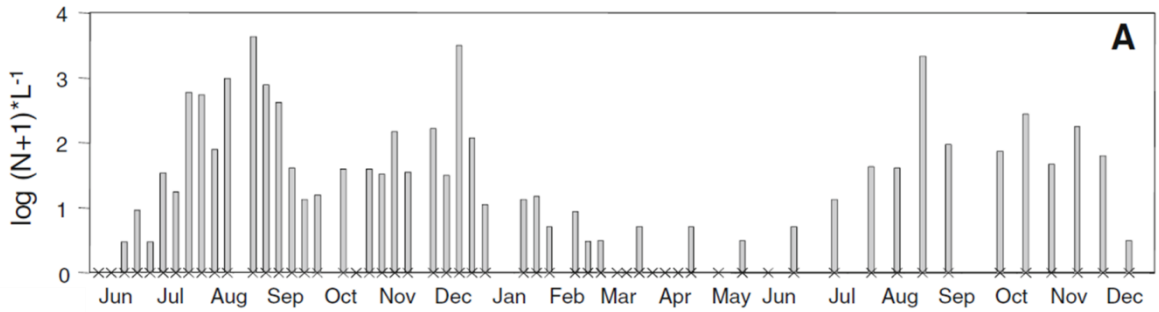

Fig. 2: Log-transformed abundances of free-living *Vibrio* spp. between May 2008 and December 2009; crosses indicate sampling days (Oberbeckmann, Wichels et al. 2011)

In addition to rising temperature, other abiotic and biotic factors have been described influencing the abundance of Vibrios, like salinity, nutrient concentrations, and the presence of other aquatic organisms such as plankton. However, temperature and salinity have been recognized as the major predictive factors in Vibrio occurrence (Randa, Polz et al. 2004; Hsieh, Fries et al. 2007; Wetz, Blackwood et al. 2008), correlating with the fact that *Vibrio* spp. grow preferentially in warm (>15 °C), low-salinity (<25 ppt NaCl) sea water (Baker-Austin, Stockley et al. 2010).

The worldwide globalization is also a central factor, which promotes the spread of pathogenic *Vibrio* spp.. The highly pathogenic *V. parahaemolyticus* pandemic clone O3:K6 caused outbreaks of sever gastroenteritis worldwide. It was first detected in South Asia (Okuda, Ishibashi et al. 1997) and spread from there to the United States (Daniels, Ray et al. 2000), America (Gonzalez, Cachicas et al. 2005; Cabanillas-Beltrán, LLausás-Magaña et al. 2006), Africa (Ansaruzzaman, Lucas et al. 2005) and even reached Europe (Martinez-Urtaza, Lozano-Leon et al. 2004). Quite recently, isolation of the pandemic *Vibrio parahaemolyticus* from Pacific oysters, harvested in Southern England has been reported (Powell, Baker-Austin et al. 2013). This finding highlights the expanding geographical distribution of highly

pathogenic *Vibrio* spp. and the current need to develop a fast and reliable monitoring approach to detect potentially pathogenic Vibrio strains in environmental samples. Because of the clinical relevance of pathogenic Vibrios for humans and marine organisms, and the current distribution trend to northern latitudes, this thesis concentrates on identification and differentiation of potentially pathogenic *Vibrio* spp. by PCR-DHPLC-protocol, which can be used to monitor relative abundance of Vibrio communities in marine waters.

1.4. Identification of *Vibrio* spp.

Currently, many different approaches exist to identify and characterize Vibrios from environmental samples. A classical approach to identify members of the *Vibrionaceae* family is the application of physiological tests, e.g. the analytical profile index (API) 20E for pure cultures. But an accurate identification of *Vibrio* spp. with this method is problematic, because of the great variability in biochemical characteristics described for this bacterial group (Thompson, Iida et al. 2004). Another standard method is the cultivation on selective agar. Two agars are presently applied for selective cultivation and quantification of *Vibrio* spp.. TCBS Agar (Thiosulfate Citrate Bile Salts Sucrose Agar) is regularly known for isolation of pathogenic Vibrios and is highly selective for the isolation of *V. cholerae* and *V. parahaemolyticus* (Kobayashi, Enomoto et al. 1963). More recently, a chromogenic agar is used for a sensitive differentiation of major pathogenic *Vibrio* species (Hara-Kudo, Nishina et al. 2001). But sole application of selective agar for the identification of potentially pathogenic strains is limited, because of the cultivation of mixed samples, including bacteria besides the *Vibrionacea* family as well as the missing detection of pathogenicity factors.

In addition, molecular methods based on the 16S rRNA gene were established for identification and characterization of potentially pathogenic Vibrio strains. An evaluation of different molecular methods was recently accomplished at the department of Microbial Ecology of the Alfred-Wegener-Institute Helgoland (Oberbeckmann, Wichels et al. 2011). The analysis included classification of environmental Vibrio isolates by mass spectrometry fingerprinting (MALDI-TOF), where species differentiation is based on the protein composition, as well as screening for virulence associated genes *toxR, tdh* (thermostable direct hemolysin) and *trh* (tdh-related hemolysin) (Bauer and Rorvik 2007) and repetitive sequence-based PCR (Hulton, Higgins et al. 1991; Versalovic, Koeuth et al. 1991) to identify intraspecific variability of *V. parahaemolyticus* and *V. alginolyticus.*

Another approach for accurate identification of potentially pathogenic Vibrios is the sequencing of the 16S rRNA- and *rpoB*-gene (encoding for the β-subunit of the RNA polymerase). 16 S rRNA sequencing is well established and essential for confident identification of bacterial isolates (Woo, Lau et al. 2008). Also comparison of 16 S rRNA sequences is standardized since sequence information of *Vibrio* spp. is widely represented in public databases. More recently, *rpoB*-gene analyses emerged as an essential method for phylogenetic analyses and identification of bacteria, especially in studying closely related isolates (Adekambi, Drancourt et al. 2009). *RpoB* sequence analysis was the first time defined by Mollet et al. in 1997 as a new powerful procedure for bacterial identification compared to the typically used 16S rRNA gene analysis (Mollet, Drancourt et al. 1997). A recent study of Ki et al. corroborates the hypothesis that *rpoB* is a potential biomarker to overcome the high conservation of the 16S rRNA and emphasize its discriminating power in identifying Vibrios (Ki, Zhang et al. 2009). In this connection also Oberbeckmann and coworkers (2011) could show, that *rpoB* sequence analysis is a more reliable method to distinguish even closely related *Vibrio* species (*V. alginolyticus* and *V. paraheamolyticus*). But generally, 16 S rRNA and *rpoB* sequencing is still cost and time intensive.

A fast and cost-efficient alternative for identification of bacterial strains is provided by the denaturing gradient gel electrophoresis (DGGE) and DHPLC. Via DGGE and DHPLC it is possible to differentiate between bacterial species in mixed samples (Muyzer, Waal de et al. 1993; Goldenberg, Herrmann et al. 2007). Using these methods, DNA-fragments with similar lengths, but different sequence polymorphism can be separated. Both methods are highly sensitive and were successfully applied for the analyses of complex microbial communities based on the 16 S rRNA (Muyzer, Waal de et al. 1993; Goldenberg, Herrmann et al. 2007; Wagner, Malin et al. 2009). In this study, we evaluated the first PCR-DHPLC approach targeting *rpoB*-gene fragments for detection and separation of potentially pathogenic *Vibrio* spp. in environmental samples.

1.5. Characterization of *Vibrio* spp. by *rpoB*-DHPLC (*Denaturing High Performance Liquid Chromatography*)

Denaturing high performance liquid chromatography is a relatively new, analytical method for identification, separation and monitoring of bacterial communities. It has been primarily used for detection of genetic mutations, such as insertions, deletions or single nucleotide polymorphism (SNPs) in human clinical diagnostics (Barlaan, Sugimori et al. 2005). But

more recently, DHPLC-protocols were adapted for the field of microbial ecology to differentiate bacterial species in mixed cultures or environmental samples based on genetic sequence variations in the 16 S rRNA gene (Goldenberg, Herrmann et al. 2007) . In this study we used DHPLC to differentiate between closely related *Vibrio* species based on the genetic variability of the *rpoB*-gene, encoding the ß-subunit of RNA polymerase. However, separation of the PCR-amplified *rpoB* fragments in DHPLC is based on the elution of partially melted DNA molecules. Likewise all chromatographically methods, this ion-pair reverse-phase chromatography consists of a stationary phase, composed of polystyrene beads, and a mobile phase, which contains triethylammonium acetate (TEAA) and acetonitrile (ACN). As a first step, the sample comprising the PCR products is injected into the flow path containing TEAA and ACN (Fig.3). To induce the partial denaturation of the PCR fragments, a specific temperature is set in the oven. During the flow path, the hydrophobic portion of the TEAA interacts with hydrophobic beads in the cartridge (Fig. 3). Then, the negatively charged phosphate backbone of the partially melted DNA fragment is getting attached to the positively charged ammonium group of the TEAA. An increasing concentration of acetonitrile reduces the TEAA/DNA attraction and the fragments elute off the cartridge (Fig. 3).

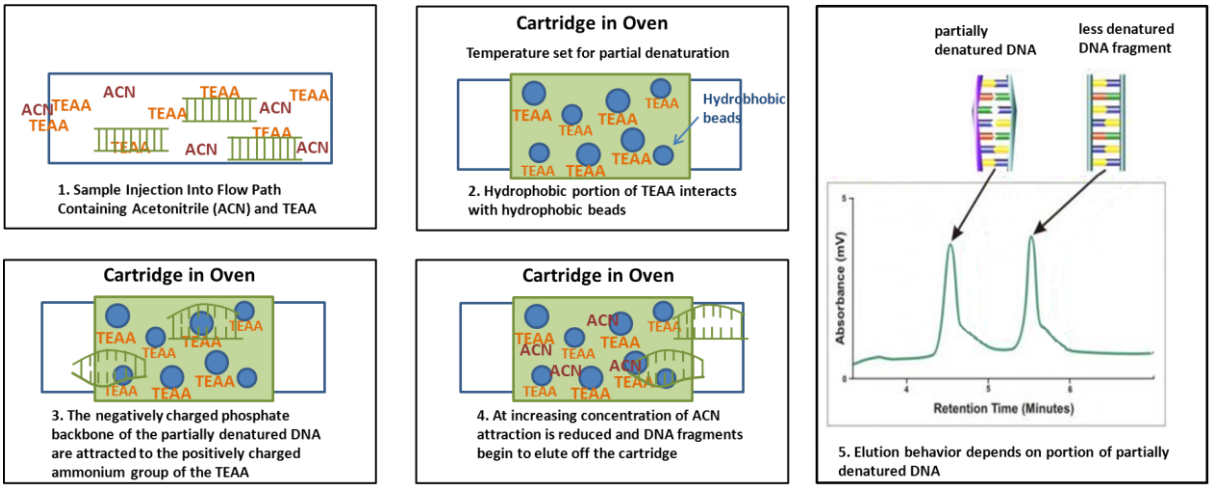

Figure 3: Principle of the DHPLC-system (modified after http://www.transgenomic.com)

Different elution behavior is based on internal base pair sequence variants and melting domains. At a semi denaturing temperature condition, DNA show structural differences in the helical fraction - fragments with a greater portion of partially denatured DNA elute earlier than more stable fragments (Fig. 3). The ammonium groups of the TEAA interacts preferably with the negatively charged phosphate of double-stranded DNA and so binding to the column

is more constant (Barlaan, Sugimori et al. 2005). The released DNA fragments pass through the UV Detector, which measures the DNA-concentration in the elution-buffer by absorbance at 260 nm. Sending data to the software program, fragments are represented by peaks in a chromatogram (Fig. 3).

In this study we developed a new *rpoB*-DHPLC protocol, including primer evaluation, optimizing PCR conditions and adaptation of DHPLC temperature and separation gradient for the profiling of Vibrio communities and species identification.

1.6. Research Aims

Rising sea water temperatures due to global warming enhances the conditions for potential pathogenic *Vibrio* spp. to grow and disperse even in temperate waters of the North and Baltic Sea. Because of the increased incidence of Vibrio infections in the last years, a fast and cost-efficient method is required to analyze and identify complex *Vibrio* spp. populations, specifically potential pathogenic *Vibrio* species, in environmental samples. Therefore, the aim of this master thesis is to develop a PCR-DHPLC-assay based on the *rpoB*-gene of the genus Vibrio, which is a promising method to not only identify but also separate *Vibrio* spp. in mixed samples due to the different running behavior of amplified PCR products in the DHPLC-system. For this purpose, following tasks were defined:

1. Development of Vibrio-specific primer-sets, targeting different regions of the *rpoB*-gene.
2. Optimizing PCR conditions with the *rpoB*-primer-sets for DNA amplification of different *Vibrio* species.
3. Optimizing DHPLC conditions by adaptation of column temperature and acetonitrile gradient for optimal separation of *Vibrio* species.

2. Material and Methods

2.1. Material

2.1.1. Equipment and consumables

Equipment	Description	Manufacturer
Autoclave	Systec VX-75	Systec GmbH
Autoclave	Systec VX-150	Systec GmbH
Centrifuge	Centrifuge 5417 R	Eppendorf
Centrifuge	Centrifuge 5430	Eppendorf
Clean bench	Heraeus HERAsafe KS	Kendro GmbH
Clean room bench	Mars Safety Class 2	Scanlaf
Deep freezer		Liebherr
DHPLC-System	Wave System 4500	Transgenomic
Electrophor gel chamber		Peqlab
Electrophor gel tray and comb		Peqlab
Gas burner	Fuego SCS basic	WLD-Tec GmbH
Incubator	MIR-253	Sanyo
Incubator	MIR-553	Sanyo
Microbank		Pro-Lab Diagnostics
Microchip Electrophoresis System	MCE-202 MultiNA	Shimadzu
Microtiter Spectrophotometer	Infinite M200	Tecan Austria GmbH
Microwave		Samsung
Molecular Imager	Chemidoc™ XRS	Biorad
Nanopure water system	Milli-Q A10	Millipore
PCR-Thermo cycler	Mastercycler gradient	Eppendorf
pH meter	Microprocessor pH meter	Nfl. GmbH
Pipettes	2 µl – 5 ml	ABIMED Gilson
Precision balance	LE 225 D	Sartorius
Scale	BP 6100	Sartorius
Stirring hot plate	MR 3002 IKAcombimag	Heidolph MGH
Thermo mixer	Thermomixer comfort	Eppendorf
Ultrasonic bath	Sonorex Super RK514	Bandelin
Refrigerator		Bosch
Vortex	K-550-GE	Bender & Hobein
Water bath		Köttermann

Consumables	Description	Manufacturer
Inoculation loop	Inoculation loop 1 µl	Sarstedt
Microwell Plate		Costar
PCR-tubes	0.5 ml PCR soft tubes	Biozym
PCR-tubes	0.2 ml PCR soft tubes	Biozym
PCR-tubes	0.2 ml 8-strip tubes	Eppendorf
Petri dishes		Omnilab
Pipet tips	200 µl, 1000 µl	Brand
Pipet tips	10 µl	Gilson
Reaction vessel	2 ml	Eppendorf
Reaction vessel	0.5 ml, 1,5 ml	Sarstedt

2.1.2. Chemicals

Chemicals	Manufacturer
Agar	Merck
Agarose	Biomol
Bacillol 25	Sigma
Bacto-Peptone	Difco
Bromphenole blue	Merck
Chloroform	Merck
dNTP-Mix 100 mM (dATP, dCTP, dGTP, dTTP)	Promega
DNA-Sizer II peqGOLD	Peqlab Biotechnologie GmbH
Ethanol absolute	Merck
Ethylenediaminetetraacetic acid (EDTA)	Calbiochem
GelRed Nucleic Acid Gel Stain 3 x in water	Biotium
Hydrochloric acid (HCl)	Merck
Isopropyl alcohol	Merck
Iron(III)phosphate ($FePO_4$ x $4H_2O$)	Merck
Magnesium chloride [$MgCl_2$]	5 Prime
Phenol	Qbiogene
Sodium chloride (NaCl)	Sigma
Sodium dodecyl sulfate (SDS)	Merck
Sucrose	
Taq buffer (10x)	Eppendorf
Tris ultra pure	Biomol
Water (nanopure)	Millipore
Yeast Extract	Difco

2.1.3. Complete Systems

DHPLC	Wave DNA Sizing Control (Transgenomic)
	Wave High-Range Mutation Marker (Transgenomic)
	Wave Low-Range Mutation Marker (Transgenomic)
	Wave Optimized TEAA-Buffer A, aqueous solution of 0.1 M TEAA, pH 7.0 (Transgenomic)
	Wave Optimized TEAA-Buffer B, aqueous solution of 0.1 M TEAA, pH 7 with 25% (v/v) acetonitrile (Transgenomic)
	Wave Optimized Solution D, 75% acetonitrile and 25% water (Transgenomic)

Multi-NA	Cleaning Solution (Shimadzu)
	DNA Ladder 500 bp (Invitrogen)
	DNA Ladder 1000 bp (Promega)
	DNA Separation Buffer 500/1000 (Shimadzu)
	Marker Solution DNA-500/DNA-1000 (Shimadzu)
	GelStar (Lonza)
	SYBR® Gold (Invitrogen)

2.1.4. Culture Media

Marine Broth 2216 (modified)	5 g Peptone 1 g Yeast Extract 15 g Agar 0.01 g $FePO_4$

The powder was suspended in 500 ml distilled water and 500 ml sea water, heated with frequent agitation and boiled till the powder was completely dissolved. The pH value was adjusted to 7.6 and autoclaved at 121°C for 20 minutes.

2.1.5. Buffer

SDS-Tris-EDTA	20 mM EDTA 50 mM TrisHCl 20 % (w/v) SDS pH8 sterile filtrated
STE Buffer	1 mM EDTA 50 mM Tris ultra pure 6.7 % Sucrose pH 8 sterile filtrated
TAE Buffer (50 x)	2 M Tris ultra pure 1 M Acetic acid 50 mM EDTA

Tris-EDTA	250 mM EDTA 50 mM Tris HCl pH 8 sterile filtrated
5 x Loading Buffer	0.25 % Bromphenole blue Sodiumsalt 50 % Glycerol 50 mM Tris ultra pure pH 7.9 sterile filtrated

2.1.6. Enzymes

Lysozyme, 10 mg/ml	Sigma
Taq Polymerase 2 U/µl	Eppendorf

2.1.7. Software

Software	**Manufacturer**
ARB (a software environment for sequence data)	(Ludwig, Strunk et al. 2004)
AlignIR™ 2.0	LI-COR Biosciences
Clustal X	(Larkin, Blackshields et al. 2007)
Geneious® 5.6.5	Biomatters
i-control 1.4 (for NanoQuant Plate)	Tecan Austria GmbH
MEGA5 (Molecular Evolutionary Genetics Analysis)	(Tamura, Peterson et al. 2011)
MultiNA Control Software 1.6	Shimadzu
MultiNA Viewer 1.6	Shimadzu
Quantity One	BioRad
SigmaPlot	Systat Software, Inc.
SVARAP (Sequence VARiability Analysis Program)	(Colson, Tamalet et al. 2006)
Wave Navigator Software	Transgenomic

2.1.8. Oligonucleotides

Table 1: Primers and GC-clamp used in this thesis

Primer	Sequence (5´to 3´)	Position (5´to 3´)		Primer length (bp)	Reference
*rpoB*458F	aggcgtgttcttcagcagcgataa	0	24	24	Hazen *et* al. (2009)
PrV295F	ttacgttgagcaaggtcgtcg	296	317	21	this study
PrV295R	cgacgaccttgctcaacgtaa	296	317	21	this study
PrV592F	tttctgcattggtag	592	607	15	this study
*rpoB*1110F	gtagaaatctaccgcatgatg	603	624	21	Tarr *et* al. (2007)
*rpoB*1110R	catcatgcggtagatttctac	603	624	21	Tarr *et* al. (2007)
PrV610F	tctaccgcatgatgcgccct	610	630	20	this study
PrV610R	agggcgcatcatgcggtaga	610	630	20	this study
PrV973R	gtcttgaggcataa	973	987	14	this study
PrV1023F	tctttggctcttcacag	1023	1040	17	this study
PrV1023R	ctgtgaagagccaaaga	1023	1040	17	this study
PrV1053F	tttatggaccagaac	1053	1068	15	this study
PrV1245F	tttgcgcgttgtaacgagtacg	1245	1267	22	Ki *et* al.(2009), qVb-F1731 modified in this study
PrV1245R	cgtactcgttacaacgcgcaaa	1245	1267	22	Ki *et* al.(2009), qVb-F1731 modified in this study
PrV1358F	tatcgctcaggcgaacg	1358	1375	17	this study
PrV1358R	cgttcgcctgagcgata	1358	1375	17	this study
PrV1423F	cgtcagaaaggtgaatygg	1423	1442	19	this study
PrV1423R	ccrattcacctttctgacg	1423	1442	19	this study
PrV1506R	aacgggataagcgatgc	1506	1523	17	this study
*rpoB*2105R	cggctacgttacgttcrataccag	1618	1642	24	Hazen *et* al. (2009)

GC-clamp	Sequence (5' to 3')	Reference
40-bp GC-clamp	CgCCCgCCgCgCCCCgCgCCCggCCCgCCgCCCCCgCCCC	Muyzer *et* al. (1993)

2.1.9. Bacterial Strains

Table 2: Vibrio strains

Strains	Vibrio Net No.	Geographical Location	Owner	Strain ID
Vibrio aestuarianus	VN2866	Germany, Baltic Sea	AWI	RE-78
Vibrio agarivorans	VN3809	Spain, Mediterranean Sea	DSMZ	DSM-13756
Vibrio alginolyticus I	VN2514	Germany, Helgoland Roads	AWI	SO14
Vibrio alginolyticus II	VN2756	Germany, Helgoland Roads	AWI	SO202
Vibrio navarrensis	VN2580	Germany, Helgoland Roads	AWI	SO92
Vibrio cholerae	VN0255	Germany, Baltic Sea	LAGUS	MV 66c2
Vibrio cholerae	VN3132	Denmark, Kattegat	AWI	HE-32
Vibrio cholerae	VN3405	Germany, North sea	KLIWAS	AU-105
Vibrio coralliilyticus	VN3810	Indian Ocean near Zanzibar	DSMZ	DSM-19607
Vibrio diazotrophicus	VN3811	Canada, Nova Scotia	DSMZ	DSM-2604
Vibrio ezurae	VN3812	Japan	DSMZ	DSM-17533
Vibrio fluvialis	VN3176	Denmark, North Sea	AWI	HE-76
Vibrio fortis	VN3813	Ecuador	DSMZ	DSM-19133
Vibrio gazogenes	VN3814	-	DSMZ	DSM-21264
Vibrio gigantis	VN3815	France	DSMZ	DSM-18531
Vibrio harveyi	VN3801	-	DSMZ	DSMZ 2165
Vibrio kanaloae	VN3817	France	DSMZ	DSM- 17181
Vibrio mediterranei	VN3819	Spain	DSMZ	DSM- 19502
Vibrio mimicus	VN3505	Germany, North Sea	KLIWAS	AU-209
Vibrio navarrensis	VN3826	Germany	DSMZ	DSM- 15800
Vibrio pacinii	VN3820	China, Laizhou	DSMZ	DSM-19139
Vibrio parahaemolyticus	VN2502	Germany, Helgoland Roads	AWI	SO2
Vibrio parahaemolyticus	VN3321	Germany, North Sea	KLIWAS	AU-21
Vibrio parahaemolyticus	VN3858	England, English Channel	CEFAS	V06/002
Vibrio parahaemolyticus	VN3933	Germany, Baltic Sea	LAGUS	43M1c2
Vibrio superstes	VN3823	Australia	DSMZ	DSM- 16383
Vibrio tasmaniensis	VN3824	Australia, Tasmania	DSMZ	DSM-17182
Vibrio vulnificus	VN0232	Germany, Baltic Sea	LAGUS	MV 9a3
Vibrio vulnificus	VN3114	Denmark, Skagerrak	AWI	HE-14
Vibrio vulnificus	VN3378	Germany, North Sea	KLIWAS	AU-78
Vibrio xuii	VN3825	China, Dahua	DSMZ	DSM-17185

Table 3: Reference group

Strain	Genus	Class
Zo20	Alteromonas	Gammaproteobacteria
Ex11	Ruegeria	Alphaproteobacteria
Ex7	Roseobacter	Alphaproteobacteria
Ex9	Psychroserpens	Bacteroidetes
Zo12	Pseudoalteromonas	Gammaproteobacteria

2.1. Methods

2.2.1. Molecular Comparison of Vibrio sequences

An approximately 1600 bp *rpoB* fragment was amplified with the primers *rpoB*458F and *rpoB*2105R according to Hazen *et* al. (2009). Sequencing of the partial *rpoB*-gene was performed with primers *rpoB*458F, *rpoB*2105R and *rpoB*1110F (Tarr, Patel et al. 2007) using the chain-determination method developed by Sanger and colleagues (1977). All partial DNA *rpoB* sequences were assembled with the AlignIR^{TM} Software 2.0.

Phylogentic comparison was conducted with all available 24 Vibrio *rpoB*-sequences based on the Vibrio isolate set used in this work (see chapter 2.1.9.). We constructed an alignment using Geneious Multiple Alignment (2.1.7.) with the default settings for gap open penalty and extension penalty. The hyper-variable sites at the 5´and 3´ends of the *rpoB* sequences were excluded from the alignment. J-modeltest was used for statistical selection of the best-fit model of nucleotide substitution for tree construction provided by MEGA 5 (Tamura, Peterson et al. 2011). The phylogentic tree was inferred using the neighbor joining algorithm with the Tamura-Nei distance model in MEGA, supported by bootstrap analysis with 1.000 replications.

2.2.2. Development of Vibrio *rpoB*-targeting PCR primers

To facilitate the identification of potential human-pathogenic species, we designed Vibrio specific primers using the Primer design tool of the ARB software package (2.1.7.). We created primers targeting different regions of the partial *rpoB*-gene, which is described as a core gene to differentiate even closely related *Vibrio* species (Ki, Zhang et al. 2009; Oberbeckmann, Wichels et al. 2011).

First, a phylogenetic tree was constructed with a total number of 174 full length *rpoB* sequences of six different *Vibrio* species (*V. alginolyticus I, V. alginolyticus II, V. parahaemolyticus, V. harveyi, V. mimicus, V. cholera* and *V. vulnificus*) and a reference group including next relatives outside of the Vibrionacea family. These sequences were provided by a representative Vibrio data base assembled by the department of Microbial Ecology of the Alfred-Wegener-Institute Helgoland. The database consists of all published *rpoB* sequences of the γ-proteobacteria group provided by GenBank in 2011 (National Center for Biotechnology Information, NBCI).

In the next step, all possible primer sequences that are specific for Vibrios were identified and potential primer sequences which contain self-complementary regions with more than three nucleotides were excluded. The default parameters were modified by choosing primer-lengths

of 14-25 bp, amplicon-sizes of 100-450 bp, GC ratio of 10-60 % and annealing-temperatures of 30-80 °C.

The specificity of new primers and those selected from literature (see chapter 2.1.8.), was tested with the Basic Local Alignment Search Tool (BLAST) algorithm. Further, the melting temperatures were calculated by considering the oligonucleotide lengths and GC-contents according to the formula $T_m = \ G + C \ x4 + \ A + T \ x2$, where $G + T$ describes the GC-content and $A + T$ describes the AT-content. This formula is specifically appropriate for primers with a length of about 20 bp and for this reason applicable to the designed primers (Mülhardt 2009). Melting temperature is defined as the temperature at which 50 % of the DNA region is in a double stranded configuration (Ririe, Rasmussen et al. 1997).

To ensure the discrimination of species of Vibrio strains used in this work (2.1.9.), the binding behavior of the selected primers and the amount of variability bordered by these primer-sets was tested. To this end, a variability map was created with a tool for quantitative analysis of nucleotides (SVARAP; 2.1.7.). All existing sequences were aligned using Geneious Multiple Alignment (2.1.7.) with the default settings for gap open penalty and extension penalty. For the entropy plot, only the conserved regions of the alignment were used and additionally converted in GDE format with Clustal X (2.1.7.). The SVARAP tool computed automatically the absolute number of different nucleotides and their frequencies (in percentage) for each nucleotide position in the alignment in correspondence to the consensus sequence. The formula for variability was constructed by Colson and coworkers (2006) as follows:

$$100 - \ maximum\ frequency\ for\ each\ of\ the\ four\ nucleotides\ at\ a\ given\ position$$

Finally, the highest and lowest variability in the *rpoB*-gene was visualized using SigmaPlot (2.1.6.).

2.2.3. Cultivation

To maintain Vibrio strains in a growth phase condition, bacterial colonies were transferred every second week to marine broth agar (2.1.4.) and incubated at 37 °C for 24 hours. Colonies of overnight cultures were used for further DNA extraction occasionally.

Generally, Vibrio cultures were stored in liquid nitrogen in a microbank-storage system. The microbank sterile vials contain porous beads, which serve as carriers to support microorganisms. For recovery of stored cultures, an inoculated bead was transferred to solid marine broth medium (2.1.4) and incubated over night at 37 °C.

2.2.4. DNA extraction

Total genomic DNA was isolated by lysozyme/SDS lysis and phenol-chloroform extraction followed by isopropanol precipitation, using a modified protocol of Anderson and McKay omitting the DNA-DNA denaturation step (Anderson and McKay 1983). An inoculation loop of overnight bacterial culture was homogenized in 380 µl STE Buffer and supplemented with 100 µl Lysozyme (10 mg/ml). After an incubation step at 37 °C for 30 minutes, cells were disintegrated by adding 50 µl Tris-EDTA and 30 µl SDS-Tris-EDTA and incubated again at 50 °C for 60 minutes. Subsequently, lysed cells were extracted by addition of 700 µl phenol-chloroform (1:1) and 70 µl sodium chloride (5 M). After centrifugation at 10 600 x g for 20 minutes, the supernatant containing the nucleic acid was transferred to a new sterile tube and precipitated on ice with one volume isopropyl alcohol. Samples were stored over night at -20 °C. After an additional centrifugation at 10 6000 x g for 20 minutes, the supernatant was discarded. The dry pellet was dissolved in 30 µl H_2O and stored at -20 °C.

2.2.5. Quantification and analysis of genomic DNA

For DNA quantification and quality control of DNA-products, we used Tecan invinite M200 including a NanoQuant Plate and the Tecan i-controll 1.4 software (2.1.1.). Average concentration of DNA was determined in a spectrophotometer by measuring the absorbance at 260 nm. The purity of DNA was verified by comparing the ratio of absorbance values 260 nm to 280 nm. Generally, a ratio between 1.6 and 2.2 is accepted for pure DNA.

In addition, agarose gel electrophoresis was used for size-dependent separation of DNA-products. The negatively charged nucleic acid molecules move in an electric field from cathode to anode, whereat small molecules can move more quickly through the gel than larger ones. By using Gel-Red (2.1.2), which intercalates into the major groove of DNA, molecules are visualized under UV-light.

Therefore, 0.8 % agarose gels were prepared in 1 x TAE buffer. For each DNA sample a 25 µl approach was mixed including 18 µl PCR- H_2O, 5 µl Loading Buffer and 2 µl DNA. Additionally, 10 µl DNA-Sizer II was added for size depending correlation. Separation of genomic DNA was achieved by electrophoresis at 80 V for 60 minutes. After staining with GelRed (3 x in water) for 30 minutes, DNA was visualized under UV-light (602 nm). Digitale images were taken using the ChemiDoc XRS system within the software Quantity One.

2.2.6. Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is defined as a technique to amplify DNA-fragments with a distinct length (Saiki, Gelfand et al. 1988). In the first step of the reaction, the targeted double

stranded DNA is heated at 94 °C – 98 °C for denaturation into two single strands. In the second step, forward and reverse primers anneal to their complementary sequence on each template. Hybridization takes place at temperatures between 50 °C and 70 °C. Finally, the DNA polymerase extends the primer using the providing nucleotides in 5´to 3´direction. By replication of the three thermal cycles (denaturation, annealing, elongation) millions of copies of DNA sequence are generated. In this study, PCR was performed for amplification of different regions of the *rpoB*-gene of potentially pathogenic *Vibrio* spp.

2.2.6.1. Vibrio specific PCR

For evaluation of Vibrio specific primer sets, which amplify fragments of different amplicon sizes, PCR conditions were optimized for each set individually. Optimal annealing temperature and Mg^{2+} concentration were therefore determined in a gradient PCR. According to the melting temperature of the primer sets, annealing conditions were categorized in three different gradient groups (see chapter 3.4.1., table 11). Commonly, annealing temperatures of approximately 5 °C below the melting temperatures are used (Innes 1990).

DNA of four Vibrio isolates was selected as template: *V. alginolyticus I* (VN2514), *V. alginolyticus II* (VN2756), *V. parahaemolyticus* (VN2502), *V. navarrensis* (VN2580) (see chapter 2.1.9.). Generally, PCR was carried out in a 25 µl reaction mix using optimized conditions listed below (see table 4). In order to test possible contaminations, a negative control was accompanied in each run.

Table 4: PCR reaction mix of a 25 µl approach

PCR reaction mix	25 µl approach [µl]
Taq Puffer	2.5
dNTP [10 mM]	1.5
Primer1 [20 µM]	0.4
Primer2 [20 µM]	0.4
$MgCl_2$ [25 mM]	1/2
Taq [5 Units]	0.3
Template [50 ng]	1
Water	adjust to 25

Gradient PCR was performed on a Thermo Mastercycler (see chapter 2.1.1.) with a specific cycling program for amplification of *rpoB* fragments:

Table 5: *rpoB*-program for gradient PCR

Step	Time	T [°C]	Cycles
1. Melting	3'	95	1
2. Melting	30"	95	
3. Annealing	30"	T_a G = 10°C	25
4. Extension	30"	68	
5. Extension	5 '	68	1
6. Hold	hold	5	1

After adaptation of annealing temperature and $MgCl_2$ concentration for standard-PCR (see chapter 3.4.1., table 12), a touchdown-PCR was used for 1 primer-set with attached GC-clamp to minimize nonspecific products and increase amplicon concentration (Barlaan, Sugimori et al. 2005). For the first synthesis cycles, annealing temperatures of 5 °C above optimal annealing temperatures for standard-PCR were chosen with a gradient of G = 10 °C. The annealing temperature was then decreased for every subsequent step of cycles (see table 6).

Tabelle 6: *rpoB*-program for touchdown-PCR

Step	Time	T [°C]	Cycles
1. Melting	5'	94	1
2. Melting	1'	94	
3. Annealing	1'	T_a G = 10 °C (-0.5/-0.3 per cycle)	20
4. Extension	1'	68	
5. Melting	1'	94	
6. Annealing	1'	T_a	12
7. Extension	1'	68	
8. Extension	6'	68	1
9. Extension	1'	67 (-5 °C per cycle)	3
10. Hold	hold	5	1

Subsequently, DNA of all Vibrio isolates (2.1.9.) was amplified with optimal annealing temperature and $MgCl_2$ concentration (see chapter 3.4.1., table 12), following the PCR programs (without gradient) previously described.

2.2.7. Microchip electrophoresis

Amplified PCR fragments were quantified and sized on the MultiNA MCE-202 microchip electrophoresis system (see chapter 2.1.1.). The MultiNA system uses microchip technology to facilitate capillary electrophoresis for nucleic acid analysis. In this study, PCR products

were analyzed using the DNA 500 and the DNA 1000 kit from Shimadzu (see chapter 2.1.3.). For detection of PCR products, microchips are getting filled with a Separation Buffer containing SYBR Gold or Gel Star (see chapter 2.1.3.) for detection of nucleic acids. In an electric field the negatively charged DNA moves from cathode to anode. Separation occurs size-depending in microseparation channels. To point the beginning and end of a run, a marker solution was additionally applied to the microchips. Besides, a DNA-Ladder (500/1000) was added for a precise detection of the fragment size in bp. DNA fragments were identified with a LED fluorescence detector and visualized in a virtual gel using the MultiNa control software 1.6.

2.2.8. DHPLC conditions

PCR products of Vibrio isolates were analyzed by DHPLC on a WAVE DNA Fragment Analysis System using the DNASep® technology (see chapter 2.1.1.). Separation of DNA fragments is based on the principle of a two buffer system, forming the mobile phase. Buffer A consists of an aqueous solution of 0.1 M TEAA, pH 7.0, and Buffer B consist of an aqueous solution of 0.1 M TEAA, pH 7 with 25% (v/v) acetonitrile. During the flow path, the DNA molecules are getting attached to the hydrophobic beads of the cartridge by interaction with Buffer A. Due to the increasing concentration of acetonitrile in Buffer B, fragments elute off the cartridge at different retention times depending on the portion of partially denatured DNA at semi denaturing temperature conditions.

Separation of DNA fragments by DHPLC is influenced by different factors such as column temperature, acetonitrile gradient, and pump flow rate. Separated PCR products were recorded and visualized with a UV-detector L-2400 at a wave length of 260 nm. The analyses of the chromatograms were accomplished with the Navigator Software (see chapter 2.1.7.). After each injection run, cleaning of the column was performed with Buffer D, containing 75 % acetonitrile and 25 % water.

We injected 10 µl PCR product of four *Vibrio* species; *V. alginolyticus I* (VN2514), *V. navarrensis* (VN2580), *V. parahaemolyticus* (VN2502) and *V. vulnificus* (VN3114) (see chapter 2.1.9.) separately on the DHPLC at a pump flow rate of 0.9 ml min^{-1} and systematically improved the conditions with two selected primer-sets. Optimization included empirical variation of column temperature between 55 °C – 70 °C and adaptation of the acetonitrile gradient using two different buffer programs (see table 7-8). Finally, we modified the PCR protocol by attachment of a 40 bp GC-clamp at the 5´end of the respective primer-set

and performed a touchdown-PCR to test the retention characteristics of the four amplicons on the DHPLC.

Table 7: DHPL fractional gradient for separation of *Vibrio* species

Gradient name	Time (min)	Buffer A (%)	Buffer B (%)
Loading	0	55	45
100 bp	0.5	50.2	49.8
225 bp	3.6	41.8	58.2
350 bp	6.8	38.2	61.8
475 bp	9.9	36.3	63.7
600 bp	13	35	65
Start Clean	13.1	0	100
Stop Clean	13.6	0	100
Start Equilibrate	13.7	55	45
Stop Equilibrate	14.6	55	45

Table 8: DHPLC linear gradient with Buffer B 0.5% min^{-1} for separation of *Vibrio* species

Gradient name	Time (min)	Buffer A (%)	Buffer B (%)
Loading	0	55	45
100 bp	2	46	54
600 bp	14	40	60
Start Clean	14.1	0	100
Stop Clean	14.6	0	100
Start Equilibrate	14.7	55	45
Stop Equilibrate	15.6	55	45

Additionally, sequences of the *rpoB* alignment were cropped at the 5´ and 3´ position of primer-binding points to obtain only the amplicon region. Then, predicted optimal column temperature and melting profiles of amplicon sequences were estimated using the WAVE maker Software from Transgenomic.

Finally, we compared sequence properties of *rpoB* amplicons with the peak profile in the DHPLC, including estimation of GC-content of each fragment and determining nucleotide distances between each pair of amplicons.

3. Results

3.1. Molecular Comparison

Recent studies postulate a reliable classification and discrimination of even closely related Vibrio isolates using *rpoB* sequencing (Ki, Zhang et al. 2009; Oberbeckmann, Wichels et al. 2011). After verification of *rpoB* sequences with AlignIR^{TM} for quality control and BLAST for sequence comparison, 24 of the original 31 Vibrio isolates were used for molecular evaluation. A phylogenic NJ tree was constructed in MEGA 5 from the aligned and assembled *rpoB* sequences (1560 bp), including 24 sequences of *Vibrio* species and one sequence of *Alteromonas* spp. (see chapter 2.2.1.). *Alteromonas* spp. could be phylogenetically discriminated from the *Vibrionacea* cluster. The NJ tree exhibits clear relationships among species as well as high discrimination between species. Even closely related species, e.g. *V. parahaemolyticus* and *V. alginolyticus* were clearly distinguished.

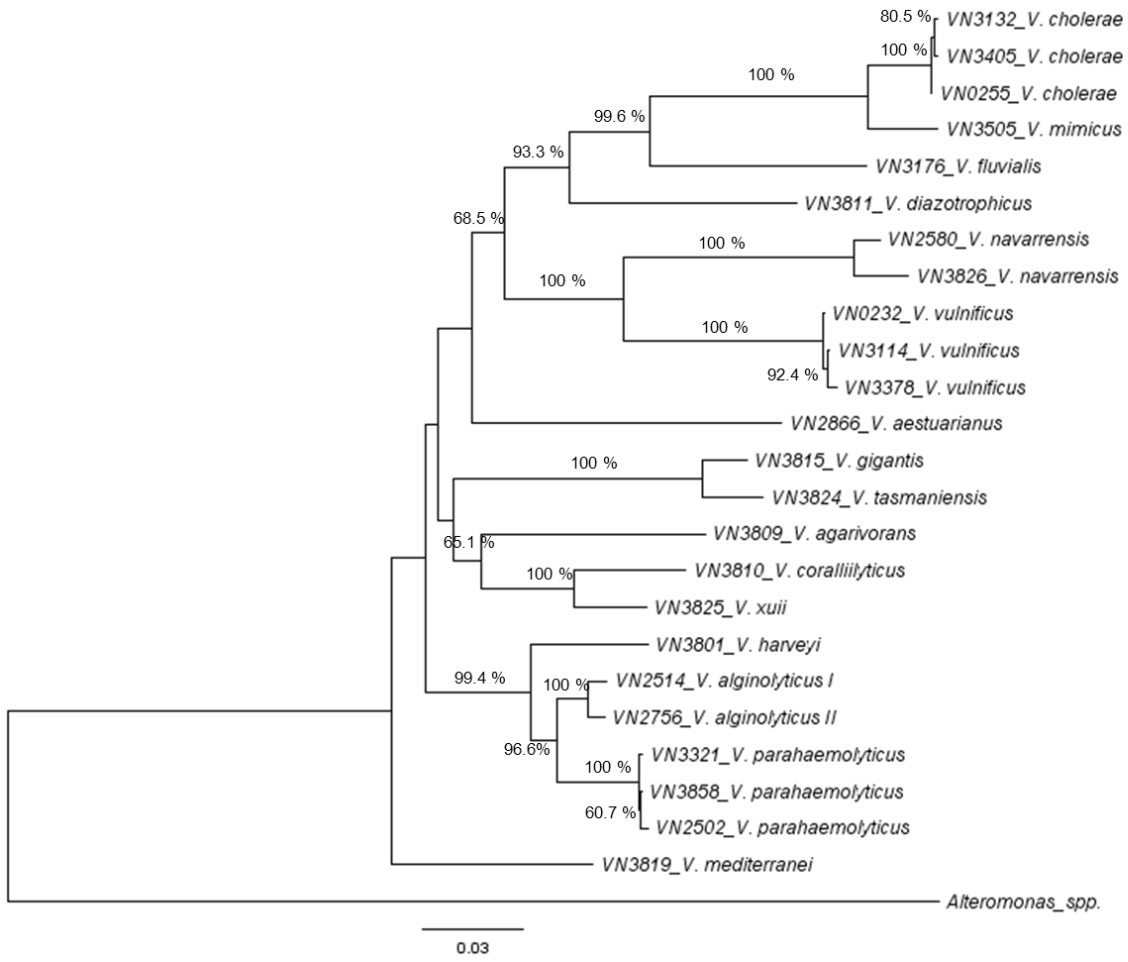

Figure 4: Neighbor-joining tree constructed in MEGA 5.1 calculated from 25 aligned *rpoB* sequences (1560 bp) using Tamura-Nei distance with the gamma model (G=0.513). The numbers on the tree indicate bootstrap values from 1.000 replications; only values greater than 50 % are shown. Branch lengths are proportional to the scale given.

3.2. Design of Vibrio specific *rpoB*-primers

For evaluation of a new *rpoB*-DHPLC protocol, 14 group-specific primers based on the genus Vibrio were successfully developed using the Primer tool of the ARB software and additionally, six published primers were chosen from literature (see chapter 2.1.8.). The designed primers and the primer combinations target different regions of the partial *rpoB*-gene to ensure exposure of polymorphic areas (Fig. 5).

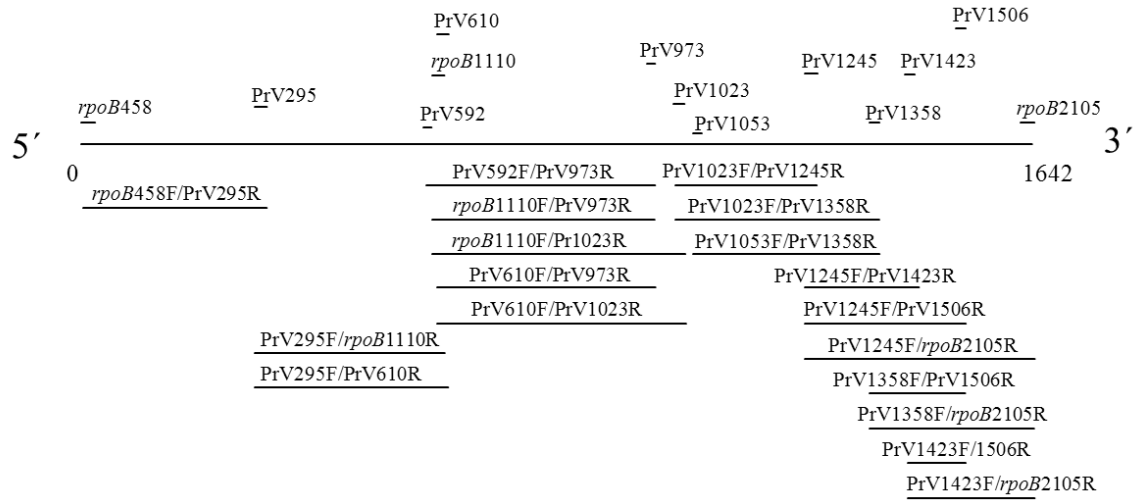

Figure 5: Partial *rpoB*-gene (1642 bp from 5´to 3´position) and primer binding sites. The *rpoB*-gene was amplified with primers *rpoB*458F and *rpoB*2105R according to Hazen *et* al. (2009).

All primers selected for this study fulfill the criteria of a GC-content between 40-60 %, primer-lengths of 14-24 bp and melting-temperatures between 40-73 °C. Furthermore, potential hairpin formation and potential self- annealing sites could be excluded. Overall, 18 primer-sets were chosen for PCR-analysis with similar melting-temperatures and amplicon-lengths of 100-400 bp.

Table 9: Primer and Primer-set facilities

Primer-sets		**Primer length (bp)**		**Primer GC-content (%)**		**Primer T_m (°C)**		**Amplicon size (bp)**
Forward	Reverse	Forward	Reverse	Forward	Reverse	Forward	Reverse	
*rpoB*458F	PrV295R	24	21	50	52	72	64	317
PrV295F	*rpoB*1110R	21	21	52	43	64	60	328
PrV295F	PrV610R	21	20	52	60	64	64	334
PrV1245F	PrV1423R	22	19	50	53/58	66	59	197
PrV1245F	*rpoB*2105R	22	24	50	50/54	66	73	397
PrV1358F	*rpoB*2105R	17	24	59	50/54	54	73	284
PrV1423F	PrV1506R	19	17	53/58	53	59	52	100
PrV1423F	*rpoB*2105R	19	24	53/58	50/54	59	73	219
PrV1023F	PrV1245R	17	22	47	50	50	66	244
PrV1023F	PrV1358R	17	17	47	59	50	54	352
PrV1245F	PrV1506R	22	17	50	53	66	52	278
PrV1358F	PrV1506R	17	17	59	53	54	52	165

*rpoB*1110F	PrV973R	21	14	43	43	60	40	384
PrV610F	PrV973R	20	14	60	43	64	40	377
PrV610F	PrV1023R	20	17	60	47	64	50	430
*rpoB*1110F	PrV1023R	21	17	43	47	60	50	437
PrV592F	PrV973R	15	14	40	43	42	40	395
PrV1053F	PrV1358R	15	17	33/40	59	41	54	322

To check the annealing behavior of the selected primer-sets for the Vibrio strains used in this work, a variability plot was drawn (Fig. 6). Therefore, an alignment of all available 24 Vibrio sequences was constructed using Geneious Multiple Alignment (see chapter 2.2.2.), which served as basis for variability analysis. Our investigation showed that the full-length *rpoB*-gene is a mosaic of conserved and variable regions (Fig. 6). Representative, four primer-sets are displayed in the map. The primer binding sites indicate positions of a low variability, while the targeted region is polymorphic (Fig. 6). Analyzing the variability in detail, 12 of the collectively 20 primers point highly conserved regions with a mean variability of 1.0-9.9 %, five primers target average conserved regions with a mean variability of 10-13 % and one primer target relatively polymorphic regions with a mean variability of 15 % (see table 10). Two primers do not match the alignment and could not be analyzed. The mean variability of the fragments, amplified by the different primer-sets, ranges between 8.5-12.7 %.

Table 10: Identification of the mean variability of primers and amplicons in the *rpoB* alignment of 24 Vibrio sequences. Positions of primers were calculated using Geneious pairwise alignment. Mean variability was calculated with the SVARAP-tool.

Primer-sets		**Position (5´- 3´)**				**Mean variability primer binding site (%)**		**Mean variability amplicon (%)**
Forward	Reverse	Forward		Reverse		Forward	Reverse	
*rpoB*458F	PrV295R	-*	-*	265	286	-	11.5	-
PrV295F	*rpoB*1110R	265	286	572	593	11.5	1.0	11.7
PrV295F	PrV610R	265	286	579	599	11.5	1.9	10.7
PrV1245F	PrV1423R	1214	1236	1390	1410	8.5	10.7	12.7
PrV1245F	*rpoB*2105R	1214	1236	-**	-**	8.5	-	-
PrV1358F	*rpoB*2105R	1372	1344	-**	-**	9.1	-	-
PrV1423F	PrV1506R	1390	1410	1475	1492	10.7	10.5	11.4
PrV1423F	*rpoB*2105R	1390	1410	-**	-**	10.7	-	-
PrV1023F	PrV1245R	996	1013	1214	1236	8.3	8.5	8.5
PrV1023F	PrV1358R	996	1013	1372	1344	8.3	9.1	9.6
PrV1245F	PrV1506R	1214	1236	1475	1492	8.5	10.5	12.5
PrV1358F	PrV1506R	1372	1344	1475	1492	9.1	10.5	13
*rpoB*1110F	PrV973R	572	593	942	956	1.0	14.9	10.4
PrV610F	PrV973R	579	599	942	956	1.9	14.9	10.6
PrV610F	PrV1023R	579	599	996	1013	1.9	8.3	10.6
*rpoB*1110F	PrV1023R	572	593	996	1013	1.0	8.3	10.4
PrV592F	PrV973R	561	576	942	956	7.8	14.9	10.4
PrV1053F	PrV1358R	1022	1037	1372	1344	9.4	9.1	9.5

Remark: * Primer does not match the alignment at 5´position **Primer does not match the alignment at 3´position

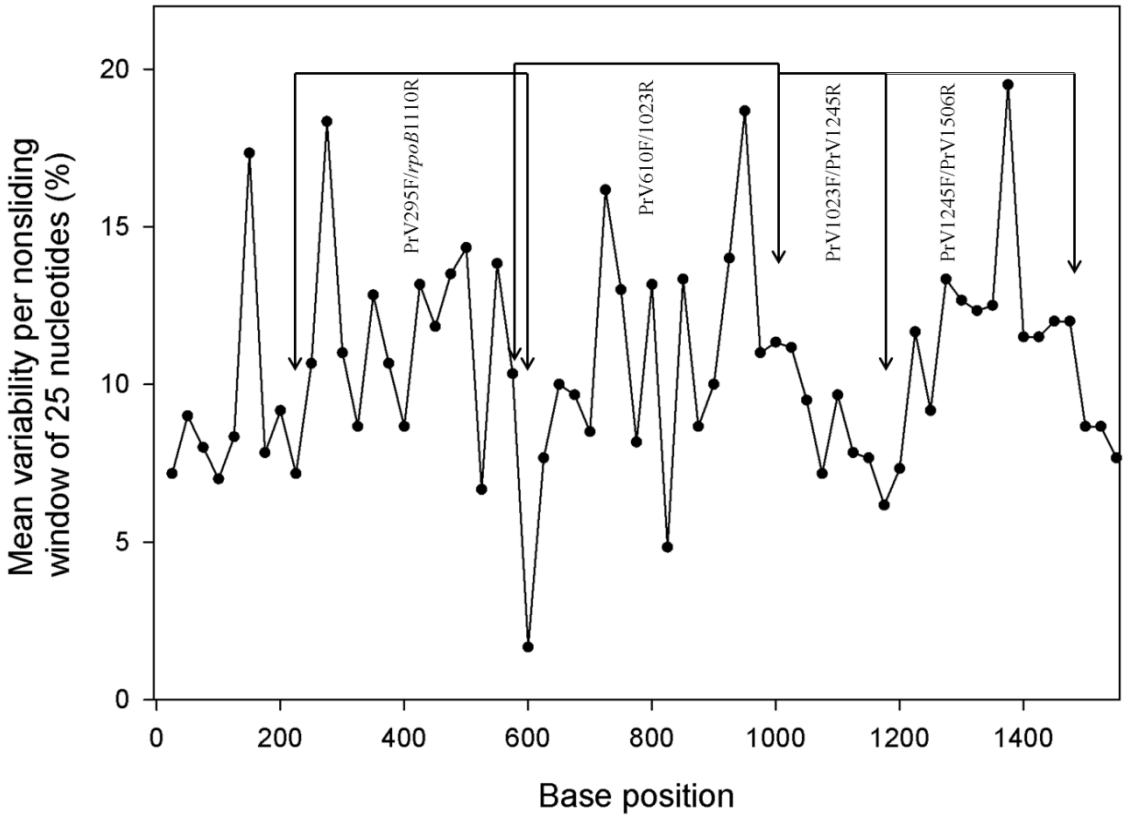

Figure 6: Identification of variable and conserved regions of *rpoB*-gene in *Vibrio* spp.. Mean variability based on the alignment of 24 Vibrio *rpoB* sequences per non-sliding windows of 25 nucleotides was calculated with the SVARAP-tool (Colson et al., 2006) and transferred to SigmaPlot. Arrows indicate primer binding sites of four primer-sets exemplarily.

For successful discrimination of Vibrios in *rpoB*-DHPLC, primers must target conserved nucleotide regions bordering highly variable areas. The identification of primer binding positions and amplicon variability were compromised to determine the best primer-combinations for PCR-DHPLC.

3.3. DNA Extraction

To utilize Vibrio specific primers for further PCR and DHPLC analyses, verification of the primer-sets by PCR was necessary. Therefore, genomic DNA of a total of 31 Vibrio strains and five reference strains (2.1.9.) was isolated according to a modified protocol of Anderson and McKay with the phenol-chloroform method (see chapter 2.2.4.). Figure 7 shows exemplarily an agarose gel of DNA products isolated from seven Vibrio strains. Concentration of total genomic DNA varied between 50 – 2000 ng/µl. For PCR-analyses, the DNA was diluted in water to a final concentration of 50 ng/µl. To assess the purity of nucleic acids, the absorbance at 280 nm and 260 nm was measured. For all isolated DNA samples, the ratio ranked between 1.8 and 2.1.

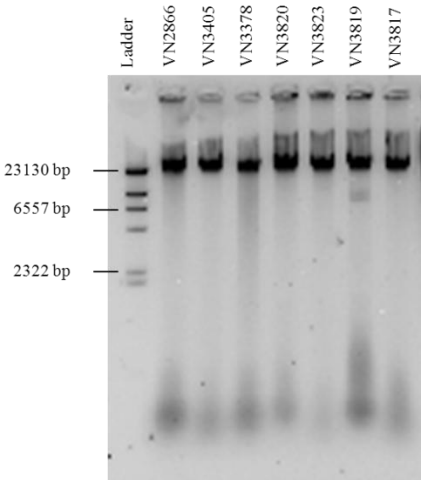

Figure 7: Representative agarose gel (0.8 %) showing genomic DNA of seven Vibrio isolates signified by their VN-No. Ladder: peqGOLD DNA-Sizer II. Genomic DNA produces bands at approximately 23 kb. Running conditions: 80 V, 60 min.

3.4. PCR

3.4.1 Optimization of PCR parameters

For specific amplification of different regions of the *rpoB*-gene, PCR conditions were optimized including variation of annealing temperature and $MgCl_2$ concentration. A gradient PCR served to determine the optimal annealing temperature for all tested primer-sets. According to the melting temperature of 18 primer sets, annealing conditions were categorized in three different gradient groups (see table 11). Theoretically, annealing temperatures should be selected approximately 5 °C below the considered melting temperature of the primer.

Table 11: Selected annealing temperatures for gradient PCR

primer-sets		**T_m (°C)**		**T_a (°C)**
Forward	Reverse	Forward	Reverse	gradient conditions
*rpoB*458F	PrV295R	72	64	50-65 °C
PrV295F	*rpoB*1110R	64	60	
PrV295F	PrV610R	64	64	
PrV1245F	PrV1423R	66	59	
PrV1245F	*rpoB*2105R	66	73	
PrV1358F	*rpoB*2105R	54	73	
PrV1423F	PrV1506R	59	52	
PrV1423F	*rpoB*2105R	59	73	
PrV1023F	PrV1245R	50	66	40-55 °C
PrV1023F	PrV1358R	50	54	
PrV1245F	PrV1506R	66	52	
PrV1358F	PrV1506R	54	52	
*rpoB*1110F	PrV973R	60	40	
PrV610F	PrV973R	64	40	
PrV610F	PrV1023R	64	50	
*rpoB*1110F	PrV1023R	60	50	
PrV592F	PrV973R	42	40	35 -50 °C
PrV1053F	PrV1358R	41	54	

Additionally, to define the exact temperatures, empirical determination is essential. For optimization of PCR conditions, four DNA samples of different Vibrio strains; *V. alginolyticus I* (VN2514), *V. alginolyticus II* (VN2756), *V. paraheamolyticus* (VN2502), *V. navarrensis* (VN2580) (see chapter 2.1.9) were selected for each primer-set and amplified by gradient PCR with individual parameters (see table 11). Figure 8 indicates representative virtual gels with PCR products of four Vibrio isolates amplified by the primer-set PrV295F/PrV610R at seven different temperatures with a selected gradient of G=10 °C. For VN2514 and VN2756 only minimal differences were observable. But for the isolates VN2580 and VN2502 unspecific secondary bands for temperatures between 50.2 °C and 57.8 °C were formed. Considering all isolates, the best conditions were found at a temperature of 65.1 °C.

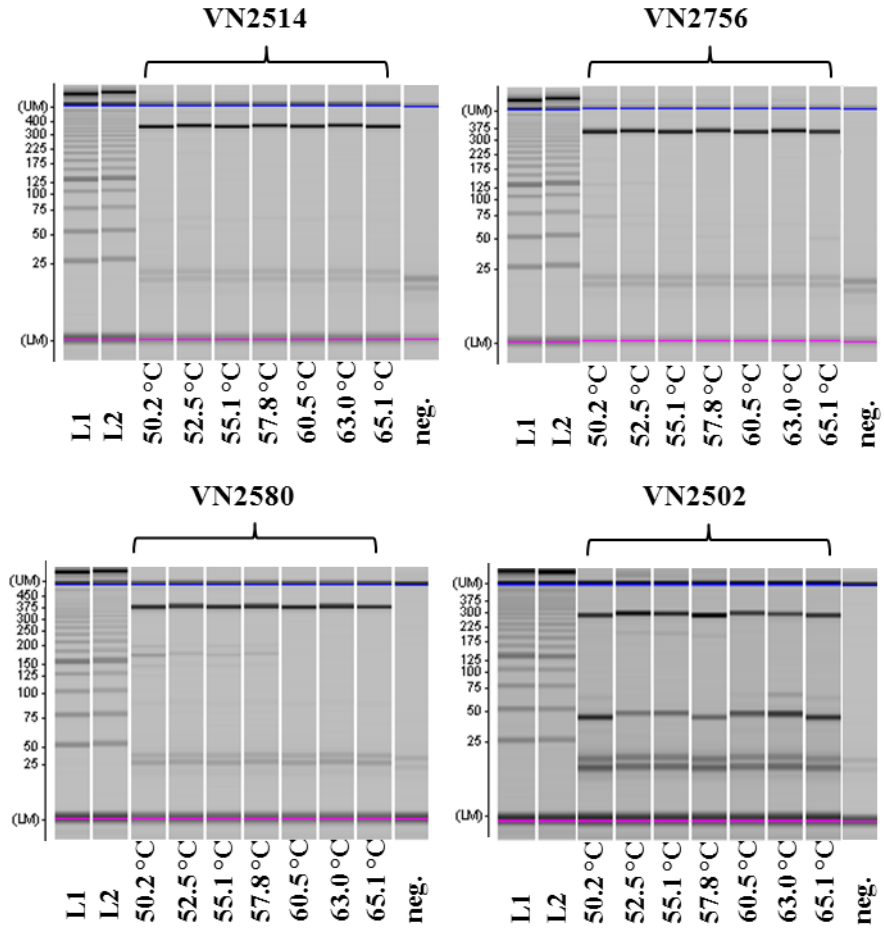

Figure 8: Gradient PCR showing PCR products of four Vibrio strains amplified by primer-set PrV295F/PrV610R at seven different annealing temperatures between 50.2 C and 65.1 °C. Best conditions were found at 65.1 °C considering all isolates. MultiNa virtual gel was produced with the DNA Kit 500 and DNA bands were visualized by application of SYBR® Gold.

Additionally, $MgCl_2$ concentration was adapted using 1 mM or 2 mM $MgCl_2$ in a 25 µl PCR reaction mix. Optimal conditions were developed for 13 of the collectively 18 primer-sets. For 5 primer-sets no annealing temperature was considered suitable (see table 12).

Table 12: Optimal annealing temperature and $MgCl_2$ concentration for each primer-set

Primer-sets		**T_a [°C]**	**$MgCl_2$ [mM]**
Forward	Reverse		
*rpoB*458F	PrV295R	60.5	2
PrV295F	*rpoB*1110R	63	1
PrV295F	PrV610R	65	1
PrV1245F	PrV1423R	60.5	1
PrV1245F	*rpoB*2105R	65	1
PrV1358F	*rpoB*2105R	63	1
PrV1423F	PrV1506R	-*	-*
PrV1423F	*rpoB*2105R	-*	-*
PrV1023F	PrV1245R	53	1
PrV1023F	PrV1358R	53	1
PrV1245F	PrV1506R	53	2
PrV1358F	PrV1506R	53	2
*rpoB*1110F	PrV973R	-*	-*
PrV610F	PrV973R	-*	-*
PrV610F	PrV1023R	53	1
*rpoB*1110F	PrV1023R	55	2
PrV592F	PrV973R	-*	-*
PrV1053F	PrV1358R	48	1

Remark: * no ideal annealing temperature and magnesium concentration were found for this primer-set

3.4.2. Amplification of Vibrio isolates

After determination of ideal PCR conditions for respectively 13 primer-sets (see table 12) a total of 31 Vibrio isolates and five isolates of a reference group (2.1.9) were amplified using the optimized PCR-protocol. The objective was to define the most suitable primer-sets for DHPLC analyses by specific amplification of all Vibrio strains excluding the reference group. Therefore, PCR results were analyzed according to the following criteria: specific amplification +, weak amplification (+), no amplification -, nearly no amplification (-) and unspecific amplification #. Figure 9 represents an example of PCR analysis using the primer-set PrV1245F/PrV1506R .

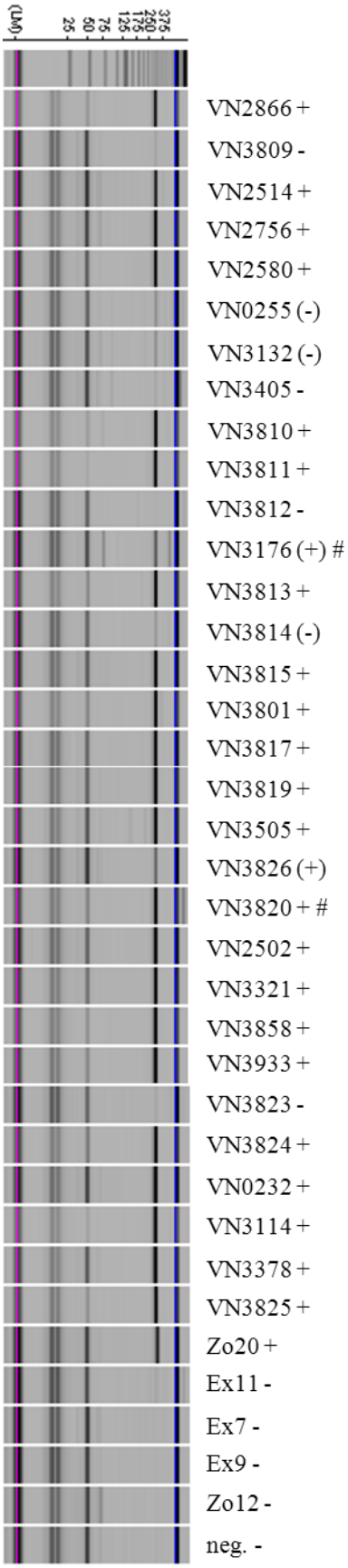

Figure 9: MultiNa virtual gel showing PCR products of 31 Vibrio isolates and five reference strains signified by their member code (2.1.9.). DNA was amplified by primer-set PrV1245F/PrV1506R with optimized PCR conditions. Specific amplification was achieved for 21 Vibrio isolates; reference group was negative except for Alteromonas (Zo20). MultiNa: DNA kit 500, DNA dye SYBR® gold.

In summary, five primer-sets (P1 – P5) produced best PCR results, showing the highest number of specific amplification of different *Vibrio* spp. and excluding the reference group. Altogether, 20-23 *Vibrio* spp. were successfully amplified using P1-P5 from a total number of 31 different species (see table 13). The selected primers target different regions of the *rpoB*-gene. Three of the five primer-sets include highly polymorphic regions to ensure the separation within the DHPLC-system (see table 13).

Table 13: Primer-sets offering best PCR results with highest specific amplification rate

Primer-set	P1 (PrV295F/ *rpoB*1110R)	P2 (PrV295F/ PrV610R)	P3 (PrV1023F/ PrV1245R)	P4 (PrV1023F/ PrV1358R)	P5 (PrV1245F/ PrV1506R)
Number of specific amplification of total of 31 Vibrio isolates	19	23	21	20	21
Light amplification	5	3	2	2	1
No amplification	1	1	4	3	4
Nearly no amplification	2	1	3	4	3
Unspecific amplification	4	3	1	2	2
Reference group	negative	negative	negative	weak amplification of Zo12	amplification of Zo20
Remark	-	-	-	-	no detection of *V. cholerae*
Mean variability amplicon	11.7 %	10.7 %	8.5 %	9.6 %	12.5 %
Position 5'- 3'	296/624	296/630	1023/1267	1023/1375	1245/1523
Length	328 bp	334 bp	244 bp	352 bp	278 bp

For optimization of DHPLC parameters, we chose P1 and P5, because these sets target the beginning and accordingly the ending region of the *rpoB*-gene and show the highest mean variability in the amplified fragments.

3.5. Optimization of DHPLC parameters

To find the optimal parameters for the analyses of the *rpoB*-gene fragments of Vibrio isolates in the DHPLC-system, primary tests were accomplished. These test included empirical determination of the column temperature and variation of acetonitrile gradient. PCR products of four Vibrio isolates amplified by P1 and P5 were used to optimize DHPLC conditions; *V. alginolyticus I* (VN2514), *V. navarrensis* (VN2580), *V. parahaemolyticus* (VN2502) and *V. vulnificus* (VN3114) (see chapter 2.1.9).

3.5.1. Variation of column temperature

We assumed that variation of column temperature plays a central role in optimizing DHPLC conditions. Hence, we estimated the predicted optimal column temperature of four Vibrio amplicon sequences for the two primer-sets (P1 and P5) using the Wave Navigator Software from Transgenomic. Ideal column temperature equates to the melting temperature of an amplicon at which 50 % of the double stranded DNA is dissociated into single strands. The partially denatured fragments exhibit different binding behavior to the column and therefore elute at different retention times. The predicted optimal temperatures for PCR fragments amplified by P1 ranged between 58.9 °C and 59.9 °C. Whereas, calculated temperatures for PCR fragments amplified by P5 varied between 60.4 °C and 61.0 °C (see table 14).

Table 14: Predicted optimal column temperature for separation of Vibrio PCR-products

Amplicons	**T_m P1 [°C]**	**T_m P5 [°C]**
VN2514	58.9	60.7
VN2580	59.6	61.0
VN2502	59.9	60.9
VN3114	59.4	61.4
mean value	59.45	61

With a basic knowledge about optimal temperature calculated by the Wave Navigator Software, we tried to find the exact column temperature due to an empirical procedure. Because all parameters can influence binding and elution of PCR products, it was necessary to manipulate DHPLC justifications systematically. To accomplish this, first temperature was exclusively verified with a gradient of 55 to 70 °C. Additionally, a fixed fractional acetonitrile gradient starting at 45 % Buffer B for 0 min to 65 % B for 13 min (see chapter 2.2.8., table 7), a pump flow rate of 0.9 ml min^{-1} and an injection volume of 10 µl were chosen. Column temperatures were at the beginning adjusted in 5 °C increments to find a roundabout region, where all PCR-fragments are partially denatured. Then, a fine scaling of temperature variation was accomplished in 1.5 °C steps to find an area providing distinct separation between different isolates. Influence of column temperature on the degree of partial denaturation of DNA and thus on the changing peak profile is exemplarily given in Figure 10. On the left site a full DHPLC run is shown, including injection peak (I), retention peak (II) and the column wash (III). A section representing 5 minutes of the full DHPLC run is given on the right site. At a column temperature of 61 °C the PCR product is completely denatured, producing a distinct high absorbance peak (A). At 62.5 °C a major peak and a smaller shoulder in front of

the major peak is visible, indicating partial denaturation of the amplicon (see arrow Fig. 10, B).

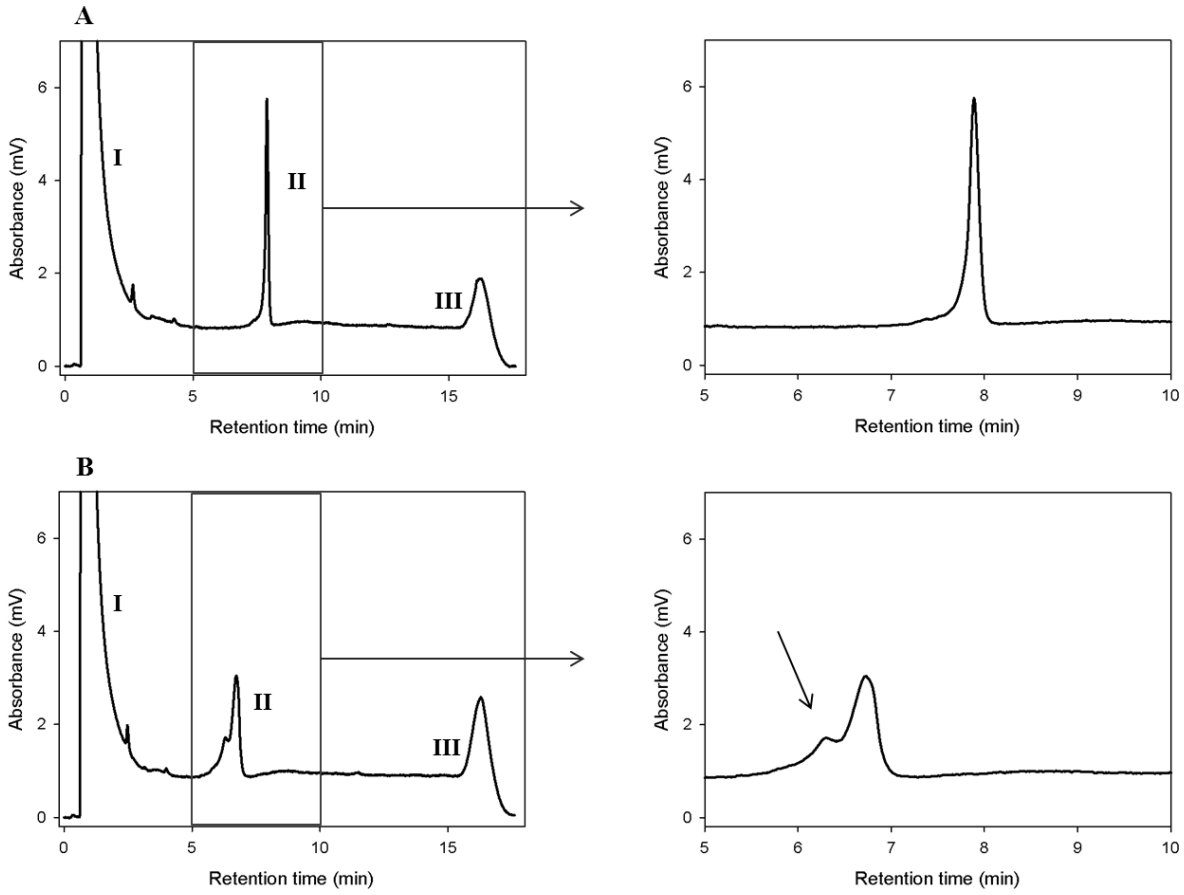

Figure 10: Influence of column temperature on the DNA helical structure and elution behavior in the DHPLC-system. Shown are exemplarily chromatograms of one PCR-product (VN2580) amplified by P5 detected by DHPLC at 61 °C (A) and 62.5 °C (B). Arrow marks the peak corresponding to the partially denatured DNA molecule. The conditions under which the experiment was performed were a stepwise acetonitrile gradient: 45 % Buffer B for 0 min (loading), 49.8 % B for 0.5 min to 65 % B for 13 min at a flow rate 0.9 ml/min and an injection volume of 10 µl.

We investigated that temperatures between 60 °C - 65 °C provides the area of interest, where best separation conditions between different PCR-fragments were found. In the following graphs only 5 minutes sections of the DHPLC run are displayed. Figure 11 shows the influence of temperature variation in 1.5 °C steps on DHPLC retention time for PCR fragments amplified by P1 of four different Vibrio isolates. Even fine scale temperature variation produces significant difference in DHPLC peak profile. Although complete separation could not be achieved, the best discriminative conditions were found at a temperature of 63 °C.

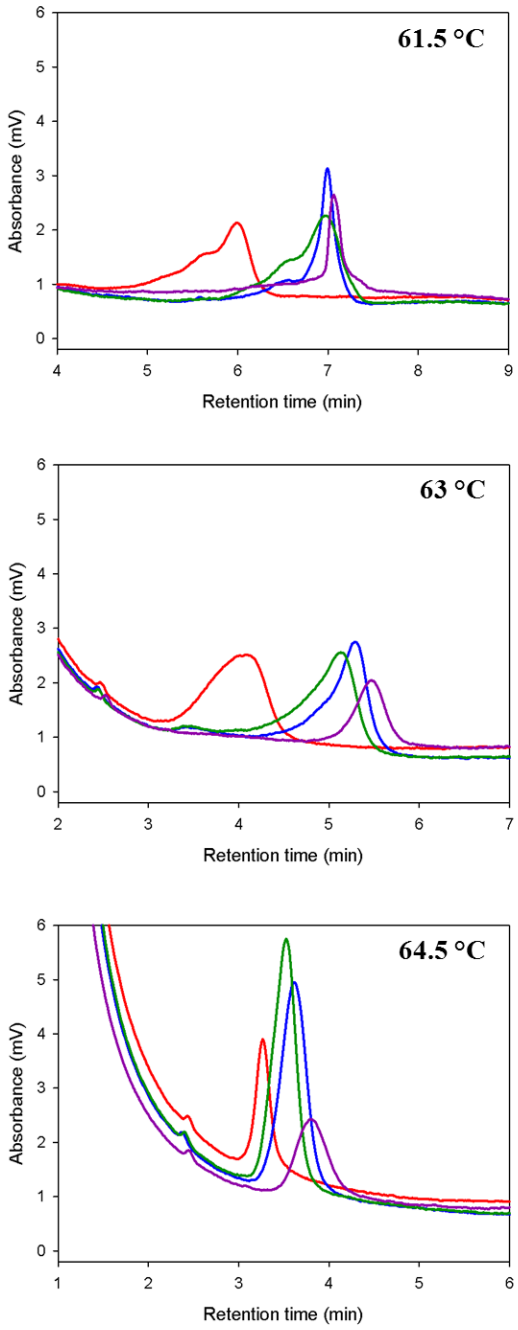

Figure 11: DHPLC retention behavior of P1-amplicons of four different Vibrio isolates: *V. alginolyticus I* (VN2514) red peak, *V. navarrensis* (VN2580) blue peak, *V. parahaemolyticus* (VN2502) green peak and *V. vulnificus* (VN3114) purple peak. Best temperature conditions for separation of DNA fragments were found at 63 °C. All separations shown were produced with a stepwise acetonitrile gradient: 45 % Buffer B for 0 min (loading), 49.8 % B for 0.5 min to 65 % B for 13 min at a flow rate 0.9 ml/min and an injection volume of 10 µl.

For PCR-fragments amplified with P5 best discriminative conditions were found at a temperature of 62.5 °C, where partial denaturation of DNA molecules is clearly visible (Fig. 12). This temperature was used to further optimize DHPLC conditions by adjustment of the acetonitrile gradient.

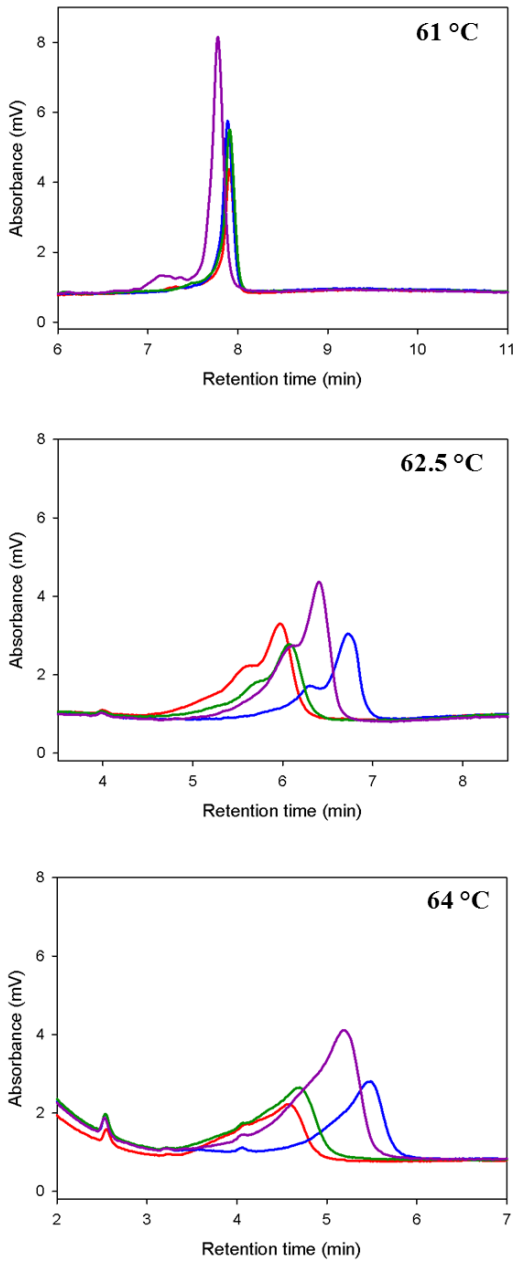

Figure 12: DHPLC retention behavior of P5-amplicons of four different Vibrio isolates: *V. alginolyticus I* (VN2514) red peak, *V. navarrensis* (VN2580) blue peak, *V. parahaemolyticus* (VN2502) green peak and *V. vulnificus* (VN3114) purple peak. Best temperature conditions for separation of DNA fragments were found at 62.5 °C. All separations shown were produced with a stepwise acetonitrile gradient: 45 % Buffer B for 0 min (loading), 49.8 % B for 0.5 min to 65 % B for 13 min at a flow rate 0.9 ml/min and an injection volume of 10 µl.

3.5.2. Variation of the acetonitrile gradient

To achieve better separation results for the four *Vibrio* species at optimal temperature condition, the acetonitrile gradient was decelerated. Aim of the modification was to induce a shift in the peak profile caused by a slow increasing level of acetonitrile as a function of time. Therefore, a linear Buffer gradient was created with an increasing concentration of 0.5 %

Buffer B (aqueous solution of 0.1 M TEAA with 25% acetonitrile) per minute, started with 45 % Buffer B for 0 min (loading), 54 % Buffer B for 2 min to 60 % Buffer B for 14 min at 63.5 °C (see chapter 2.2.8.). Figure 13 shows the DHPLC analysis for the adapted running conditions at 63 °C for PCR products amplified by P1 (A) and 62.5 °C for PCR products amplified by P5 (B). Generally, the DHPLC results indicate loss in peak intensity and a shift in retention time after adaptation of the acetonitrile gradient. Peak profile of P1-amplicons resembles the analyses of the first DHPLC protocol including only temperature variation. In contrary, distinct alteration in retention behaviour is observable for PCR fragments amplified by P5 (see arrow Fig. 13 B). P5-PCR products of *V. vulnificus* (VN3114) and *V. navarrensis* (VN2580) are clearly distinguishable, but P5-PCR products of *V. alginolyticus* (VN2514) and *V. parahaemolyticus* (VN2580) (see chapter 2.1.9.) were not detectable.

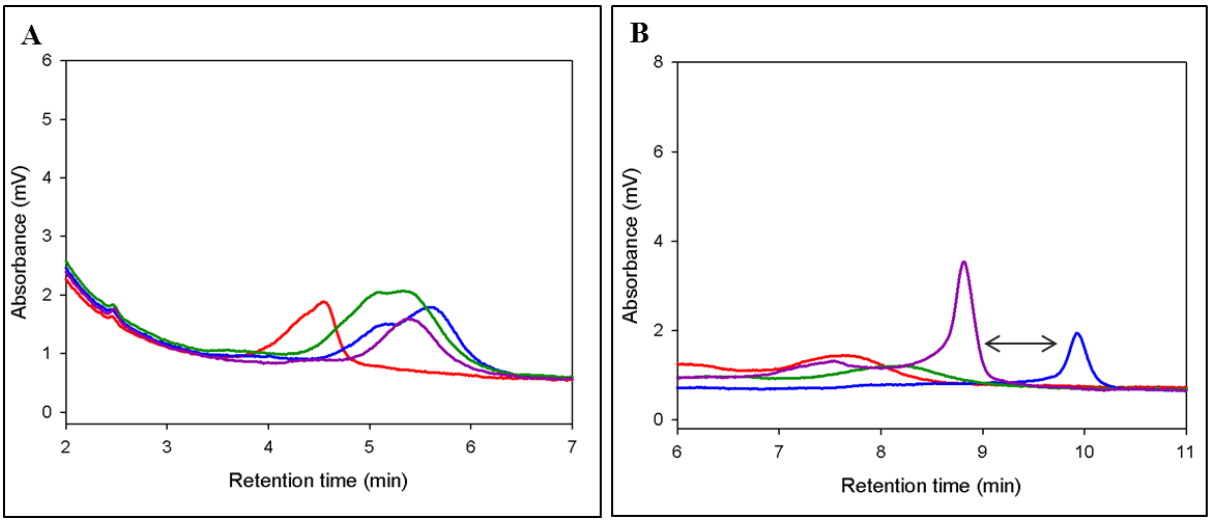

Figure 13: DHPLC analysis of 4 individual PCR-products of *V. alginolyticus I* (VN2514) red peak, *V. navarrensis* (VN2580) blue peak, *V. parahaemolyticus* (VN2502) green peak and *V. vulnificus* (VN3114) purple peak amplified with primer-set 1 and 5 with at A) 63 °C and B) 62.5 °C. Acetonitrile gradient was adapted to: 45 % Buffer B for 0 min (loading), 54 % B for 2 min to 60 % B for 14 min at 63.5 °C; flow rate 0.9 ml/min; injection volume 10 µl.

Briefly, best separation conditions in the DHPLC-system were found for PCR-products amplified by P5 at a column temperature of 62.5 °C with a linear acetonitrile gradient of 45 % Buffer B for 0 min (loading), 54 % Buffer B for 2 min to 60 % Buffer B for 14 min at a flow rate of 0.9 ml min^{-1} with an injection volume of 10 µl. But using these conditions only two of four Vibrio isolates were detected by DHPLC.

3.5.3. Adaptation of PCR-DHPLC protocol by attachment of a 40 bp GC-clamp

To overcome the loss in peak intensity, PCR protocol was modified including attachment of a GC-clamp to 5'end of the respective P5-primer and a touchdown-PCR. After attachment of

the 40 bp GC-clamp, loss in amplicon concentration was observable using the Vibrio specific *rpoB*-PCR adapted for P5 (Fig. 14 A). Application of a touchdown-PCR was essential to increase the amplicon concentration for a reliable detection in DHPLC. Best annealing temperatures for touchdown PCR were found by conducting a gradient PCR (see chapter 2.2.4.1). Optimal amplification was realized at annealing temperatures of 60 °C to 50 °C with -0.5 °C per cycle and 2 mM $MgCl_2$ (Fig. 14 B).

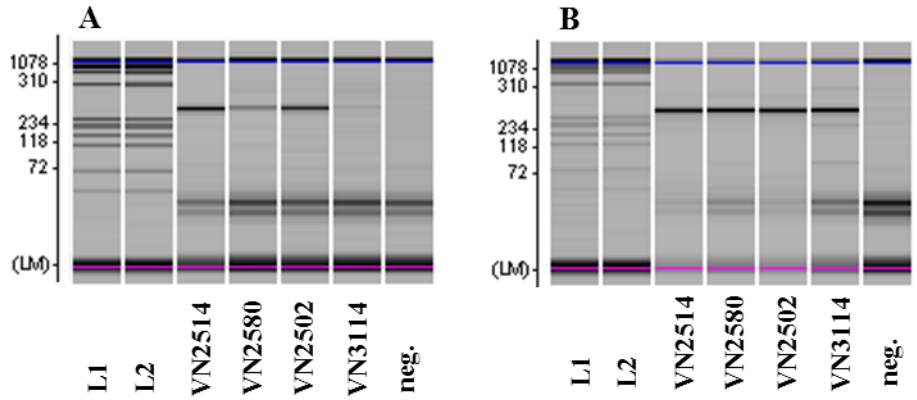

Figure 14: MultiNa virtual gel showing PCR products of four different Vibrio isolates amplified by P5 with attached GC-clamp using a standard *rpoB*-PCR (A) and touchdown *rpoB*-PCR (B). Amplification with standard *rpoB*-PCR is insufficient for further DHPLC analyses; application of an optimized touchdown-PCR protocol yielded high concentrated amplicons. MultiNa: DNA kit 1000, DNA dye SYBR® gold.

Under optimized touchdown conditions (see chapter 2.1.9) distinct amplification was successful for 21 of 31 Vibrio isolates. Results were the same as for P5 without GC-clamp (see chapter 3.4.2, Fig. 9).

For comparability, DHPLC conditions were optimized as for PCR products amplified by P5 without GC-clamp, including variation of column temperature and subsequent adaption of acetonitrile gradient.

3.5.3.1. Variation of column temperature

Column temperatures were initially adjusted in 5 °C increments starting at 55 °C to 75 °C and then justified in 1.5 °C steps. Figure 15 indicates the influence of temperature variation in 1.5 °C steps on DHPLC retention time for PCR fragments amplified by P5 with an attached GC-clamp for four different Vibrio isolates. Best discriminative DHPLC conditions were found at a temperature of 63.5 °C, one degree above optimal temperature condition for PCR fragments amplified by P5 without GC-clamp (Fig. 15).

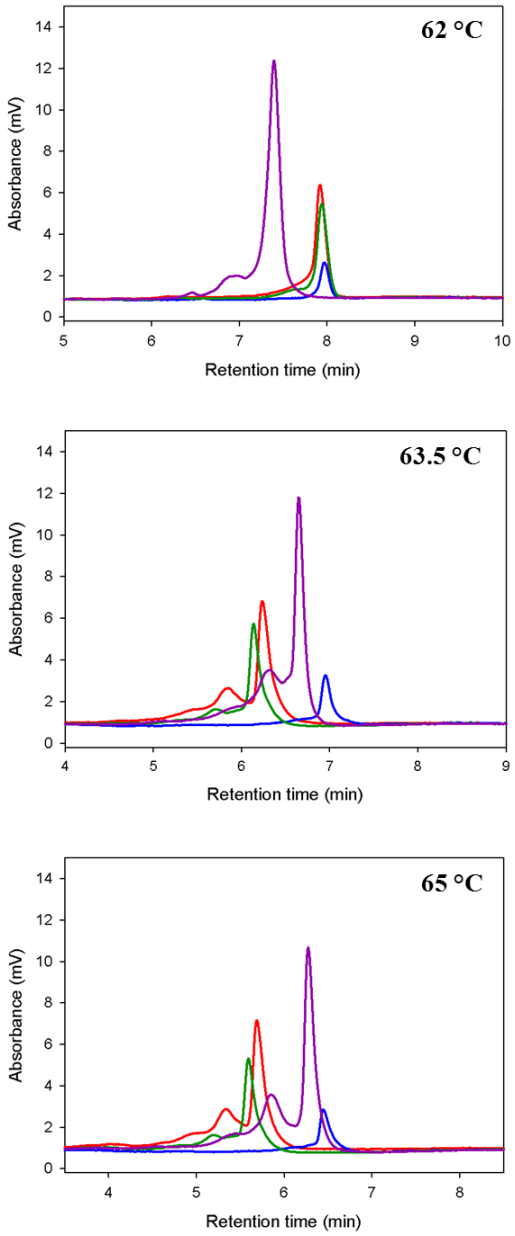

Figure 15: DHPLC retention behavior of PCR fragments amplified by P5 with an attached GC-clamp of four different Vibrio isolates: *V. alginolyticus I* (VN2514) red peak, *V. navarrensis* (VN2580) blue peak, *V. parahaemolyticus* (VN2502) green peak and *V. vulnificus* (VN3114) purple peak. Best temperature conditions for separation of DNA fragments were found at 63.5 °C. All retention peaks shown were produced with a stepwise acetonitrile gradient: 45 % Buffer B for 0 min (loading), 49.8 % B for 0.5 min to 65 % B for 13 min at a pump flow rate of 0.9 ml/min and an injection volume of 10 µl.

Comparing the two PCR-DHPLC protocols in detail, attachment of a GC-clamp to 5'end of P5 and performance of touchdown-PCR was essential to achieve a distinct and high absorbance DHPLC peak profile (see arrow Fig. 16).

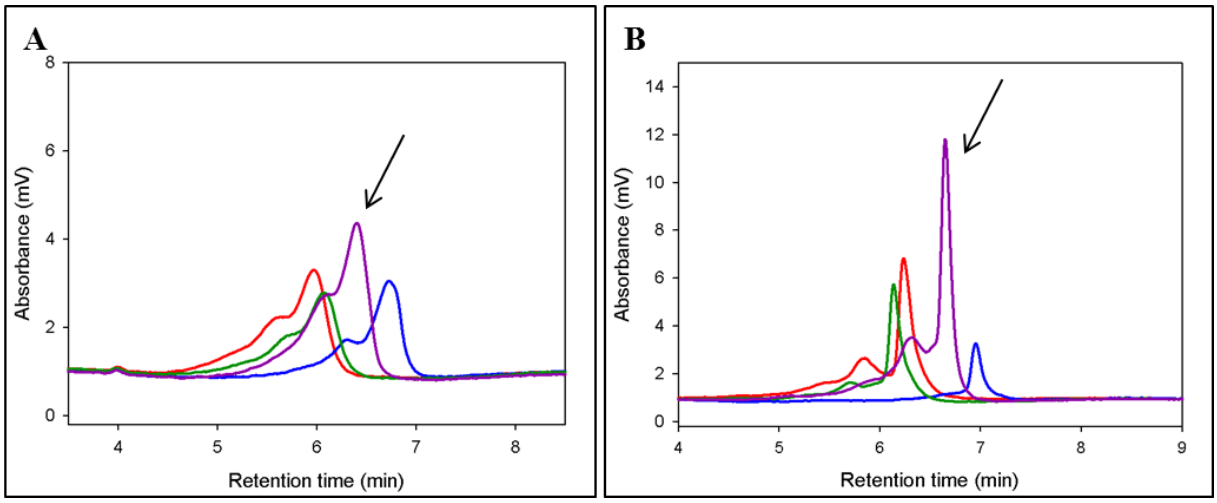

Figure 16: DHPLC analysis of 4 PCR-fragments using forward primer without (A) and with 40-bp GC-clamp (B). Arrows indicate increase in peak intensity after adaptation of the PCR protocol. All retention peaks shown were produced with a stepwise acetonitrile gradient: 45 % Buffer B for 0 min (loading), 49.8 % B for 0.5 min to 65 % B for 13 min at a pump flow rate of 0.9 ml/min and an injection volume of 10 µl.

3.5.3.2. Variation of acetonitrile gradient

As a second step we modified the acetonitrile gradient for better separation of the four Vibrio isolates following the method previously described (see chapter 3.5.1.). Figure 17 shows the DHPLC analysis for the adapted acetonitrile gradient at 63.5 °C for PCR products amplified by P5 without GC-clamp (A) and with attached GC-clamp (B). In comparison, detection of all Vibrio isolates could be accomplished using the modified acetonitrile gradient (see arrow Fig. 17 B). Furthermore, PCR products of *V. vulnificus* (VN3114) and *V. navarrensis* (VN2580) are clearly distinguishable, but definite separation of the closely related *V. alginolyticus* (VN2514) and *V. parahaemolyticus* (VN2580) isolates could not be achieved so far.

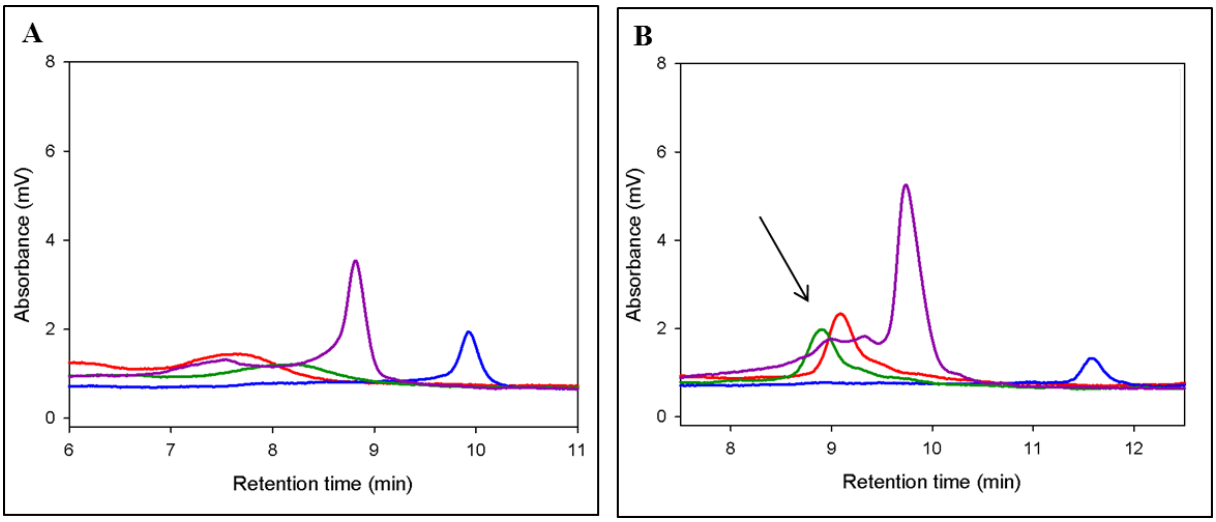

Figure 17: DHPLC analysis of 4 individual PCR-products: *V. alginolyticus I* (VN2514) red peak, *V. navarrensis* (VN2580) blue peak, *V. parahaemolyticus* (VN2502) green peak and *V. vulnificus* (VN3114) purple peak amplified with primer-set 5 with attached GC-clamp at 63.5 °C. All retention peaks shown were produced with a linear acetonitrile gradient: 45 % Buffer B for 0 min (loading), 54 % B for 2 min to 60 % B for 14 min at 63.5 °C at a pump flow rate of 0.9 ml/min and an injection volume of 10 µl.

Compendiously, the best PCR-DHPLC protocol included amplification of *rpoB* fragment by P5 with an attached GC-clamp at the 5` respective end and corresponding DHPLC conditions including column temperature of 63.5 °C, a linear acetonitrile gradient of 45 % Buffer B for 0 min (loading), 54 % Buffer B for 2 min to 60 % Buffer B for 14 min at a pump flow rate of 0.9 ml min^{-1} and an injection volume of 10 µl.

3.5.4. Application of all Vibrio isolates

With the establishment of optimized PCR-DHPLC conditions, we applied 22 Vibrio *rpoB* fragments, including 16 different species, separately into the flow path of the DHPLC system (see table 15). All of the 22 Vibrio isolates produced distinct peaks in the DHPLC system under partial denaturing conditions. The retention time ranged between 4 – 13 min and peak intensity varied between 0.5 – 12 mV. PCR products of three Vibrio isolates, *V. aestuarianus* (VN2866), *V. tasmaniensis* (VN3824) and *V. xuii* (VN3825) produced unambiguous peaks at two different retention times (data not shown), but for data interpretation we considered only the second major peak.

Table 15: Comparison of DHPLC retention time and sequence characteristics of P5-amplicons between Vibrio strains detectable by PCR-DHPLC

Vibrio strains	DHPLC retention time [min]
VN3815 *V. gigantis*	5.2
VN2866 *V. aestuarianus*	5.25
VN3824 *V. tasmaniensis*	5.55
VN3817 *V. kanaoloe*	5.6
VN3825 *V. xuii*	6.4
VN3820 *V. pacinii*	6.8
VN3811 *V. diazotrophicus*	7.2
VN3810 *V. coralliilyticus*	7.55
VN3819 *V. mediterranei*	8.4
VN3813 *V. fortis*	9.1
VN2502 *V. parahaemolyticus*	9.2
VN3321 *V. parahaemolyticus*	9.2
VN3858 *V. parahaemolyticus*	9.2
VN3933 *V. parahaemolyticus*	9.2
VN2514 *V. alginolyticus I*	9.45
VN2756 *V. alginolyticus II*	9.6
VN0232 *V. vulnificus*	9.9
VN3114 *V. vulnificus*	9.9
VN3378 *V. vulnificus*	9.9
VN3801 *V. harveyi*	11.6
VN2580 *V. navarrensis*	13.15
VN3505 *V. mimicus*	13.15

Examine the table results in detail, different PCR products of the same species show peak profiles at similar retention times. Figure 18 represents the peak profiles of different PCR-products belonging to the same species.

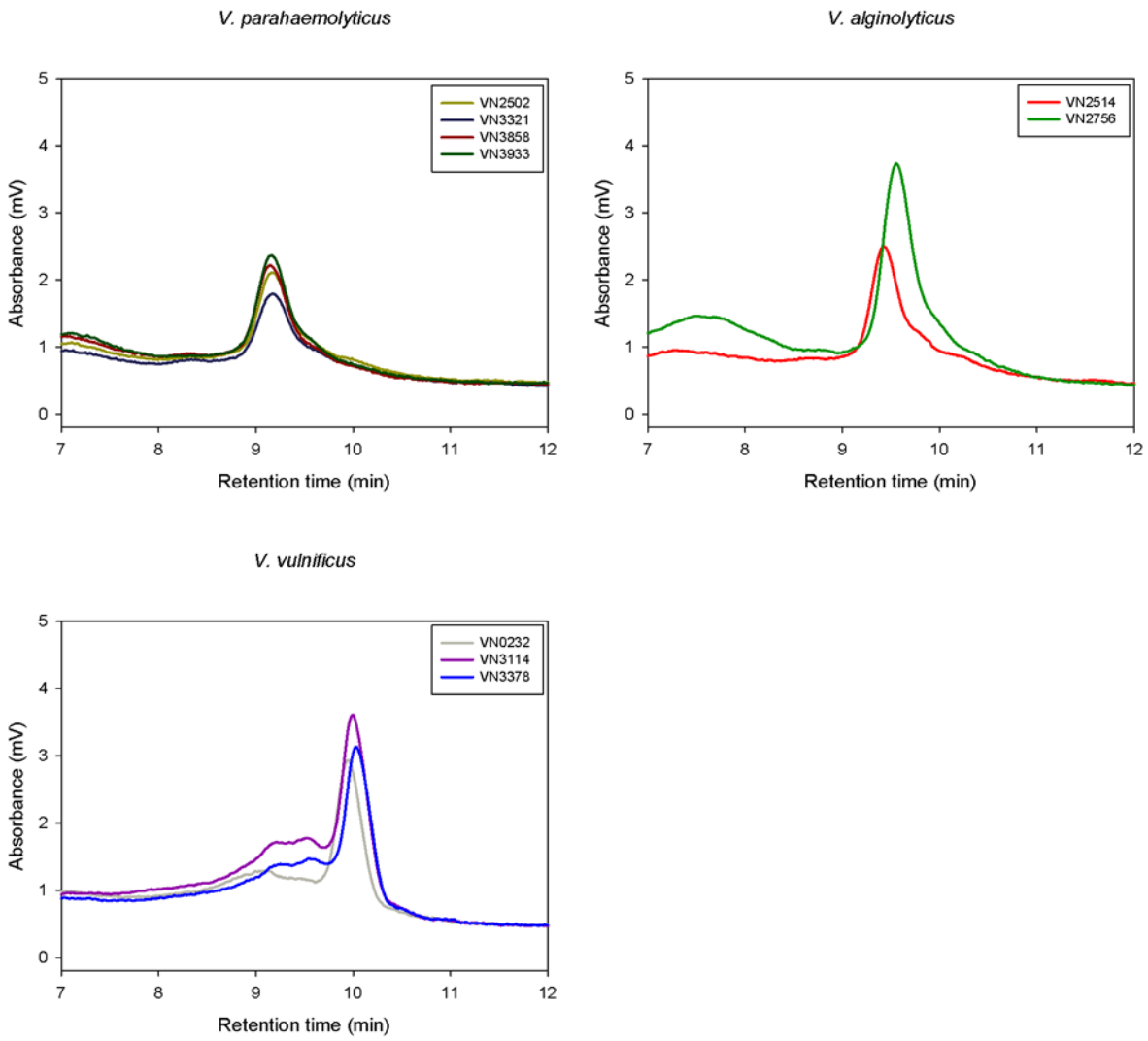

Figure 18: DHPLC peak profiles representing elution behavior of different PCR-products belonging to the same species. PCR-products were amplified with P5 (with GC-clamp) and injected into the flow path of DHPLC. Linear acetonitrile gradient: 45 % Buffer B for 0 min (loading), 54 % B for 2 min to 60 % B for 14 min at 63.5 °C; flow rate 0.9 ml/min; injection volume 10 µl.

Furthermore, we investigated that separation of at least 6 different *Vibrio* species was successful according to their different retention characteristics using the optimized PCR-DHPLC protocol. But still, separation by DHPLC was not possible for closely related *Vibrio* species, e.g. *V. parahaemolyticus* and *V. alginolyticus.*

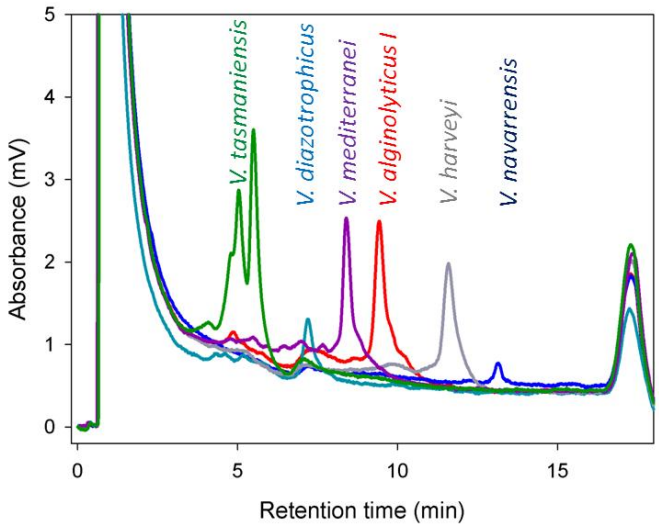

Figure 19: DHPLC analysis of 6 individual PCR-products amplified with P5 with attached GC-clamp. DHPLC linear acetonitrile gradient: 45% B for 0 min, 54% B for 2 min, and 60% B for 14 min at 62.5 °C; flow rate 0.9 ml/min; injection volume 10 µl.

In summary, PCR-DHPLC offered detection of 22 different Vibrio isolates of a total number of 31 using P5 and amplification with an optimized touchdown-protocol. Those 22 isolates included 16 different *Vibrio* species. By adjustment of column temperature and acetonitrile gradient, we separated at least 6 different *Vibrio* species according to their different elution behavior in the DHPLC. Furthermore, we observed that different PCR-products of one species produce DHPLC peaks at the same retention time.

4. Discussion

In this study we investigated potential human pathogenic *Vibrios* spp. by PCR-DHPLC based on partial *rpoB*-gene fragments. Some strains of *Vibrio* spp. induce outbreaks of gastrointestinal infections or primary septicemia in humans caused by consumption of contaminated see-food or contact with polluted water, and many species are potential pathogens for marine organisms (Thompson, Iida et al. 2004). Due to rising sea water temperature and the worldwide marine traffic conditions are enhanced for potentially pathogenic *Vibrio* spp. to grow and disperse even in temperate waters of the North and Baltic Sea (Wiltshire, Malzahn et al. 2008; Belkin 2009; Baker-Austin, Stockley et al. 2010). Because of the increased incidence of Vibrio infections in the last years, a rapid and accurate method is required to analyze and identify complex *Vibrio* spp. populations, specifically potential pathogenic *Vibrio* species, in environmental samples. A general problem in investigating Vibrios is the difficulty in differentiation between species due to their close phylogenetic relationship, especially described for *V. parahaemolyticus* and *V. alginolyticus* (Kita-Tsukamoto, Oyaizu et al. 1993; Robert-Pillot, Guenole et al. 2002). Generally molecular methods based on 16S rRNA analyses have been regularly applied for differentiation of bacterial isolates (Woo, Lau et al. 2008). But for identification of *Vibrio* spp., the 16S rRNA gene has been described as not sufficiently polymorphic to ensure their reliable identification (Thompson, Gevers et al. 2005; Tarr, Patel et al. 2007). For this reason, other biomarkers were consulted to overcome the high conservation of the 16S rRNA gene. Recently, Oberbeckmann and coworkers (2011) showed that *rpoB* sequence analysis is a more reliable method to distinguish even closely related *Vibrio* species. Many sequence based molecular methods have been established for identification and characterization of Vibrio strains during the last decade, like MALDI-TOF, repetitive sequence based PCR or DGGE (Eiler and Bertilsson 2006; Oberbeckmann, Wichels et al. 2011). Most recently, DHPLC has been applied to separate mixtures of PCR amplicons derived from different organisms. In this study, we report the first development of a PCR-DHPLC approach targeting *rpoB*-gene fragments for detection and separation of potentially pathogenic Vibrios, which could be used to monitor abundance of microbial communities in environmental samples.

4.1. Design of Vibrio specific Primers

Foundation for development of the *rpoB*-DHPLC approach was the evaluation of Vibrio specific Primers, targeting different regions of the *rpoB*-gene. In recent studies *rpoB*-based molecular methods were described as a reliable tool for classification of Vibrio isolates, rather

than 16S rRNA based methods (Tarr, Patel et al. 2007; Ki, Zhang et al. 2009; Oberbeckmann, Wichels et al. 2011). In consistence with these studies, our phylogenetic comparison of the around 1600 bp *rpoB* partial gene of the genus Vibrio exhibited a high discrimination between species and even a clear separation between *V. parahaemolyticus* and *V. alginolyticus* (see chapter 3.1. Fig. 4). Separation of species-specific PCR products by DHPLC requires that amplicons have a short length and a high variability of the base compositions between species. Studies to date have successfully applied 200 – 500 bp PCR fragments for separation by PCR-DHPLC (Barlaan, Sugimori et al. 2005; Goldenberg, Herrmann et al. 2007; Troedsson, Lee et al. 2008). In this study we were able to develop 10 forward and 10 reverse primers (see chapter 2.1.8.) combining to 18 primer-sets, which amplify fragments of 100-400 bp and cover different regions of the partial *rpoB*-gene.

4.2. Cultivation and DNA extraction

For identification and characterization of bacterial strains by *rpoB*-DHPLC we used a set of 31 Vibrio isolates. The first step for development of this new approach was the cultivation of bacterial strains on marine broth medium and DNA extraction by lysozyme/SDS lysis and phenol-chloroform extraction followed by isopropanol precipitation, using a modified protocol of Anderson and McKay (1983). This culture method and extraction of genomic DNA has been successfully applied for severe potentially human pathogenic *Vibrio* spp. from environmental samples (Oberbeckmann, Wichels et al. 2011). All of the 31 Vibrio isolates were extracted in this study with an A260/A280 ratio between 1.8 and 2.1. Ideally, the ratio of the absorbance measured at A260/A280 should be 1.8 – 2.0. Ratios less than 1.8 indicate protein contamination, while ratios greater than 2.0 indicate the presence of RNA (Kowalchuk 2004). Concentration of total genomic DNA varied between 50 – 2000 ng/µl. The modified phenol-chloroform extraction relies on phase separation resulting in an upper aqueous phase, containing nucleic acids (RNA, DNA) and a lower organic phase, containing mainly phenol. Between the aqueous and organic phase, an interface is formed enclosing proteins and membrane residues. To gain pure DNA only the upper phase was transferred into a new sterile tube. Difference of DNA concentrations may depend on the volume transferred from the aqueous phase. For PCR-analyses, we used a final DNA concentration of 50 ng/µl. Despite variation in DNA concentration, we had enough material for further PCR-analyses.

4.3. Optimization of PCR parameters

The PCR technique became an important tool for amplification of nucleic acids purified from microorganisms and potential microbial pathogens (Bej and Mahbubani 1992). In this study

we developed 18 Vibrio specific Primer-sets for amplification of different regions of the *rpoB*-gene. Aim of using the PCR methodology was the detection and specific amplification of 31 Vibrio isolates for DHPLC-analysis and simultaneously absence in detection of the reference group. The determining factor for effective amplification of *Vibrio* spp. by PCR is optimization of the PCR reaction and thermal cycling parameters. In this study, improvement of PCR included adaptation of the $MgCl_2$ concentration and annealing temperatures by performance of a gradient PCR. The gradient PCR generally serves to identify optimal PCR conditions providing efficient and specific amplification for each primer-set. Optimal annealing temperatures were found for 13 of the original 18 primer-sets (see chapter 3.4.1., table 12). Three of the five primer-sets, which did not work in the laboratory included primer PrV973. This oligonucleotide primer had a low melting temperature of 40 °C. Generally, annealing temperatures should be selected 5 °C below the lowest T_m of a primer-pair, resulting in a T_a of 35 °C (Innes 1990). But the preferable T_a value for each primer is between 60-70 °C (Bej, Mahbubani et al. 1991). As a consequence of having a too low T_a, primers will anneal to sequence regions other than the true target, as internal single-base mismatches or partial annealing may be tolerated. Furthermore, PrV973 showed the highest mean variability at the primer binding position in the *rpoB*-gene with 14.9 %. Respectively, amplification with PrV973 leaded to nonspecific PCR products or no PCR product.

The other two primer-sets, which produced unspecific or no PCR products, were PrV1423/PrV1506 and PrV1423/*rpoB*2105. One explanation for this result is the difference in T_m values of 14 °C for Primer-set PrV1423/*rpoB*2105 (see chapter 3.4.1., table 11). Primers with a difference in T_m greater than 5°C can result in inadvertent preferential or no amplification. Another explanation for an ineffective PCR is an incorrect application of the $MgCl_2$ concentration. The concentration of $MgCl_2$ affects primer annealing, DNA melting temperature, and enzyme activity. A low $MgCl_2$ concentration can lead to missing PCR-products and a high $MgCl_2$ concentration can affect unspecific PCR products. A range of 1 – 4 mM magnesium ion can be used to optimize PCR reactions (Kowalchuk 2004). In our study, we tested only $MgCl_2$ concentrations between 1 mM – 2 mM. Application of higher $MgCl_2$ concentration could have yield specific PCR-products for the primer-sets PrV1423/PrV1506 and PrV1423/*rpoB*2105.

4.4. Amplification of Vibrio isolates

For a reliable identification of Vibrio strains from environmental samples by *rpoB*-DHPLC it is important that the designed Primers amplify specifically the diverse group of Vibrio species, but not closely related bacteria outside of the Vibrionacea family. In total five primer-

sets fulfill these conditions. Using the optimized PCR parameters P1-P5 exhibited sufficient amplification of Vibrio species with 20-23 distinct PCR-products of Vibrio isolates from a total number of 31. For the DHPLC-approach, we selected two primer-combinations (P1 and P5), showing the highest mean variability of the amplicon region. The sequence based variation is the foundation for an adequate separation by DHPLC.

4.5. DHPLC

In recent times the DHPLC, originally developed to detect gene mutations and single nucleotide polymorphisms, has been successfully adapted for separation of bacterial PCR-amplified fragments of complex microbial communities and is thus a time- and cost-efficient alternative to other molecular methods, like full length sequencing (Wagner, Stoppa-Lyonnet et al. 1999; Goldenberg, Herrmann et al. 2007). Furthermore, *rpoB*-gene analysis have already been described as a powerful method for discrimination of even closely related Vibrios, e.g. *V. alginolyticus* and *V. parahaemolyticus* (Ki, Zhang et al. 2009; Oberbeckmann, Wichels et al. 2011). For the first time, we combined these approaches to develop a new *rpoB*-DHPLC assay for identification and characterization of potential pathogenic Vibrios strains. This study focused on the evaluation and optimization of DHPLC-conditions, including adaptation of the column temperature and acetonitrile gradient, for a successful identification of Vibrio species.

4.5.1. Variation of the column temperature and acetonitrile gradient

Separation by DHPLC can only occur in case of partial denaturation of the PCR molecules, because PCR products with a greater degree of partial denaturation have a lower bond to the column and elute earlier off the cartridge than more stable fragments (see chapter 1.5.). Hence, we assumed that the column temperature is one of the key factors contributing the detection of genetic variants in the DHPLC-system.

Applying P1, best separation conditions for four different Vibrio isolates were empirically found at 63 °C, around 3.5 °C above the predicted, average optimal column temperature calculated by the Wave Navigator Software (see chapter 3.5.1.). Using P5, best separation was achieved at 62.5 °C, 1.5 °C above the predicted, average optimal temperature. These results revealed that despite detection of the computed optimal temperatures, empirically variation is necessary to find the exact column temperature for optimal separation. The defined ideal temperatures were then used for further optimization of DHPLC conditions by modification of the acetonitrile gradient. Our expectation was a better separation of Vibrio isolates as a result of a decelerated acetonitrile gradient. Applying four PCR products

amplified with P1 the peak profile was consistent after adaptation of the acetonitrile gradient. However, a clear shift in the retention characteristics was observable for PCR products amplified with P5, but using these conditions only two of four isolates were detectable (see chapter 3.5.2.). To gain a better understanding of the different elution behavior in the DHPLC, we generated melting profiles of the four *rpoB* amplicon-sequences at 63 °C for P1 and 62.5 °C for P5 using the WAVE Navigator Software. The melting profiles show the amount of α-helicity at different points of the amplicon and provide information about GC- and AT- rich regions in the fragments.

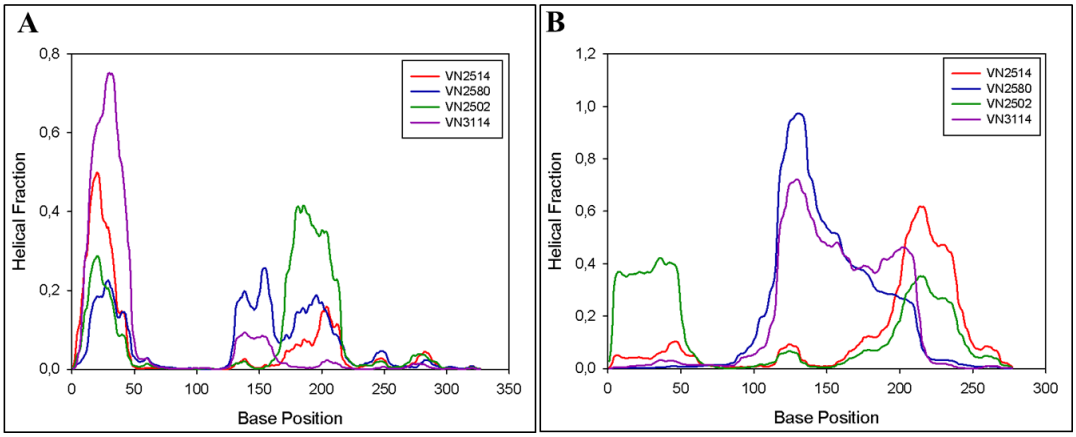

Figure 20: Wave Navigator Software-generated melting profiles for *rpoB* sequences of four Vibrio isolates at the best temperature conditions found empirically. Similar melting profiles are represented for P1 sequences at 63 °C (A), whereas P5 sequences exhibit different melting profiles at 62.5 °C (B).

Melting domains of fragments amplified with P1 were nearly similar, but distinct differences were observable for fragments amplified with P5. The results indicate that for efficient differentiation of Vibrio species in the DHPLC not only the amount of variability in the *rpoB*-gene is the discriminative factor, also differences in melting-domains and accordingly the distribution of AT-rich and GC-rich regions along the amplicon fragment play an important role. Our finding is in agreement with a previous review, in which the correlation between the content and distribution of AT base pairs and the behaviour of DNA fragments on the DHPLC is described (Xiao and Oefner 2001). In addition, it has been reported that with other techniques, such as DGGE, separation of PCR-products depends on melting-domains rather than sequence variants (Muyzer and Smalla 1998; Kisand and Wikner 2003). A second difference between P1 and P5, which might influence the retention characteristics of the PCR-products, is the length of the amplicon region with 328 bp for P1, and 278 bp for P5. Studies to date have generally applied around 200-500 bp PCR fragments for separation by PCR-DHPLC (Barlaan, Sugimori et al. 2005; Goldenberg, Herrmann et al. 2007; Troedsson, Lee et al. 2008). But, in a recent study it has been investigated that the DHPLC is able to

discriminate short amplicons (~100 bp without primers) based on the sequence differences and even if the fragments only differ only in a small number of nucleotides (Epp, Stoof-Leichsenring et al. 2011).

4.5.2. Adaptation of PCR-DHPLC by attachment of a 40 bp GC-clamp

Most efficient separation in the DHPLC-system was found for PCR-products amplified by P5 at a column temperature of 62.5 °C and with a decelerated acetonitrile gradient (see chapter 2.2.8., table 8) at a flow rate of 0.9 ml min^{-1} and with an injection volume of 10 µl. But these optimized separation conditions induced complete denaturation of the PCR products of *V. alginolyticus* (VN2514) and *V. vulnificus* (VN3114), which resulted in a loss of peak intensity and failure of detection in the DHPLC (see chapter 3.5.2.). The denaturation might be consequence of the increased acetonitrile level of 54 % Buffer B at the starting point of the adapted program compared to acetonitrile level of 49.8 % Buffer B used initially (see chapter 2.2.8.). Xiao and coworkers reported that acetonitrile has a denaturing function itself and that an increasing level of acetonitrile of 0.8 % equates to an increase of temperature of 1 °C (Xiao and Oefner 2001). To overcome the loss in peak intensity, we modified the PCR-protocol by attachment of a 40 bp GC-clamp at the 5´end of P5 and a touchdown PCR. A GC-clamp is commonly used to prevent denaturation of amplicons and has been applied for appropriate identification and separation of PCR products by DGGE (Sheffield, Cox et al. 1989). In recent studies, the GC-clamp has also been applied for improvement of DHPLC separation (Barlaan, Sugimori et al. 2005; Epp, Stoof-Leichsenring et al. 2011). For example, Barlaan and co-workers (2005) testified that an incorporation of a 40- bp GC clamp into the amplification primer was essential to surmount insufficient PCR products and to effectively discriminate genetic differences in DHPLC. After attachment of the GC-clamp, performance of a touchdown PCR was necessary to avert loss in amplicon concentration emerged by using the *rpoB*-PCR adapted for P5 (see chapter 3.5.3. Fig. 14). A touchdown PCR is generally used to increase specificity in PCR amplification and amplicon concentration (Korbie and Mattick 2008) and has also been effectively applied for PCR-DHPLC (Barlaan, Sugimori et al. 2005). However, using the modified PCR protocol, detection of all Vibrio isolates (*V. alginolyticus I* (VN2514), *V. navarrensis* (VN2580), *V. parahaemolyticus* (VN2502) and *V. vulnificus* (VN3114)) was successful (see chapter 3.5.3.2. Fig. 17).

4.5.3. Application of all Vibrio isolates

After determination of most efficient separation conditions for four Vibrio isolates in the DHPLC, we applied 22 PCR-products of potentially pathogenic Vibrio strains on the system.

Basically, we could show that different PCR products of the same species showed identical peak profiles (see chapter 3.5.4. Fig. 18), which is essential to distinguish between different Vibrio isolates in mixed or environmental samples. But PCR products of three Vibrio isolates, *V. aestuarianus* (VN2866), *V. tasmaniensis* (VN3824) and *V. xuii* (VN3825) produced unambiguous peaks at two different retention times (data not shown), which is problematically for a reliable identification of Vibrio strains in mixed samples. A previous study from Troedsson and colleagues (2008) hypothesizes that the helical structure of the amplicon influences occurrence of multiple peaks. It has been demonstrated that amplicons with predicted helical fractions greater than 95% produces single peaks, while fragments with a lower predicted helical fraction generates multiple peaks (Troedsson, Lee et al. 2008). According to this finding, the column temperature needed to be chosen in correspondence to a predicted DNA helicity above 95 %, calculated by the Wave Navigotor Software. But variation of temperature might influence the separation condition of the PCR products by DHPLC. A similar incidence in separation of PCR products based on their sequence differences has been described for DGGE-analyzes, where double bands impeded reliable identification of bacteria or eukaryotes (Janse, Bok et al. 2004). The artifactual double bands, as consequence of a false amplification, were decreased in intensity by extending the final elongation step up to 30 min (Janse, Bok et al. 2004).

Nevertheless, we were able to clearly separate six different Vibrio isolates due to their different running characteristics in the DHPLC (see chapter 3.5.4. Fig. 19). To gain better understanding of the elution behavior in the DHPLC, we compared the retention time of individual PCR-products with their sequence characteristics. For this purpose, we determined the total number of nucleotide distances between each pair of Vibrio strains detected by PCR-DHPLC for the 278 bp *rpoB* fragment bordered by P5 (see table 16). Of the original 21 Vibrio isolates, 17 *rpoB* fragments were available for sequence analysis. Vibrio strains were tabulated due to their retention time observed in DHPLC analysis at 63.5 °C, starting with *V. gigantis* (5.2 min) to *V. navarrensis* (13.15 min) (see table 16). The heat map illustrates the amount of distinctness between Vibrio strains, in which red fields indicate a high level of distinctness and blue fields a low level of distinctness.

Table 16: Total number of nucleotide distances between each pair of Vibrio strains calculated for the 278 bp P5-amplicon

Retention time [min]	Isolates	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
5.2	1. VN3815 *V. gigantis*	0																
5.25	2. VN2866 *V. aestuariuanus*	29	0															
5.55	3. VN3824 *V. tasmaniensis*	1	30	0														
6.4	4 .VN3825 *V. xuii*	35	38	35	0													
7.2	5. VN3811 *V. diazotrophicus*	34	28	34	41	0												
7.55	6. VN3810 *V. coralliilyticus*	36	42	36	9	34	0											
8.4	7. VN3819 *V. mediterranei*	33	36	34	36	35	39	0										
9.2	8. VN2502 *V. parahaemolyticus*	41	37	42	37	40	40	33	0									
9.2	9. VN3858 *V. parahaemolyticus*	39	35	40	35	38	38	31	2	0								
9.2	10. VN3321 *V. paraheamolyticus*	39	35	40	35	38	38	31	2	0	0							
9.45	11. VN2514 *V. alginolyticus I*	38	39	39	34	41	39	33	8	6	6	0						
9.6	12. VN2756 *V. alginolyticus II*	38	39	39	33	40	38	30	7	5	5	3	0					
9.9	13. VN0232 *V. vulnificus*	42	34	42	38	32	43	36	34	32	32	34	33	0				
9.9	14. VN3114 *V. vulnificus*	42	33	42	38	32	43	35	33	31	31	35	34	1	0			
9.9	15. VN3378 *V. vulnificus*	42	33	42	38	32	43	35	33	31	31	35	34	1	0	0		
11.6	16. VN3801 *V. harveyi*	39	38	40	36	41	43	27	16	14	14	18	15	42	41	41	0	
13.15	17. VN2580 *V. navarrensis*	51	45	50	43	46	45	39	39	37	37	41	40	32	32	32	42	0

We assumed that *rpoB*-genes from closely related organisms would elute off the cartridge with similar retention times, while amplicons from more distantly related species would be expected to be more widely separated. Accordingly, highest numbers of nucleotide substitutions with 51 nucleotide differences were found between *V. navarrensis* and *V. gigantis*, which showed also most distant retention peaks in the DHPLC (see table 16). A low level of substitutions were correspondingly found within species, including the *V. parahaemolyticus*, *V. alginolyticus* and *V. vulnificus* cluster. But also low distances between *V. parahaemolyticus*, *V. alginolyticus* and *V. harveyi* were observable (see table 16). But the hypothesis of a direct correspondence between nucleotide distances and retention time was not attested to be true in all cases. *V. harveyi* and *V. vulnificus* for example showed a high level of distinctness, but eluted next to each other (*V. vulnificus*: 9.9 min; *V. harveyi*: 11.6 min; table 16). In conclusion, nucleotide distances do not describe different retention characteristics of individual Vibrio strains in the DHPLC alone.

Additionally, we plotted the GC-content of *rpoB*-amplicons against the retention time at 63.5 °C and found a significant positive correlation ($R=0.9341$, $p<0.0001$) between those parameters (Fig. 21). Moreover, we observed that differentiation of Vibrio isolates is possible if GC-variability is higher than 0.5 %.

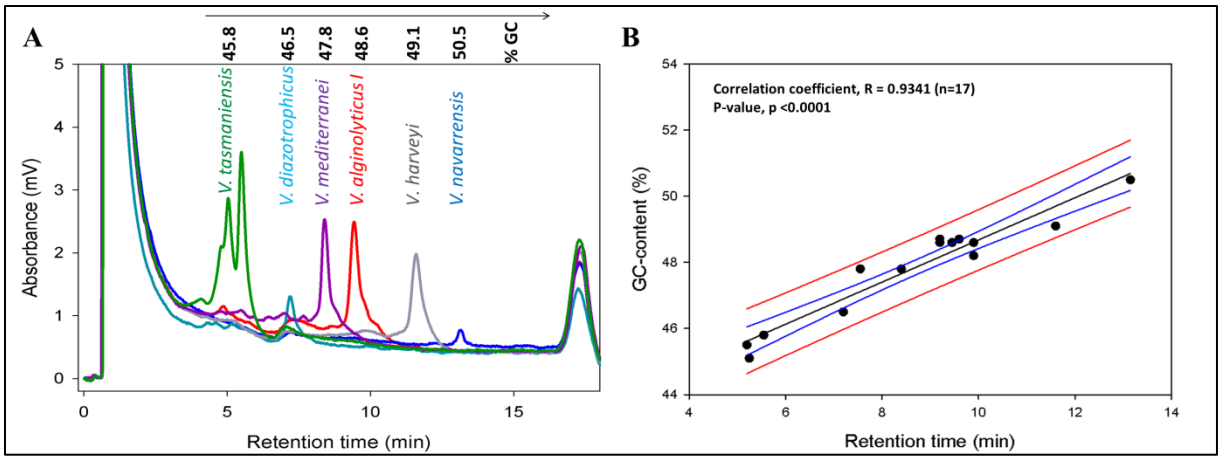

Figure 21: DHPLC analysis of 6 individual PCR-products amplified with P5GC at 62.5 °C (A). The retention time observed in the DHPLC analysis (A) was compared with the GC-content of the *rpoB* sequences (B). Correlation coefficient, R = 0.9654; p-value<0.0001 (n=17).

By examining the correlation between GC-content and retention time in the DHPLC for all *rpoB*-amplicons applied on DHPLC, our set of amplicons exhibited GC-values ranging between 45.1 % - 50.5 %. But closely related Vibrio species showed highly similar GC-contents, for example GC-contents of *V. parahaemolyticus* and *V. alginolyticus* only differed in 0.1 % (see table 17). Hence, separation by DHPLC was not possible for closely related *Vibrio* species due to their similar GC-content.

Table 17: Comparison of DHPLC retention time and GC-content of P5-amplicons of all Vibrio strains detected by PCR-DHPLC

Vibrio strains	Retention time [min]	GC-content [%]
VN3815 *V. gigantis*	5.2	45.5
VN2866 *V. aestuarianus*	5.25	45.1
VN3824 *V. tasmaniensis*	5.55	45.8
VN3817 *V. kanaoloe*	5.6	-
VN3825 *V. xuii*	6.4	47.8
VN3820 *V. pacinii*	6.8	-
VN3811 *V. diazotrophicus*	7.2	46.5
VN3810 *V. coralliilyticus*	7.55	47.8
VN3819 *V. mediterranei*	8.4	47.8
VN3813 *V. fortis*	9.1	-
VN2502 *V. parahaemolyticus*	9.2	48.7
VN3321 *V. parahaemolyticus*	9.2	48.6
VN3858 *V. parahaemolyticus*	9.2	48.6
VN3933 *V. parahaemolyticus*	9.2	-
VN2514 *V. alginolyticus I*	9.45	48.6
VN2756 *V. alginolyticus II*	9.6	48.7
VN0232 *V. vulnificus*	9.9	48.2
VN3114 *V. vulnificus*	9.9	48.6
VN3378 *V. vulnificus*	9.9	48.6
VN3801 *V. harveyi*	11.6	49.1

VN2580 *V. navarrensis*	13.15	50.5
VN3505 *V. mimicus*	13.15	-

Our analysis demonstrates that the GC-content is the main factor contributing the elution behavior in the DHPLC, because it has an influence of the relative thermostability of the DNA molecules (Lipsky, Mazzanti et al. 2001). In this connection, PCR products with a higher amount of AT-rich regions denatured more and eluted earlier than PCR products with a higher GC-content (Xiao and Oefner 2001). For other factors, such as the variability of DNA fragments not in all cases a direct correlation was found. Our study is furthermore in agreement with a recent report, which corroborates the hypothesis that DHPLC is a suitable method for the identification and separation of DNA molecules of similar sizes but with different GC-contents (Belda, Sentandreu et al. 2004).

4.6. Conclusion and Outlook

In this study we have evaluated a new *rpoB*-DHPLC approach to identify and separate potential pathogenic *Vibrio* species to offer monitoring of bacterial communities in environmental samples. There are, however, important key factors for a successful discrimination of *Vibrio* species, including primer design, optimization of the PCR protocol and adaptation of DHPLC parameters. For a reliable identification by *rpoB*-DHPLC, we evaluated 13 primer-sets, which were specific for the genus Vibrio. Overall, five primer-sets were verified by distinct amplification of 20-23 Vibrio isolates of a total number of 31 strains, and two primer-sets were selected for DHPLC-analyzes, showing the highest amount of mean variability in the amplicon-region. We investigated that in the DHPLC-system adaptation of temperature and acetonitrile gradient were essential to yield optimal separation of Vibrio isolates. Additionally, we modified the PCR-protocol by attachment of a 40 bp GC-clamp and a touchdown-PCR to stabilize DNA-molecules and permit identification of all Vibrio isolates at optimal temperature conditions. According to these improvements, ideal PCR-DHPLC conditions were found for PCR-products amplified by P5 with an attached GC-clamp and for DHPLC parameters including column temperature of 63.5 °C, a linear acetonitrile gradient of 45 % Buffer B for 0 min (loading), 54 % Buffer B for 2 min to 60 % Buffer B for 14 min at a pump flow rate of 0.9 ml min^{-1} and an injection volume of 10 µl. Using these optimized PCR-DHPLC protocol, we were able to separate six potentially pathogenic Vibrio strains due to their different retention characteristics in the DHPLC, but closely related *Vibrio* species, e.g. *V. parahaemolyticus* and *V. alginolyticus* could not be separated due to their highly similar GC content (48.6% and 48.7%). To reach efficient separation of all Vibrio isolates, further

improvements are necessary. Our investigations showed that in addition to sequence variants the GC-content in the amplicon-sequences is the discriminative factor contributing the retention characteristics of Vibrio isolates. Furthermore, melting-domains and amplicon-length can influence the separation efficiency in the DHPLC. On this ground, further investigations would include the design of new primer-pairs, which provide a high GC-variability of amplicons with a threshold > 0.5 %, different melting domains and short lengths (~100-300 bp) of the PCR-fragments. Within the characteristics of those primer-sets, it may be possible to further refine the *rpoB*-DHPLC approach to allow separation mixed samples or assessment of complex microbial populations in environmental samples.

5. Literature

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6. Appendage

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