Genotypic and phenotypic characterization of clinical and environmental *Vibrio vulnificus* isolates of the North Sea and Baltic Sea region

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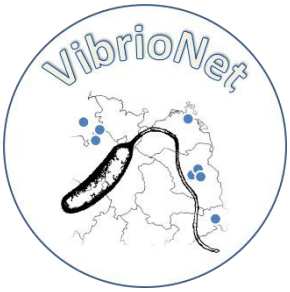

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List of Abbreviations

bp	base pairs
CDC	Centers for Disease Control and Prevention
CFU	colony forming units
C-genotype	clinical genotype
CHEF	clamped homogenous electric field
COVIS	Cholera and Other *Vibrio* Illness Surveillance system
CPCA	cellobiose-polymyxin B-colistin agar
CPS	capsular polysaccharide
ctxA$^+$	positive for the cholera toxin gene *ctxA*
CVA	CHROMagar™ *Vibrio*
dNTP	deoxynucleoside triphosphate
ECOFF	epidemiological cut-off values
E-genotype	environmental genotype
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agriculture Organization
KLIWAS	Klima Wasser Schifffahrt
LD_{50}	50% lethal dose
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MALDI-TOF MS	matrix-assisted laser desorption ionization–time of flight mass spectrometry
MARTX	multifunctional autoprocessing repeats in toxin
Mb	mega base pairs
MIC	minimal inhibitory concentration
MLST	multilocus sequence typing
MOI	multiplicity of infection
MPN	most probable number
NAB	nonulosonic acid biosynthesis
NaCl	sodium chloride
NulO	nonulosonic acid
OMV	outer membrane vesicles

OD_{600}	optical density at 600 nm
PCR	polymerase chain reaction
PFGE	pulse field gel electrophoresis
ppt	parts per thousand
RAPD-typing	randomly amplified polymorphic DNA typing
rep-PCR	repetitive extragenic palindromic PCR
RKI	Robert-Koch-Institute
RL32	ribosomal protein 32
RTX	repeats in toxin
SAC	sialic acid catabolism
SAT	sialic acid transport
ST	sequence type
TCBSA	thiosulfate-citrate-bile-sucrose agar
TRAP	tripartite ATP-independent periplasmic
USA	United States of America
VBNC	viable but non-culturable
vcg	virulence correlated gene
Vibrio spp.	*Vibrio* species
VvhA	*V. vulnificus* hemolysin A
Vvpdh	*V. vulnificus* potentially dangerous for humans
VvpE	*V. vulnificus* metalloprotease E
VvpM	*V. vulnificus* metalloprotease M
WHO	World Health Organization

1 Introduction

1.1 Taxonomy and Biochemical Characteristics of *Vibrio vulnificus*

Vibrio vulnificus belongs to the genus *Vibrio* of the family *Vibrionaceae* within the class of Gammaproteobacteria (Thompson and Swings, 2006). This family comprises of eight genera of Gram-negative, facultative anaerobic, straight or curved rods that are mostly oxidase-positive, halophilic, and motile by at least one polar flagellum (Farmer III and Janda, 2004). *Vibrio* spp. are ubiquitously distributed in marine and aquatic environments as free-living bacteria or as part of the microbiota of aquatic organisms. Many species are bioluminescent and show high tolerance towards salt (NaCl >3%), alkaline pH (pH >8), and bile salts (Gomez-Gil and Roque, 2006). These characteristics support the growth of *Vibrio* spp. in alkaline peptone water or on thiosulfate-citrate-bile-sucrose agar (TCBSA). *V. vulnificus* forms mostly green but sometimes also yellow colonies on TCBSA depending on the sucrose-fermenting ability of the strains. Among many others, further selective media are CHROMagar™ Vibrio (CVA) and cellobiose-polymyxin B-colistin agar (CPCA) on which *V. vulnificus* appears green-blue/turquoise-blue and yellow with a yellow diffusion halo, respectively (Strauch and Dieckmann, 2012). The latter one and its modifications are suggested to be the most suitable media for isolation of *V. vulnificus* from environmental samples (Oliver, 2006).

Most vibrios are symbionts or commensals of aquatic organisms, but important human and fish pathogens are also found within the genus *Vibrio*. Ten *Vibrio* species are regarded as potential human pathogens as they have been detected in human clinical specimens (Nishibuchi, 2006; Alter, 2012). These species are *Vibrio vulnificus, Vibrio alginolyticus, Vibrio cholerae, Vibrio cincinnatiensis, Vibrio furnissii, Vibrio fluvialis, Vibrio metschnikovii, Vibrio mimicus, Vibrio parahaemolyticus,* and *Vibrio harveyi*. Human infections caused by *Vibrio* spp. range from gastrointestinal, wound, or ear infections to severe septicemia. *V. vulnificus, V. cholerae*, and *V. parahaemolyticus* are considered as the most clinically relevant human pathogens within this genus (Daniels and Shafaie, 2000). The best known and most studied *Vibrio* species is *V. cholerae.* Toxigenic (*ctxA*$^+$) strains of this species belonging to the serogroups O1 or O139 are the causative agents of Cholera, a still endemic disease in many Asian and African countries with symptoms of severe watery diarrhea, vomiting, and dehydration (Kaper *et al.*, 1995; Sack *et al.*, 2004).

Taxonomy of vibrios was originally defined based on morphological features that were complemented by growth or biochemical characteristics. In the late 1980s, taxonomy has been

revolutionized by application of genomic techniques, such as sequencing of 16S rDNA or diverse housekeeping genes (e.g., *recA, rpoA, sodA,* and *rpoB*) (Thompson and Swings, 2006). Since then the number of *Vibrio* spp. with similar biochemical properties has been increasing. Consequently, biochemical differentiation at the species level becomes more complicated and can often lead to misidentification. For example, the main biochemical characteristic originally used to distinguish *V. vulnificus* from other *Vibrio* spp. was its ability to ferment lactose (Baumann and Schubert, 1984). Nowadays, however, lactose fermentation is not regarded as a reliable taxonomic trait as this ability is also found among other *Vibrio* spp. and moreover not exhibited by all *V. vulnificus* strains (Clergé *et al*., 2003). For this reason, species identification is more and more replaced or complemented by PCR-based analyses of species-specific gene targets (Nishibuchi, 2006). One possibility to discriminate between *V. cholerae*, *V. parahaemolyticus,* and *V. vulnificus* is, for example, a multiplex PCR allowing amplification of species-specific regions within the *toxR* gene homologues (Bauer and Roervik, 2007). *ToxR* is widely distributed within the family of *Vibrionaceae* and encodes a global regulator for virulence and adaption to changing environmental conditions (Nishibuchi, 2006). Another target gene used for species identification of *V. vulnificus* is *vvhA,* encoding a hemolysin/cytolysin present in all *V. vulnificus* strains (Nishibuchi, 2006).

V. vulnificus strains can be subdivided into three biotypes according to different biochemical characteristics (e.g., sorbitol fermentation, indole formation, or growth on Simmons' citrate agar) (Clergé *et al.*, 2003). Nowadays, biotypes can also be distinguished on the genetic level with PCR-based methods (Sanjuan and Amaro, 2007). Biotype 1 and 2 are comprised of diverse serotypes whereas biotype 3 constitutes of one single serotype (Amaro *et al.,* 1992; Bisharat *et al.,* 2007a; Sanjuán *et al*., 2011). Human infection is mostly associated with biotype 1 and only sporadically caused by biotype 2 *V. vulnificus*, which is predominantly a pathogen for fish and eel (Veenstra *et al*., 1992; Amaro and Biosca, 1996; Dalsgaard *et al*., 1996). Biotype 3 seems to be geographically restricted to Israel, where it was responsible for an outbreak of human infection among fish farmers and consumers after handling of infected tilapia (Bisharat *et al*., 1999; Nishibuchi, 2006; Alter, 2012).

To date, complete or draft genome sequences are available for 31 *V. vulnificus* strains[1]. The genome of *V. vulnificus* is organized in two circular chromosomes (about 3.3 Mb and 1.8 Mb) (Chen *et al*., 2003). Vibrios commonly possess two chromosomes (Okada, *et al*., 2005). While most of the genes required for cell growth are located on the large chromosome, the small

[1] http://www.ncbi.nlm.nih.gov/genome/genomes/189

chromosome preferentially harbors genes for adaptation on changing environmental conditions (Iida and Kurokawa, 2006). However, both chromosomes are required for survival, as essential genes for growth are located on each one of them.

1.2 Ecology of *Vibrio vulnificus*

V. vulnificus can ubiquitously be found in marine ecosystems as part of the autochthonous microbial community and prefers coastal, estuarine or brackish environments with moderate salinity (Thompson and Polz, 2006; Oliver, 2006). *V. vulnificus* can be isolated from seawater and sediment. In addition, it is also found within and associated to the surface of bivalve mollusks, crustaceans, plankton, and fish. The occurrence can vary significantly and correlates with salinity and seawater temperature (Oliver, 2006).

V. vulnificus concentrations in seawater are generally low, ranging from <1 to 50 CFU/mL (Pfeffer *et al*., 2003; Oliver, 2006), but can also be elevated to 10^4 CFU/mL (Vanoy *et al*., 1992; Heidelberg *et al*., 2002 a,b; Oliver, 2006). Depending on the studied area and season, bacterial counts in sediments range from <1 to 10^5 CFU/g (Oliver, 2006). Compared to the surrounding environment, *V. vulnificus* can be enriched to >10^6 CFU/g in filter feeding bivalve mollusks. The bacterial load in oysters can vary significantly, even in oysters from the same sampling site ranging from "undetectable" to >10^5 CFU/g (Birkenhauer and Oliver, 2003). A mean level of 2300 CFU/g of *V. vulnificus* in oysters was reported from the Gulf coast and Atlantic coast between May and October (Motes *et al*., 1998). While *V. vulnificus* is generally not present in fish from open sea water, high levels of 10^6 CFU/g were observed in fish from coastal areas (DePaola *et al*., 1994). Cell numbers of 10^5 to 10^7 cells/m^3 were found associated to zooplankton (Heidelberg *et al*., 2002a). While *V. vulnificus* constitutes only a small percentage of the overall *Vibrio* population in seawater (0.15%) (Pfeffer *et al*., 2003) and fish (0.3%) (Yano *et al*., 2004), it accounts for a large proportion of the whole *Vibrio/Photobacterium* population associated to plankton (57%) (Heidelberg *et al*., 2002a; Oliver, 2006).

As mentioned above, the occurrence of *V. vulnificus* in marine environments shows seasonal and regional variation and strongly depends on seawater temperature and salinity. Although *V. vulnificus* has sporadically been found in seawater at temperatures as low as 8 °C (Wright *et al*., 1996), most studies reported detection of *V. vulnificus* at seawater temperatures higher than 15 °C (Oliver, 2006). *V. vulnificus* has been identified predominantly in brackish or coastal environments with salinity levels between 8 ppt and 23 ppt (Oliver, 2005a). For *V. vulnificus* to survive in seawater, an optimal temperature range of 13 to 22 °C (at 10 ppt) and

an optimal salinity range of 5-25 ppt (at 14 °C), or 5-15 ppt (at 21 °C) was determined under laboratory conditions (Kaspar and Tamplin, 1993). An optimal salinity range of 5 to 10 ppt was suggested by Randa *et al.,* as they found the highest occurrence of *V. vulnificus* within this salinity range (Randa *et al*., 2004). Motes *et al.* (1998) observed increasing *V. vulnificus* levels in oysters with rising seawater temperature up to 26 °C, but numbers remained constant at higher temperatures. The numbers of *V. vulnificus* in oysters were high at salinity levels between 5 and 25 ppt, while decreased levels were found at a salinity above 28 ppt (Motes *et al*., 1998).

The low detection rate of *V. vulnificus* in seawater at temperatures below 15 °C can partly be explained by its entry into the "viable but non-culturable" (VBNC) state (Oliver, 2005a). It is assumed that low temperatures (approximately <13 °C) or nutrient deficiencies can trigger the entry of *V. vulnificus* into the VBNC state, in which cells are dormant with reduced metabolic activity and display increased stress resistance (Oliver, 2006). This represents a diagnostic problem since VBNC cells remain undetected by culture-based approaches, but still pose an infection risk (Oliver, 2006). They can resuscitate when more favorable conditions arise, for example, by temperature and nutrition upshift and can then cause fatal infections in animals (Oliver and Bockian, 1995; Oliver, 2006). However, lack of genetic evidence and reproducibility in resuscitation still keep a debate ongoing, whether entering the VBNC state actually is a regulated stress response or simply a consequence of dying cells (McDougald and Kjelleberg, 2006).

However, the extremely low frequency of *V. vulnificus* isolation at low seawater temperatures cannot entirely be explained by its entry into the VBNC state, as some studies proved the absence of *V. vulnificus* with PCR-based techniques (Randa *et al*., 2004; Collin and Rehnstam-Holm, 2011). Several studies assumed a strong decline of the *V. vulnificus* population in the water column during winter with a suggested hideaway in sediments (Vanoy *et al*., 1992; Randa *et al*., 2004; Böer *et al*., 2013; Brennholt *et al*., 2014). Mixing events in spring and summer may lead to a renewed inoculation of the water column and to an initiation of the summer bloom, when more favorable conditions arise. Indeed, the isolation from sediments (Vanoy *et al*., 1992; Böer *et al*., 2013) and bottom-dwelling fish (DePaola *et al*., 1994) was possible during winter, while *V. vulnificus* was not detectable in the surrounding seawater. Besides, sediments were often reported to contain *V. vulnificus* concentrations that were one to two orders of magnitude higher compared to the surrounding water column (Vanoy *et al*., 1992; Randa *et al*., 2004; Oliver, 2006; Böer *et al*., 2013; Brennholt *et al*., 2014). Further studies describe the isolation of *V. vulnificus* from mussels at low seawater temperatures of 8 °C

(Hoi *et al*., 1998) or 10.8 °C (Motes *et al*., 1998). These observations suggest that sediments and marine organisms represent a refuge where *V. vulnificus* are sheltered from harsh conditions, such as nutrient deficiencies or cold temperatures in winter.

The preference of *V. vulnificus* for low to moderate salinity and warm temperature is also reflected by the seasonal and spatial distribution pattern in German coastal waters (Hauk *et al*., 2010; Böer *et al*., 2013; Brennholt *et al*., 2014; Huehn *et al*., 2014), where *V. vulnificus* has generally been detected in seawater with a salinity range of 2.3-17.2 ppt and a temperature range of 13.9-26.5 °C (Hauk *et al*., 2010; Böer *et al*., 2013). The North Sea and Baltic Sea coasts are characterized by a specific *Vibrio* species composition, which is probably influenced by the different prevailing salinity ranges. The dominant *Vibrio* spp. along the North Sea coast with salinity levels up to 34 ppt (Baker-Austin *et al*., 2012b) was *V. alginolyticus*, followed by *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* non-O1/non-O139. In contrast, the most prevalent species along the Baltic Sea coast with salinity levels below 18 ppt (Baker-Austin *et al*., 2012b) was *V. cholerae* non-O1/non-O139, followed by *V. vulnificus, V. alginolyticus* and *V. parahaemolyticus* (Brennholt *et al*., 2014). *V. vulnificus* was isolated with a frequency of 14 to 77% between 2004 and 2007, and with levels up to 10^6 CFU/L in 2008 from seawater along the Mecklenburg-Western Pomeranian coastline of the Baltic Sea (Hauk *et al*., 2010). Along the German North Sea coast, occurrence of *V. vulnificus* seems to be confined to mesohaline environments of the river estuaries of Weser and Ems with salinity levels below 18 ppt (Brennholt *et al*., 2014). Between 2009 and 2011, *V. vulnificus* was detected with a frequency of 5% positive samples in the North Sea with levels up to $4.8*10^4$ CFU/kg in sediments and $4.6*10^2$ CFU/L in seawater (Böer *et al*., 2013).

Mussel harvesting areas for primary production in Germany are exclusively located at the North Sea coastline. Probably due to higher salinity, a low prevalence of *V. vulnificus* was observed in blue mussels (*Mytilus edulis*) from harvesting areas in the Wadden Sea (Huehn *et al*., 2014). In 2012 and 2013, *V. vulnificus* was sporadically found with a frequency of 2.1% and 1.4% positive blue mussel samples. Even in the hot summer of 2010, *V. vulnificus* levels ranged from <1 to $4*10^2$ MPN/g (Bartelt, 2014), which is still one order of magnitude lower than concentrations associated with human infection (Jackson *et al*., 1997). A higher prevalence was found in mussels from the Baltic Sea at the South Coast of Sweden, where *V. vulnificus* was detected in 80% of sampled *Mytilus edulis* during the hot summer of 2006 but exclusively at seawater temperatures above 17 °C (Collin and Rehnstam-Holm, 2011). Although some studies have described the detection of *V. vulnificus* in mussels at low seawater temperatures (Hoi *et al*., 1998; Motes *et al*., 1998), only temperatures above 20 °C are regarded as a high risk

for multiplication to clinically relevant numbers in mussels (Shapiro *et al*., 1998; Nishibuchi and DePaola, 2005).

Interestingly, *V. vulnificus* could not be detected in German coastal waters until a critical temperature threshold of 20 °C had been exceeded. However, it has repeatedly been found for a long time afterwards, even at low temperatures of 10 °C (Hauk *et al*., 2010; Böer *et al*., 2013; Huehn *et al*., 2014). In the course of climate change, an increase in the occurrence of this pathogen is expected for European coastal waters. Rising sea surface temperatures, a higher frequency of heat waves, and decreasing salinity due to heavy rainfall events may favor proliferation of *V. vulnificus*. In addition, rising sea levels accompanied by flooding of coastal areas may expand low-salinity environments and thus may promote a further dissemination of *V. vulnificus* (Lowe *et al*., 2009; Baker-Austin *et al*., 2010, 2012b Martinez-Urtaza *et al*., 2010; Brennholt *et al*., 2014). The Baltic Sea is especially regarded as a high-risk area, as it is a low-salinity intracontinental sea that is described as one of the fastest warming marine ecosystems on Earth (Baker-Austin *et al*., 2012b). The number of months with sea surface temperatures above 17 °C - known for favoring the occurrence of *Vibrio* spp. - is expected to increase at the German Baltic Sea coast from one month (1970-1999) up to three months in the distant future (2070-2099) (Brennholt *et al*., 2014). The mean annual seawater temperature of the North Sea is predicted to rise about 2.7 °C until 2099, resulting in an estimated increase of *V. vulnificus* occurrence up to 30% in the estuaries. Moreover, increased fresh water flow after rainfall events and a resulting shift of brackish water zones may lead to a further dissemination of *V. vulnificus* beyond the estuaries of the North Sea (Brennholt *et al*., 2014).

1.3 Disease and Epidemiology of *Vibrio vulnificus*

There are two ways to get infected by *V. vulnificus*, which typically result in one of three clinical manifestations: gastroenteritis, primary septicemia, or wound infection. One way of infection is through the consumption of raw or undercooked seafood or by swallowing seawater loaded with the pathogen (Shapiro *et al*., 1998; Oliver, 2006). This can result in a rarely reported and relatively mild gastroenteritis with symptoms of diarrhea, abdominal cramps, nausea, vomiting, and fever (Johnson *et al*., 1986; Oliver, 2006). More frequently however, foodborne infections are reported to result in a life-threatening primary septicemia. This is typically accompanied by symptoms of hypotension and development of characteristic secondary cutaneous lesions on the extremities, which can progress to necrotizing fasciitis (Shapiro *et al*., 1998; Oliver, 2006). Septicemic shock with systolic blood pressure below 85 mm Hg is a serious complication and

a strong indication of a fatal outcome when occurring within the first 12 hours of hospital admission (Tacket *et al*., 1984; Klontz *et al*., 1988; Oliver, 2006).

The other transmission route involves wounds exposed to seawater during aquatic activities or injuries incurred by handling or processing of seafood (Klontz *et al*., 1988; Oliver, 2006). Even small wounds due to insect bites can be entry points for *V. vulnificus* leading to fatal infections (Klontz *et al*., 1988; Oliver, 2005a, 2006). It is also assumed that *V. vulnificus* can persist on the skin for some time and is able to infect wounds acquired 24 hours after exposition to seawater (Colodner *et al*., 2002). Entry of *V. vulnificus* through skin lesions can result in severe skin and soft-tissue infections (Oliver, 2005a, 2006). Such infections can be first observed as swelling and erythema with intense pain and characteristic bullae. Wound infections can rapidly progress to cellulitis, necrotizing fasciitis, and finally to secondary septicemia (Klontz *et al*., 1988; Oliver, 2005b; Horseman and Surani, 2011). In addition, atypical and rare manifestations of *V. vulnificus* infection, such as peritonitis, pneumonia, endometritis, meningitis, septic arthritis, osteomyelitis, endophthalmitis, and keratitis have been reported, exceptionally (Horseman and Surani, 2011).

First symptoms of primary septicemia are usually developed within 36 hours after consumption of seafood. However, incubation periods ranging from seven hours to ten days have also been described (Oliver, 2005a, 2006). For wound infections, incubation periods ranging between the extremes of three hours and twelve days have been reported, but first symptoms usually occur within the first 24 hours (Oliver, 2005a, 2006). Treatment of *V. vulnificus* infections includes therapy with antimicrobial agents, which should be administered as early as possible. Patients suffering from primary septicemia generally do not survive, if no antimicrobial therapy is administered within the first 72 hours (Klontz *et al*., 1988; Oliver, 2006). Moreover, the fast progression of the disease often makes surgical debridement and amputation necessary in early stages of infection (Daniels and Shafaie, 2000).

An epidemiological study of 442 cases of *V. vulnificus* infection occurring between 1988 and 1996 in the United States reported an overall mortality rate of 39% for the three major syndromes combined, with death occurring within a mean time of three days (Shapiro *et al*., 1998). Specifically primary septicemia showed the highest case fatality rate of 61% while that of wound infections was reported to be lower (17%) (Shapiro *et al*., 1998). One fatality has also been reported for a case of gastroenteritis in an 89-year-old woman (Hlady and Klontz, 1996).

Of those patients with primary septicemia, 97% were predisposed by at least one underlying disease, such as chronic liver diseases or immunocompromising conditions, while this was only the case for 68% and 35% of patients suffering from wound infection and

gastroenteritis, respectively (Shapiro *et al*., 1998). Regular alcohol abuse resulting in liver damage, or chronic liver diseases, such as hepatitis, hemochromatosis, and cirrhosis are regarded as the most important host-related risk factors favoring *V. vulnificus* infection (Shapiro *et al*., 1998; Oliver, 2005a). It is assumed that higher serum iron levels resulting from these diseases contribute to an increased susceptibility to the pathogen. There are two hypotheses to explain this circumstance. On the one hand, excess serum iron can lead to reduced neutrophil activity and thus to immunocompromising conditions in the host. Another explanation is an increased growth rate of the pathogen due to the better availability of iron (Jones and Oliver, 2009). It has already been shown *in vitro* that higher iron levels increase the ability of *V. vulnificus* to grow in human serum (Wright *et al*., 1981; Simpson and Oliver, 1987). Additionally, chronic diseases (diabetes, renal diseases, heart diseases, and cancer), chronic use of antacids, and immunocompromising conditions (e.g., corticosteroid treatment, chemotherapy, and AIDS) are associated with a higher risk for *V. vulnificus* infection (Shapiro *et al*., 1998; Oliver, 2005a). Wound infections often occur in individuals without any identifiable risk factor (Shapiro *et al*., 1998). However, progression of wound infection to septicemia with a fatal outcome is highly associated with these predisposing diseases (Shapiro *et al*., 1998; Oliver, 2005a). Most cases of primary septicemia or wound infection occurred in males with a median age of 54 and 59, respectively (Shapiro *et al*., 1998). It is suspected that estrogen could protect women from the LPS endotoxin in a so far unknown mechanism (Merkel *et al*., 2001; Oliver, 2005a).

Human infections occur mainly in summer months, when high seawater temperatures favor proliferation of the pathogen (Anonymous, 2011). The frequency of infections mirrors the seasonal distribution of *V. vulnificus* in the environment. A study assessing *V. vulnificus* levels in oysters over a 3-year period in Florida revealed that oyster-related cases of primary septicemia occurred only when oysters presented a minimum load of 10^3 CFU/g (Jackson *et al*., 1997). In an illustrative case report of primary septicemia in a 25-year-old male with underlying hepatitis, the total amount of ingested cell numbers from eating six raw oysters was estimated to be $6*10^5$ CFU. Remarkably, these were able to grow up to $1.1*10^8$ CFU/mL in the blood of the patient, who died in less than 24 hours after hospitalization (Jackson *et al*., 1997).

The incidence rate of *V. vulnificus* infections worldwide is relatively low, but has increased in recent years. Hsueh *et al*. reported that the mean annual case rate in Taiwan raised from four cases in the period from 1985 to 1993 up to 14 cases in the following years of 1994 to 2000 (Hsueh *et al*., 2004). In the United States, *V. vulnificus* is the leading cause of seafood-associated deaths (95%) (Oliver, 2005a) and infections demonstrate the highest case-fatality

rate of any vibriosis and even of any food-borne disease (Mead *et al*., 1999; Scallan *et al*., 2011; Newton *et al*., 2012). Surveillance data indicated that the annual incidence of *V. vulnificus* infections and of other vibrioses in the United States is increasing (Newton *et al*., 2012). In the period from 1988 to 1996, a mean annual number of 47 *V. vulnificus* infections was reported to the Centers for Disease Control and Prevention (CDC) (Shapiro *et al*., 1998). This number has more than doubled to an average of 103 cases between 1996 and 2010 (Newton *et al*., 2012). Of 422 *V. vulnificus* infections reported to the CDC between 1988 and 1996, 45% were wound infections and 43% were cases of primary septicemia (Shapiro *et al*., 1998).

In Germany, on the other hand, *V. vulnificus* infections occurred only sporadically and cases were almost exclusively wound infections that were contracted during seawater activities. This may be attributed to the low prevalence of *V. vulnificus* in bivalve mollusks from German mussel harvesting areas and a generally lower seafood consumption in Germany. As notification for these infections is not mandatory in Germany, reliable epidemiological data is missing (Huehn *et al*., 2014). Literature research and unpublished information from health authorities revealed at least eleven cases of *V. vulnificus* infection in Germany between 1994 and 2011 (Table 1). For the whole Baltic Sea region, a total of 24 cases were reported in Germany, Denmark and Sweden between 1991 and 2011 (Andersen, 1991; Bock *et al*., 1994; Melhus *et al*., 1995; Bruun *et al*., 1996; Dalsgaard *et al*., 1996).

Most of these cases occurred in summers after long periods of exceptionally warm weather, which resulted in seawater temperatures above 20 °C for several weeks (in 1994, 2003, 2006, and 2010). Additional six cases of *V. vulnificus* infection were reported in Germany during the hot summer in 2014[2,3], but most of them have not been confirmed by health authorities yet (Hauk, 2014; C. Frank, RKI, Berlin, personal communication). So far, all cases occurring in Germany have been exclusively reported from the Baltic Sea. Due to the dramatic course of disease, the number of unreported cases of primary septicemia or wound infection is supposed to be low. However, it is possible that mild infections remain unreported (Scallan *et al*., 2011).

The increased frequency of infections during heat waves demonstrates the strong impact of climate anomalies on the incidence of *V. vulnificus* infections. In parallel to an increased occurrence of *V. vulnificus* in German coastal waters in the course of climate change, also a higher frequency of human infection is expected for the future. Further aspects discussed to

[2] http://promedmail.chip.org/pipermail/promed/2014-September/005308.html
[3] http://www.mz-web.de/panorama/toedliche-keime-in-der-ostsee-urlauber-an-infektion-gestorben,20642226,28270840.html

contribute to a rising incidence and clinical burden of *V. vulnificus* infection in Europe are an increasing consumption of seafood and increased bathing tourism due to extended bathing seasons during warm weather periods (Dalsgaard *et al*., 1996; Baker-Austin *et al*., 2010). Moreover, demographic change coupled with an increased occurrence of chronic diseases will lead to rising numbers of individuals susceptible to *V. vulnificus* infection (Baker-Austin *et al*., 2010).

Table 1. Cases of *V. vulnificus* infection occurring in Germany between 1994 and 2011.

Year	Location	Age /Gender	Type of Infection	Underlying Diseases	Reference
1994	Usedom	71/73/f	wound infection	none	Hoyer *et al*., 1995; Stephan and Knabner, 1996; Kuhnt-Lenz *et al*., 2004
2002	Luebeck Bay	59/f	uncertain, presented with septicemia and swellings after bathing in the Sea	immune deficiency, non-Hodgkin's lymphoma	Kuhnt-Lenz *et al*., 2004
2003	Usedom	50/m	wound infection	diabetes mellitus, heart disease, diabetic skin ulcers, and others	Ruppert *et al*., 2004; Frank *et al*., 2006
2003	Usedom	62/f	wound infection, fatal	arterial hypertension, liver cirrhosis, and others	Ruppert *et al*., 2004; Frank *et al*., 2006
2006	Usedom	57/m	wound infection	diabetes mellitus,	Frank *et al*., 2006
2006	Usedom	72/m	wound infection	coronary heart disease, chronic leg edema	Frank *et al*., 2006
2006	Stralsund	76/m	wound infection	chronic skin ulcer	Frank *et al*., 2006
2010	Rostock	58/m	wound infection	unknown	Frank, C. (RKI), personal communication; VN-288
2010	Ruegen	31/m	wound infection	unknown	Böer *et al*., 2012; Frank, C. (RKI), personal communication
2010	Ruegen	81/f	wound infection, double infection with *V. cholerae*, fatal	multiple diseases, not specified	Böer *et al*., 2012; Frank, C. (RKI), personal communication
2011	Lubmin	69/m	uncertain, presented with septicemia and ulcerations after bathing in the Sea	heart disease	Frank, C. (RKI), personal communication

1.4 Virulence Factors and Pathogenesis of *Vibrio vulnificus*

During the last decades, numerous research activities have been dedicated to the characterization of putative virulence factors, leading to the present understanding of *V. vulnificus* pathogenesis. One of the first defense mechanisms *V. vulnificus* encounters after entering the body via the oral route is the acidic pH of the stomach. *V. vulnificus* is described as highly susceptible to low pH, but can adapt by expression of enzymes, such as lysine-decarboxylase and different superoxide dismutases that help coping with acidic stress and the resulting superoxide radicals (Jones and Oliver, 2009; Horseman and Surani, 2011).

After entering the small intestine, *V. vulnificus* needs to adhere to epithelial cells to colonize the gut. The main factors involved in the adhesion process are capsular polysaccharide (CPS), type IV pili, and flagella. In addition, the outer membrane protein OmpU and the membrane-bound lipoprotein IlpA contribute to adhesion to epithelial cells (Jones and Oliver, 2009). To warrant sufficient nutrient supply and thus survival in the body, *V. vulnificus* possesses an enormous repertoire of diverse extracellular degradative toxins and enzymes. These include cytolysins, hemolysins, and RTX toxins (e.g., MARTX, RTXA2, RTXA3, VvhA, VllY, HlyIII), proteases (e.g., VvpE), lipases (e.g., phospholipase A), mucinases, chondroitinases, hyaluronidases, DNases, esterases, and sulfatases (Testa *et al*., 1984; Oliver *et al.*, 1986; Chang *et al*., 1997; Chen *et al*., 2003, 2004; Lee *et al*., 2004b; Gulig *et al*., 2005; Miyoshi, 2006; Koo *et al*., 2007; Jones and Oliver, 2009; Joseph, 2009). In this context, the ability to metabolize sialic acids is of great importance, especially for survival in the intestine, where sialic acids occur in high abundance (Jeong *et al*., 2009; discussed in more detail in Section 1.6.6).

Diarrheal symptoms of rarely reported gastroenteritis are probably caused by an enterotoxin, whose presence was assumed in culture supernatants of weakly virulent isolates, as the supernatants caused fluid accumulation in the rabbit ileal loop test (Stelma *et al*., 1988). However, the enterotoxic activity, which was not correlated with hemolytic or proteolytic activity, has not been further investigated and no responsible enterotoxin has been identified, so far.

To further reach deeper tissue and finally the blood circulation to produce systemic infection, *V. vulnificus* must invade the intestinal mucosa. It is assumed that *V. vulnificus* achieves this by active destruction of the epithelial barrier, rather than by a passive translocation mediated by host immune cells (e.g., microfold cells or dendritic cells) (Hong *et al*., 2013). The highly cytotoxic multifunctional autoprocessing repeats in toxin (MARTX; formerly RTXA or

RTXA1) is thought to be involved in this invasion process, as an *rtxA1* mutant was defective in disseminating from the intestine into the blood stream (Kim *et al*., 2008). In addition, the observed characteristics of the MARTX toxin suggest its contribution to this process (Kim *et al*., 2008). These include strong contact-dependent cytotoxicity, induction of cell rounding, and particularly the accumulation at cellular junction areas (Kim *et al*., 2008). Finally, Jeong and Satchell (2012) demonstrated that not only MARTX, but also the cytolytic hemolysin VvhA causes necrosis in epithelial cells resulting in severe tissue damage in the small intestine. Although MARTX seems to play a predominant role, only the double mutant completely lost the ability to cause necrotic intestinal tissue damage and to disseminate into the bloodstream and the liver. This implies that both enzymes act additively and complement each other in the destruction of the mucosal barrier (Kim *et al*., 2008; Jeong and Satchell, 2012).

Within the bloodstream, *V. vulnificus* needs to evade the immune system of the host. This is primarily achieved by expression of CPS, which is essential for virulence in mice (Simpson *et al*., 1987). Encapsulation prevents recognition and opsonization by the complement system, thus averting phagocytosis by macrophages (Johnson *et al*., 1984; Tamplin *et al*., 1985; Wright *et al.*, 1990). It is assumed that CPS allows evasion of the non-specific immune response and mediates resistance to the bactericidal effects of human serum by masking surface antigens (Jones and Oliver, 2009; Horseman and Surani, 2011). Lipopolysaccharide (LPS) and CPS are further shown to promote inflammation and septic shock by inducing secretion of pro-inflammatory cytokines via distinct mechanisms (Powell *et al*., 1997). It was speculated that they act synergistically during systemic *V. vulnificus* infections (Strom and Paranjpye, 2000). Another mechanism of *V. vulnificus* used to avoid the immune response, is the active killing of immune cells, for example, by cytotoxic activity that may be mediated by VvhA (Lee *et al*., 2004b), or by inducing apoptosis in macrophages (Kashimoto *et al*., 2003; Tsuchiya *et al*., 2007). In addition, MARTX has been shown to prevent phagocytosis by an unknown mechanism, other than simple destruction of phagocytes (Lo *et al*., 2011).

Survival and multiplication of *V. vulnificus* in the blood are strongly dependent on the availability of iron (Wright *et al*., 1981; Morris *et al*., 1987; Simpson and Oliver, 1987). This is underlined by the increased susceptibility of persons with higher serum iron levels due to various liver diseases. Consequently, animal experiments are often performed in an iron-overloaded mouse model in which mice are treated with ferric ammonium citrate or iron-dextran to mimic the higher iron availability in susceptible hosts (Stelma *et al*., 1992; Gulig *et al*., 2005). The significance of available iron for pathogenicity of *V. vulnificus* was confirmed by a decrease of the 50% lethal dose (LD_{50}) from 10^6 to 1 CFU in the iron-overloaded mouse

model compared to non-treated mice (Wright *et al*., 1981; Stelma *et al*., 1992). Because of a strong correlation to virulence in mice, Stelma *et al.* (1992) postulated that the ability to use transferrin-bound iron represents a critical virulence factor of *V. vulnificus.* Several iron acquisition systems contribute to virulence of *V. vulnificus* (Litwin *et al*., 1996; Jones and Oliver, 2009). These include the heme-receptor HupA (Litwin and Byrne, 1998), the iron receptors TonB und TolC (Jones and Oliver, 2009), as well as catechol and hydroxamate siderophore systems allowing acquisition of iron bound to transferrin or lactoferrin (Simpson and Oliver, 1983). One example is the siderophore system comprising the genes *venB, vuuA*, and *viuB* that mediate synthesis, uptake, and intracellular cleavage of the catechol siderophore vulnibactin, respectively (Swain, 2011). It was suggested that cleavage of transferrin by the metalloprotease VvpE is essential for vulnibactin to efficiently scavenge bound iron (Okujo *et al*., 1996). Finally, the hemolytic activities of VvhA, (Lee *et al*., 2004b), MARTX (Kim *et al*., 2008), or phospholipase A (Koo *et al*., 2007) enable *V. vulnificus* to lyse erythrocytes and to use released hemoglobin as an additional iron source.

Further dissemination in the body is accomplished by flagella-mediated motility. It has already been shown that deletions in the flagellar operon resulted in an attenuated adhesion ability, reduced biofilm formation, and virulence in mice (Kim and Rhee, 2003; Lee *et al*., 2004a). Penetration into deeper tissue is enabled by the action of diverse degradative enzymes, such as MARTX, VvhA, or VvpE that are suspected to contribute to the extremely destructive character of the pathogen. The cytolytic hemolysin VvhA was found associated to outer membrane vesicles (OMV), which allow the delivery of VvhA into the host cells, where it can fulfill its cytolytic function (Kim *et al*., 2010). Associated to OMVs, bacterial toxins can be quickly distributed throughout the entire organism within the body fluids. This association allows them to reach deeper tissues that may be not yet accessible for the whole bacterial cell (Kuehn and Kesty, 2005). Thus, VvhA coupled to OMVs may pave the way for *V. vulnificus* cells by its proven cytotoxic as well as its vasodilatory activity (Lee *et al*., 2004b). The elastolytic *V. vulnificus* metalloprotease VvpE was speculated to contribute to the generation of secondary skin lesions. Purified protease increases vascular permeability through generation of the vasodilator bradykinin, resulting in edema characteristic for *V. vulnificus* infection. Moreover, VvpE degrades type IV collagen, a major component of the vascular basement membrane, resulting in breakdown of capillary vessels, and thus in severe hemorrhagic tissue damage (Miyoshi, 2006; Jones and Oliver, 2009). Although several studies suggested a minor role of VvpE for virulence in mice (Jeong *et al*., 2000; Shao and Hor, 2000; Fan *et al*., 2001), it is essential for the swarming ability of *V. vulnificus*, which in turn is regarded as a general

virulence factor contributing to bacterial adhesion and further colonization of the host (Kim *et al*., 2007). Although a variety of potential virulence factors have been characterized and shown to contribute to virulence, the pathogenesis of *V. vulnificus* infection has not yet been fully elucidated (Gulig *et al*., 2005; Quinones-Ramirez *et al*., 2010).

1.5 Enigma of *Vibrio vulnificus*

The high abundance of *V. vulnificus* worldwide together with increasing numbers of elderly people and people with predisposing diseases are inconsistent with the low rate of *V. vulnificus* infections. For example, Klontz estimated that the number of individuals susceptible to *V. vulnificus* infection ranges between 12 and 30 million people in the United States, which represent about seven percent of the adult American population (Oliver, 2006; Anonymous, 2005). In addition, about 20 million people in the United States were estimated by the Food and Drug Administration to consume 75-80 million servings of raw oysters annually (Oliver, 2005a, 2006). However, the mean annual number of reported *V. vulnificus* septicemia after consumption of Gulf coast oysters was as low as 32 cases between 1995 and 2001 (Anonymous, 2005). Thus, the question arises, why there are so few cases with so many people at risk. One explanation could be that additional predisposing host factors have to be present to further increase the susceptibility to the pathogen and that these may be encountered in only a small proportion of the population (Oliver, 2005a, 2006). Another explanation could be that only few *V. vulnificus* strains are virulent and able to effectively cause infection in susceptible individuals (Oliver, 2005a). The latter hypothesis is supported by observations made by Jackson *et al.* (2007), who revealed a high genetic diversity among *V. vulnificus* isolates obtained from case-associated oysters. Yet, only one strain with a distinct PFGE profile was recovered from the blood of the patient (Jackson *et al*., 1997). Moreover, only this clinical isolate and oyster isolates with matching PFGE profiles were virulent in mice, while oyster isolates with a different PFGE pattern were avirulent. These experiments clearly indicate that not all strains are equally virulent and highlight the need for reliable methods to identify strains with a higher pathogenicity potential.

In this regard, many studies have been dedicated to the characterization of clinical and environmental isolates in order to reveal virulence attributes essentially required for pathogenicity. If not all strains are equally virulent, then such critical virulence factors should be present in the majority of isolates obtained from clinical specimens (in the following termed “clinical isolates”), as they have already proven to cause human infection. However, since

clinical isolates originally come from the environment, such virulence factors can also be present in non-clinical isolates (Oliver, 2005a).

In recent years, many researchers have tried to assess the virulence of *V. vulnificus* isolates in animal and cell culture models with different results depending on the cell line, animal model and the strain collection used. Generally, the majority of strains were reported to be virulent in mice and to possess similar patterns of phenotypic characteristics, regardless of their origin (Johnson and Calia, 1981; Tison and Seidler, 1981; Oliver *et al*., 1986; Tison and Kelly, 1986; Kaysner *et al*., 1987; Stelma *et al*., 1992; DePaola *et al*., 2003; Gulig *et al*., 2005; Oliver, 2005a). Differences in mouse virulence were observed among the isolates but could not be traced back to a single virulence determinant (Jackson *et al*., 1997; Garcia Moreno and Landgraf, 1998; Starks *et al*., 2000; Thiaville *et al*., 2011). The virulence-associated factors discussed in the section above are present in most of the *V. vulnificus* isolates and cannot be used to differentiate between avirulent and virulent isolates (Oliver *et al*., 1986; Strom and Paranjpye, 2000; Anonymous, 2005). The gene *vvhA*, for example, is used as a universal species marker in PCR analyses (Nishibuchi, 2006). Additionally, CPS expression was found to be essential for establishing an infection (Simpson *et al*., 1987), but not all encapsulated isolates were actually virulent in mice (Stelma *et al*., 1992; Starks *et al*., 2000). Virulence of *V. vulnificus* appears to be multifactorial and establishment of infection is accomplished by the synergistic action of diverse virulence factors (Strom and Paranjpye, 2000; Anonymous, 2005).

1.6 Clinical *versus* Environmental Isolates of *Vibrio vulnificus*

Molecular characterization of clinical and environmental *V. vulnificus* isolates using CHEF- gel electrophoresis (Buchrieser *et al*., 1995), ribotyping (Hor *et al*., 1995; Tamplin *et al*., 1996), PFGE (Tamplin *et al*., 1996), and rep-PCR typing (Chatzidaki-Livanis *et al*., 2006a) revealed high genotypic diversity. Different multilocus sequence typing analyses also showed great genetic heterogeneity among the *V. vulnificus* population but also the existence of two major lineages (Bisharat *et al*., 2005, 2007b; Cohen *et al*., 2007; Lubin *et al*., 2012; Reynaud *et al*., 2013). Additionally, *V. vulnificus* isolates can be divided into two genotypes based on polymorphisms at different gene loci. These gene variations correlate with the isolation source, suggesting that different genotypes of *V. vulnificus* possess different pathogenic potential. While environmental isolates were mainly of the E-genotype (environmental genotype associated with lower pathogenicity potential), the C-genotype (clinical genotype associated with higher pathogenicity potential) is found in the majority of clinical isolates. Consequently, the C-type allele may serve as a marker to identify highly virulent strains. Such gene

polymorphisms were found within the virulence-correlated gene *vcg* (Rosche *et al*., 2005), the 16S rDNA (Nilsson *et al*., 2003), the pilus type IV assembly gene *pilF* (Roig *et al*., 2010), the *viuB* gene encoding the vulnibactin utilization protein (Panicker *et al*., 2004), the cytolysin/hemolysin gene *vvhA* (Senoh *et al*., 2005), and the capsular polysaccharide operon (Chatzidaki-Livanis *et al*., 2006b). In addition, several more genes, such as *nanA* or genomic region XII, are found to be lineage-specific and assumed to be associated with virulence (Cohen *et al*., 2007; Lubin *et al*., 2012).

1.6.1 Multilocus Sequence Typing

Multilocus sequence typing (MLST) is a method applied for phylogenetic and epidemiological analyses of bacterial strains on a global scale. For MLST analyses, internal fragments (generally 300-500 bp) of a distinct set of housekeeping genes are sequenced and analyzed for point mutations. For every occurring unique sequence at a single locus a distinct allelic number is assigned. Allelic numbers of each locus are combined to an allelic profile, which in turn is assigned a distinct sequence type (ST). Allelic numbers and sequence types are numbered in the order of appearance.

MLST analysis of *V. vulnificus* according to Bisharat *et al*. (2005) is performed on ten housekeeping genes; five from each chromosome: *glp* (glucose-6-phosphate isomerase), *gyrB* (DNA gyrase, subunit B), *mdh* (malate-lactate dehydrogenase), *metG* (methionyl-tRNA synthetase), and *purM* (phosphoribosyl-aminoimidazole synthetase) located on the large chromosome and *dtdS* (threonine dehyrogenase), *lysA* (diaminopimelate decarboxylase), *pntA* (transhydrogenase alpha subunit), *pyrC* (dihydroorotase), and *tnaA* (tryptophanase) located on the small chromosome (Bisharat *et al*., 2005, 2007b). Sequence and profile definitions as well as primers and conditions used for amplification and sequencing are provided on the *V. vulnificus* Multilocus Sequence Typing website[4].

MLST analysis showed a division of the *V. vulnificus* population into two major clusters or lineages (Bisharat *et al*., 2005, 2007b). Environmental isolates were overrepresented in cluster I, while the majority of cluster II isolates were of clinical origin, implying a higher pathogenicity potential for the latter cluster. Furthermore, a correlation between MLST cluster II and a higher virulence in mice has been described (Thiaville *et al*., 2011). Grouping of the isolates to the different clusters did not reflect the existence of the three biotypes. Biotype 1 was present in both clusters, whereas biotype 2 was only present in Cluster I. Biotype 3 was

[4] http://pubmlst.org/vvulnificus

found at an intermediate position between the two clusters and is therefore regarded as a hybrid containing major parts from both lineages (Bisharat *et al*., 2005, 2007b). Similar observations were made based on other MLST schemes and strain collections (Cohen *et al*., 2007; Sanjuán, 2008; Thiaville *et al*., 2011; Lubin *et al*., 2012; Reynaud *et al*., 2013).

1.6.2 16S rRNA

Sequence analysis of the 16S rRNA gene revealed that polymorphisms in a total of 17 base pairs allow grouping of *V. vulnificus* isolates into two different genotypes, designated type A and type B (Aznar *et al*., 1994). A study assessing the prevalence of the two genotypes in environmental isolates from Korea, reported the majority of strains to belong to the 16S rRNA-type B (65%) (Kim and Jeong, 2001), which is in accordance to a study performed on isolates from the Italian Mediterranean Sea (Maugeri *et al*., 2006). In contrast to these results, other studies observed type A as the most abundant genotype among environmental strains from the Gulf of Mexico, the U.S. Atlantic coast (94%), and the Spanish Mediterranean coast (80%) (Nilsson *et al*., 2003; Chatzidaki-Livanis *et al*., 2006a; Arias *et al*., 2010).

The most significant observation in this context was made by Nilsson *et al*. (2003), who found that clinical isolates obtained from primary septicemia cases were mainly type B (76%). Remarkably, the frequency of type B was even higher when only fatal cases were considered (94%). The 16S rRNA-type B allele was therefore postulated as a potential indicator for strain virulence (Nilsson *et al*., 2003). Vickery *et al.* (2007) developed a Real-Time PCR assay for rapid 16S rRNA-typing of *V. vulnificus* isolates and revealed the presence of both alleles (designated as 16S rRNA-type AB) in 22% of all isolates and in 15% of clinical isolates (Vickery *et al*., 2007).

However, results from other studies put the reliability of the 16S rRNA-type B allele as marker for virulence into question. Gordon *et al*. (2008) observed an almost even A/B ratio among clinical isolates from Florida. A higher ability to cause lethal systemic infection in mice was indeed observed among 16S rRNA-type B and type AB strains, but type A strains exhibiting high virulence were also reported (Thiaville *et al*., 2011).

1.6.3 Virulence Correlated Gene (*vcg*)

DNA polymorphisms in the so called virulence correlated gene (*vcg*) of unknown function also divide *V. vulnificus* isolates into two genotypes, designated *vcg*-type C and *vcg*-type E. *Vcg* was first identified by RAPD-typing as a ~200 bp amplicon generated in all clinical isolates but in only eight per cent of the environmental isolates, implying an association with virulence

(Warner and Oliver, 1999). Sequence analyses revealed the presence of two distinct *vcg*-genotypes which can be distinguished by simple PCR amplification (Rosche *et al*., 2005). It was suggested that *vcg*-typing allows differentiation between virulent and avirulent strains, since 72% of clinical isolates were *vcgC*, while the *vcgE* allele was observed in the vast majority of environmental isolates (93%) (Rosche et al., 2005). Although occurrence of clinical strains belonging to the *vcgE*-type indicates that *vcgE* strains also possess a certain virulence potential, the authors assumed that they are less likely to cause infection (Rosche *et al*., 2005).

The genetic variations found in the *vcg* and 16S rRNA genes strongly correlated with each other and with specific MLST clusters. Generally, 16S rRNA-type A corresponds to *vcgE* and MLST cluster I (A/E-type, E-genotype), while 16S rRNA-type B corresponds to *vcgC* and MLST cluster II (B/C-type, C-genotype). The majority of 16S rRNA-type AB strains were reported to be *vcg*-type E (Chatzidaki-Livanis *et al*., 2006a; Cohen *et al*., 2007; Drake *et al*., 2010). Very few strains were reported to generate amplicons for both *vcg* alleles and these also possessed both alleles of the 16S rRNA gene (Warner and Oliver, 2008). Although typing based on the *vcg* and 16S rRNA genes may group the majority of clinical biotype 1 isolates to the C-genotype, these typing schemes failed to identify pathogenic strains of other biotypes. All human clinical biotype 2 isolates investigated by Sanjuán *et al*. (2009) possessed the E-genotype associated with a lower virulence potential and clinical biotype 3 isolates from Israel were of the AB/E-type.

1.6.4 Mannitol Fermentation and Associated Genes

The clinical genotypes *vcg*-type C and 16S rRNA-type B were further shown to correlate with the ability to ferment mannitol. Therefore, mannitol fermentation was suggested as a less expensive alternative to identify genotypes with a higher virulence potential (Drake *et al*., 2010; Froelich and Oliver, 2011). Investigations on the arrangement of two genes encoding a putative hemolysin and a TRAP-type mannitol transport system located upstream of the mannitol fermentation genes revealed interesting differences among strains of the *vcgC*-genotype. In the vast majority of clinical *vcgC* isolates (95%) the two genes were directly adjacent to each other. In contrast, the majority of the environmental *vcgC* isolates (57%) as well as all investigated *vcgE* isolates possessed a different gene arrangement (Froelich and Oliver, 2011).

1.6.5 *pilF*

Recently, another typing method has been described that allowed identification of potentially pathogenic strains irrespective of the biotype (Roig *et al*., 2010; Baker-Austin *et al*., 2012a). Differentiation between pathogenic and non-pathogenic strains is based on a variable 34 bp region within the *pilF* gene, encoding a protein involved in type IV pilus assembly. The gene polymorphism in *pilF* was originally observed during multilocus sequence analysis and divided *V. vulnificus* isolates into two genotypes (Sanjuán, 2008). Conventional PCR as well as Real-Time PCR assays were developed to amplify the *pilF* variant that has been associated with the clinical origin (referred to as *pilF*-type C or Vvpdh - *V. vulnificus* potentially dangerous for humans) (Roig *et al*., 2010; Baker-Austin *et al*., 2012a). Vvpdh was present in nearly all clinical isolates regardless of their biotype, indicating its reliable application as a marker for pathogenicity. Moreover, it was present in all environmental isolates that were classified as pathogenic based on their ability to survive in human serum (exceptions were Vvpdh-positive but serum susceptible strains due to lacking encapsulation). This highlights the usefulness of Vvpdh detection for identification of environmental isolates endangering public health (Roig *et al*., 2010; Baker-Austin *et al*., 2012a).

1.6.6 Sialic Acid Catabolism and Biosynthesis

Sialic acids are widely distributed in mammalian hosts and exhibit numerous diverse functions (Angata and Varki, 2002). For example, they are quite abundant in the human intestine as essential components of mucins (sialylated glycoproteins) and can also be found coupled to other proteins on the surface of many cells. As they represent an unlimited carbon and nitrogen source, the capability to metabolize sialic acids confers a huge selective advantage, especially for enteropathogenic bacteria. The capability to utilize *N*-acetylneuraminic acid, the most abundant sialic acid, has been shown to contribute to virulence of *V. vulnificus* (Jeong *et al*., 2009). Disruption of the *nanA* gene encoding an N-acetylneuraminate lyase, the key enzyme for sialic acid catabolism, resulted in attenuated virulence in mice, as well as in lower *in vitro* cytotoxicity, adhesion, and growth. In total, results of *in vitro* and *in vivo* analyses of a *nanA*-defective mutant demonstrated an important role of sialic acid catabolism in the pathogenesis of *V. vulnificus*, probably by supporting growth, adhesion, and survival in the intestine (Jeong *et al*., 2009). Genes mediating sialic acid catabolism (SAC) (*nanA, nanK, nanE,* and *nagA*) and transport (SAT) (*siaPQM*) in *V. vulnificus* are arranged together in the SAC and SAT region. Studies on the distribution of the *nanA* gene among *V. vulnificus* isolates demonstrated that the

presence of *nanA* and thus the ability to use sialic acid as the sole carbon source, is lineage-specific and also associated with the clinical origin (Lubin *et al*., 2012).

In addition to the utilization of sialic acids as nutrient sources, *V. vulnificus* is also capable of *de novo* biosynthesis of sialic acids (Lewis *et al*., 2011). Presented on the surface of many bacterial pathogens, sialic acid and other nonulosonic acid (NulO) containing structures are involved in numerous interactions with the host. For example, they help to evade the immune defense by mimicking the surface of eukaryotic cells (Vimr *et al*., 2004). In *Vibrionaceae*, the genes mediating nonulosonic acid biosynthesis (NAB) are arranged in *nab* gene clusters with the core components *nab1, nab2,* and *nab3* (Lewis *et al*., 2011). Phylogenetic analyses revealed that Nab1 and Nab2 amino acid sequences in the two clinical *V. vulnificus* isolates CMCP6 (CM-like alleles) and YJ016 (YJ-like alleles) are highly divergent and associated with different levels of NulO expression. Investigations on the distribution of CM-like or YJ-like *nab1* and *nab2* alleles among *V. vulnificus* isolates revealed the presence of both allele types to almost equal parts in clinical isolates. Remarkably, the majority of environmental isolates possessed the YJ-like *nab1* and *nab2* alleles, associated with the lowest levels of NulO expression. The observation of non-typeable *V. vulnificus* isolates showing intermediate NulO expression compared to strains with YJ-like or CM-like alleles, indicated the existence of further *nab* allele types (Lewis *et al*., 2011).

1.6.7 Genomic Region XII

Genome comparison revealed the presence of three species-specific genomic regions (ORFs VVA0080 - VVA0186, ORFs VVA0301 - VVA0336, and ORFs VVA1613 - VVA1636) in the two clinical *V. vulnificus* isolates CMCP6 and YJ016 that harbor several putative virulence genes (Cohen *et al*., 2007). The 33 kb region spanning ORFs VVA1613 to VVA1636 was designated region XII and was found predominantly in clinical isolates (84%) (Cohen *et al*., 2007). Moreover, it was significantly associated with the clinically relevant genotypes of the 16S rRNA and *vcg* genes (B/C-type), as well as with MLST cluster II. Cohen *et al.* (2007) speculated that the presence of region XII might contribute to the higher pathogenicity potential of these genotypes by mediating a selective advantage within the host. Among a number of hypothetical proteins, region XII encompasses an arylsulfatase gene cluster, a sulfate reduction system, two chondroitinase genes, and an oligopeptide ABC transport system. In addition to their function in nutrient acquisition, arylsulfatases and chondroitinases have been implicated in host-pathogen interactions of other bacteria (Cohen *et al*., 2007; Gulig *et al*., 2010; Morrison *et al*., 2012).

1.7 Aims of the Study

V. vulnificus infections rarely occur in Germany, but an increasing frequency of severe wound infections has been reported in recent years, correlating with unusually high temperatures in summer. Moreover, *V. vulnificus* is expected to become more abundant in German coastal waters due to impacts of climate change. Therefore, the incidence and clinical burden of *V. vulnificus* infections in Germany are predicted to rise in the future.

Against this background, the major aim of this dissertation was to characterize *V. vulnificus* isolates obtained from German coastal waters as well as from clinical cases that occurred in the Baltic Sea region. Special focus was laid on the distribution of virulence-associated phenotypic traits, putative genetic virulence determinants, as well as on antimicrobial resistance phenotypes and mechanisms. Phylogenetic relationships were explored by multilocus sequence typing of housekeeping genes.

Phylogenetic analysis combined with the characterization of virulence-associated traits and genotypes should aid to elucidate lineages of pathogenic strains. The comparison of clinical and environmental isolates aimed to evaluate the applicability of potential pathogenicity markers for identification of pathogenic strains in German coastal waters.

The development and evaluation of rapid methods for characterization and identification of pathogenic *V. vulnificus* strains should establish a basis to estimate the public health risk emanating from this pathogen in German coastal waters. In addition, methods for efficient molecular cloning in *V. vulnificus* should be evaluated as a prerequisite for functional analyses of potential virulence genes.

Finally, the distribution of potential pathogenicity markers in recent isolates from German coastal waters should be assessed with special focus on the comparison of North Sea and Baltic Sea isolates that might explain the different incidences of *V. vulnificus* infection in both areas.

2 Publications

2.1 List of Publications and Own Contribution

Publication 1: Genotypic Diversity and Virulence Characteristics of Clinical and Environmental *Vibrio vulnificus* Isolates from the Baltic Sea Region

Nadja Bier, Silke Bechlars, Susanne Diescher, Florian Klein, Gerhard Hauk, Oliver Duty, Eckhard Strauch, and Ralf Dieckmann; Applied and Environmental Microbiology, 2013, 79(12):3570-3581.
http://dx.doi.org/10.1128/AEM.00477-13

I contributed to the selection of phenotypic analyses as well as of target genes for genotypic characterization. I developed and optimized protocols for the analyses of *V. vulnificus* strains. With the exception of MLST, intact-cell MALDI-TOF mass spectrometry, and RL32 sequence analyses (performed by F. Klein and R. Dieckmann), I performed all experimental work for phenotypic and genotypic characterization with the technical assistance of Susanne Diescher. I analyzed the respective results, contributed to the overall interpretation of the data, and wrote major parts of the manuscript.

Publication 2: Multiplex PCR for Detection of Virulence Markers of *Vibrio vulnificus*

Nadja Bier, Susanne Diescher, and Eckhard Strauch; Letters in Applied Microbiology, 2015, 60(5): 414-420.
http://dx.doi.org/10.1111/lam.12394

I took major parts in the conception of the study and designed alternative primers for amplification of the 16S rRNA-type B allele. I performed experiments for optimization and validation of the multiplex PCR with technical assistance of Susanne Diescher. I analyzed the results, performed statistical analyses and wrote the major part of the manuscript.

Publication 3: Virulence Profiles of *Vibrio vulnificus* in German Coastal Waters, a Comparison of North Sea and Baltic Sea Isolates

Nadja Bier, Claudia Jäckel, Ralf Dieckmann, Nicole Brennholt, Simone I. Böer, and Eckhard Strauch; International Journal of Environmental Research and Public Health, 2015, 12(12): 15943-15959.
http://dx.doi.org/10.3390/ijerph121215031

I was involved in the study design (strain selection and selection of phenotypic and genotypic analyses) and responsible for all experimental work with the exception of the PCR amplification of MLST alleles (performed by C. Jaeckel). I assembled and analyzed the obtained MLST sequences and performed cluster and eBURST analyses. I analyzed and interpreted the obtained data by the use of different programs (e.g., MEGA6, Bionumerics, Accelrys Gene) and performed statistical analyses. I wrote the major part of the manuscript.

Publication 4: Survey on Antimicrobial Resistance Patterns in *Vibrio vulnificus* and *Vibrio cholerae* non-O1/non-O139 in Germany Reveals Carbapenemase-Producing *Vibrio cholerae* in Coastal Waters.

Nadja Bier, Keike Schwartz, Beatriz Guerra, and Eckhard Strauch; Frontiers in Microbiology, 2015, 6(1179).
http://dx.doi.org/10.3389/fmicb.2015.01179

I took major parts in the selection of antimicrobial agents and resistance genes. Experimental work to assess antimicrobial resistance of *V. vulnificus* (using disk diffusion and broth microdilution), as well as the detection of resistance genes in *V. vulnificus* were performed by myself. Additionally, I assisted in testing of *V. cholerae* isolates and performed all experiments concerning the four carbapenemase-producing *V. cholerae* strains. I interpreted the data, performed statistical analyses, and wrote the major part of the manuscript.

Publication 5: PVv3, a New Shuttle Vector for Gene Expression in *Vibrio vulnificus*.

Karina Klevanskaa, Nadja Bier, Kerstin Stingl, Eckhard Strauch, and Stefan Hertwig; Applied and Environmental Microbiology, 80(4): 1477-1481.
http://dx.doi.org/10.1128/AEM.03720-13

I screened *V. vulnificus* isolates for the presence of plasmid pVN-0126. I was responsible for molecular cloning of the *gfp* gene and the *vvhBA* hemolysin operon in *E. coli* and *V. vulnificus*. Additionally, I performed PCR and sequence analyses of transformants and contributed to the drafting of the manuscript by revising it critically and preparing figures.

2.2 Publication 1

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Genotypic Diversity and Virulence Characteristics of Clinical and Environmental *Vibrio vulnificus* Isolates from the Baltic Sea Region

Nadja Bier,[a] Silke Bechlars,[a] Susanne Diescher,[a] Florian Klein,[a] Gerhard Hauk,[b] Oliver Duty,[b] Eckhard Strauch,[a] Ralf Dieckmann[a]

Federal Institute for Risk Assessment, Department of Biological Safety, National Reference Laboratory for Monitoring Bacteriological Contamination of Bivalve Molluscs, Berlin, Germany[a]; Landesamt für Gesundheit und Soziales Mecklenburg-Vorpommern, Rostock, Germany[b]

The genetic diversity of *Vibrio vulnificus* isolates from clinical and environmental sources originating from the Baltic Sea region was evaluated by multilocus sequence typing (MLST), and possible relationships between MLST clusters, potential genotypic and phenotypic traits associated with pathogenicity, and source of isolation were investigated. The studied traits included genotyping of polymorphic loci (16S rRNA, *vcg*, and *pilF*), presence/absence of potential virulence genes, including *nanA*, *nab*, and genes of pathogenicity regions, metabolic features, hemolytic activity, resistance to human serum, and cytotoxicity to human intestinal cells. MLST generated 35 (27 new) sequence types and divided the 53 isolates (including four reference strains) into two main clusters, with cluster I containing biotype 1 and 2 isolates of mainly environmental origin and cluster II containing biotype 1 isolates of mainly clinical origin. Cluster II isolates were further subdivided into two branches. Branch IIB included isolates from recent cases of wound infections that were acquired at the German Baltic Sea coastline between 2010 and 2011 and isolates from seawater samples of the same regions isolated between 1994 and 2010. Comparing the MLST data with the results of genotyping and phenotyping showed that strains of MLST cluster II possess a number of additional pathogenicity-associated traits compared to cluster I strains. Rapid microbiological methods such as matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry combined with typing of selected virulence-associated traits (e.g., serum resistance, mannitol fermentation, *nanA*, and pathogenicity region XII) could be used for risk assessment purposes regarding *V. vulnificus* strains isolated from the Baltic Sea region.

Vibrio vulnificus is a potent bacterial pathogen present in coastal waters worldwide and is found preferentially in waters with moderate salinity. It can cause serious wound infections with lethal outcome and is also responsible for cases of death caused by consumption of contaminated seafood. In the United States, particularly oysters contaminated with *V. vulnificus* have been reported to be responsible for deadly infections (1–3). The severity of disease is strongly influenced by the health condition of exposed individuals. Immunocompromised people and persons with underlying diseases resulting in elevated serum iron levels are especially at high risk. In the case of primary septicemia, mortality rates greater than 50% have been reported, and for wound infections, approximately rates of 25% have been reported (4). Environmental factors, such as warm water and moderate salinity, are known to favor the multiplication of the pathogen. Therefore, the effect of global warming on seawater temperatures has aroused concerns that infections caused by *V. vulnificus* will increase in numbers (5–8). However, despite the frequent occurrence of the pathogen, the number of cases reported is relatively low, indicating that not all strains of *V. vulnificus* are equally virulent (9, 10).

The Baltic Sea is an intracontinental sea and a low-salinity marine ecosystem. At the same time it shows an increased rate of warming of surface water, especially in summers, which is distinctly higher than that of most other ocean seas. This led in some very hot summers (e.g., 1994, 2002, 2003, and 2006) to a significant number of reports of wound infection after contact with Baltic Sea water due to bathing or strolling along the shore line (11–16). During the summer of 2006, 67 *Vibrio* sp. infections were reported from the whole Baltic Sea region (8). Health authorities in the state of Mecklenburg-Vorpommern, Germany, have repeatedly detected *V. vulnificus* in nine out of 10 investigated German bathing locations since 2006 (15, 17). Locally acquired foodborne infections have not yet been reported in Germany, probably because oyster and mussel production does not play a role in the Baltic Sea and mandatory notification of suspected *V. vulnificus* infections does not exist.

The species *V. vulnificus* displays a high degree of intraspecies diversity and includes strains with various virulence potentials (4). Although the majority of strains are virulent in animal models (18), several investigations showed genetic divergence among strains from clinical and environmental origins. Phylogenetic and comparative genomic analyses grouped *V. vulnificus* strains into three major lineages: clade/cluster 1 includes biotype 1 (BT1) strains as well as biotype 2 (BT2) strains isolated mainly from the environment, while clade/cluster 2 strains include the majority of human clinical isolates of biotype 1 (19–21); a third lineage is comprised of strains that are biotype 3 human pathogens but seem to be regionally restricted to Israel (4). Recently, Broza et al. defined a fourth phylogenetic group, termed clade A, based on simple sequence repeats and multilocus sequence typing (MLST) (22).

Several studies developed rapid methods to distinguish clinical (C-type) from environmental (E-type) strains based on variations in the sequences of the 16S rRNA gene (23–25), of the virulence-

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correlated gene (*vcg*) (10, 26, 27), and of the *pilF* gene, encoding a protein required for pilus type IV assembly (1, 28). Other key genes that may differentiate between the genomes of clinical and environmental genotypes were recently identified using a comparative genome analysis approach (29), and Kim et al. identified further potential virulence factors by comparative transcriptomic analyses between clinical and environmental isolates using a cDNA microarray (30). Thiaville et al. analyzed the virulence properties of 33 clinical and 36 environmental isolates of *V. vulnificus* biotype 1 from the United States in a mouse model and found that higher virulence was correlated with, but not exclusive to, the clinical genotype (31). While several studies examined the distribution and characteristics of this species from tropical regions and the United States, studies on pathogenic and nonpathogenic *V. vulnificus* strains from northern temperate waters are scarce.

In the present study, we performed a detailed comparative analysis of genotypic and phenotypic traits known to be involved in or indicative for virulence of a diverse selection of *V. vulnificus* Baltic Sea strains. So far, *V. vulnificus* cases have been relatively rare in Germany, and thus a more systematic collection of strains from the Baltic Sea started only in the last few years. From the *Vibrio* strain collection of the Robert Koch Institute (RKI) and from samplings from the local authorities, 49 Baltic Sea isolates originating from wound infections and from environmental sources sampled between 1994 and 2011 and four reference strains were investigated.

MATERIALS AND METHODS

Bacterial strains. A total of 49 *V. vulnificus* isolates from the Baltic Sea region and four reference strains (ATCC 33149, ATCC 27562, MO6-24, and CMCP6) were selected. In the study, all available Baltic Sea clinical strains ($n = 19$) present in our strain collection were investigated, while environmental strains ($n = 30$) were chosen to represent the period from 1990 to the present. A detailed list of isolates is shown in Table S1 in the supplemental material. Most strains originated from the *Vibrio* strain collection of the Robert Koch Institute, Berlin (RKI), which is now located at the Federal Institute for Risk Assessment (BfR), or were collected by the Landesamt für Gesundheit und Soziales, Rostock, Germany (LAGuS). All Baltic Sea clinical strains were isolated from patients with wound infections and other nonintestinal infections after contact with seawater (recreational activities or fish handling). Four reference strains from different sources were used for comparison. Species identities of all strains were confirmed by biochemical assays (32) and species PCR as described by Bauer and Roervik (33).

MLST. Multilocus sequence typing (MLST) analyses were performed on 10 housekeeping genes from both chromosomes as described by Bisharat et al. (19, 34). PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced (both strands) through a sequencing service (Qiagen, Hilden, Germany). Chromatograms were imported, assembled, and trimmed using BioNumerics (version 6.6.4; Applied Maths, Sint-Martens-Latem, Belgium). The alleles at each locus were compared to the PUBMLST database (35). Different allele numbers were given to each distinct allele sequence within a locus, and a distinct sequence type (ST) number was attributed to each distinct profile of alleles by the administrator of the PUBMLST database. Novel allele sequences and novel allelic profiles (sequence types) were submitted to the PUBMLST database. Phylogenetic and molecular evolutionary analyses of individual gene loci and concatenated gene sequences were conducted using BioNumerics and MEGA version 5.1 (36). The DnaSP software package, version 5.10 (37), was used to estimate DNA sequence variation parameters among multialigned sequences, including total number of mutations (Eta), number of segregating polymorphic sites (S), nucleotide diversity (pi), average number of nucleotide differences (k), and mutation rate (θG). Split-tree generation for individual loci, concatenated sequences, and the pairwise homoplasy test (phi) for recombination were done using the SplitsTree 4.0 software (38).

Intact-cell MALDI-TOF MS. Sample preparation for whole-cell matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) was performed as described previously (39). In brief, colonies were directly transferred from Mueller-Hinton (MH) agar plates onto a 384-position ground steel target plate (Bruker Daltonics, Bremen, Germany) using a sterile pipette tip and immediately mixed with 1 μl of 25-mg/ml sinapinic acid (Bruker Daltonics) in 50% acetonitrile (Sigma-Aldrich, Taufkirchen, Germany), supplemented with 0.6% trifluoroacetic acid (Roth, Karlsruhe, Germany). The matrix/sample spots were crystallized by air drying. All mass spectra were acquired with an Ultraflex II MALDITOF/TOF MS (Bruker Daltonics) equipped with an all-solid-state Smartbeam laser Nd:YAG laser and operated at 100 Hz in the positive linear mode (delay, 100 ns; voltage, 25 kV; mass range, 2.2 to 20 kDa) under the control of Flexcontrol software version 3.0 (Bruker Daltonics). The spectra were obtained by averaging up to 7,000 laser shots acquired at a fixed laser power, which had been set to the minimum laser power necessary for ionization of selected samples before starting the analyses. Spectra were recorded in automatic mode. All spectra were externally calibrated by using the standard calibration mixture, protein calibration standard I (Bruker Daltonics). Sets of potentially cluster-discriminating biomarker ions were identified by visual inspection of mass spectra.

PCR genotyping. Genomic DNA was extracted using the RTP bacterial DNA kit from Stratec Molecular, Berlin, Germany. PCRs were performed using a Mastercycler EP gradient (Eppendorf, Hamburg, Germany) in a volume of 25 μl with 1× PCR buffer (2 mM $MgCl_2$), 0.2 mM each deoxynucleoside triphosphate (dNTP), 0,2 μM each primer, and 1.5 U DreamTaq DNA polymerase (Fermentas, St. Leon-Rot, Germany). Real-Time PCR experiments were performed using an Applied Biosystems 7500 real-time PCR System (Applied Biosystems, Foster City, CA). The PCR primers, target genes, and amplicon sizes used are shown in Table 1. Sequencing reactions (MLST genes, *vcg*, and *pilF*) were performed commercially at Eurofins MWG Operon (Ebersberg, Germany). Primers were synthesized by Metabion International AG (Martinsried, Germany).

Mannitol fermentation. *V. vulnificus* strains were streaked onto LB agar plates and incubated at 37°C for 24 h. Five milliliters of mannitol fermentation broth (1% mannitol, 0.0075% bromothymol blue, 1% peptone, 0.5% NaCl, pH 7.4) was inoculated using an inoculating needle, incubated at 37°C, and examined for mannitol fermentation after 24 h and 5 days.

Cytotoxicity assay. Caco-2 cells were cultivated in Dulbecco's modified Eagle medium (DMEM) (with 4.5 g/liter glucose) supplemented with 10% fetal calf serum (FCS), 1 mM sodium pyruvate, 4 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells (2.5×10^4 cells in 100 μl) were seeded in 96-well plates (TPP, Trasadingen, Switzerland) and incubated at 37°C with 5% CO_2 for 22 h to reach a confluent monolayer. Before the assay, cells were washed once with DMEM without phenol red. Bacterial cells grown in 20 ml brain heart infusion broth (BHIB) for 16 h at 37°C and 200 rpm were harvested at 5,000 × *g* for 10 min, and supernatants were filtered (0.2 μm). Filtered culture supernatants (200 μl) were added to Caco-2 cells and incubated at 37°C with 5% CO_2 for 2 h. Plates were centrifuged at 275 × *g* for 4 min, and 50 μl of the supernatants was transferred into a new plate. For determining cytotoxicity, lactate dehydrogenase (LDH) activity in the supernatant was determined using the Cytotox96 kit (Promega) according to the manufacturer's protocol. Additionally, the induced morphological changes of the Caco-2 cells were examined microscopically after 4 h. Experiments were performed at least twice.

Hemolytic activity of cells and culture supernatants. Twenty milliliters of BHIB was inoculated with the appropriate volume of *V. vulnificus* overnight cultures to give an optical density at 600 nm (OD_{600}) of 0.1 and

TABLE 1 Primers and probes used for PCR and sequencing

Primer	Gene target or designation	Sequence (5′ to 3′)[c]	Amplicon size(s) (bp)	Reference and/or source
vcg-typeC-F	Virulence-correlated gene clinical allele	AGCTGCCGATAGCGATCT		26
vcg-typeE-F	Virulence-correlated gene environmental allele	CTCAATTGACAATGATCT		26
vcg-typeC/E-R	Virulence-correlated gene	CGCTTAGGATGATCGGTG	277	26
vcg-seq-F[b]	*vcg*, partial	TGTCAAACGACATCGAAACAA		This study
vcg-seq-R[b]	*vcg*, partial	CTTAGGATGATCGGTGTAAAGG	380	This study
VVA1612F	Region XII, flanking region	ACCCTGATCGTTGGCTACTC		21
VVA1613R	Region XII	GGAGCGGTGTGATGGTGTTG	2,257	21
VVA1625F	Region XII	CGGTCTGTGGTTTATCG		21
VVA1625R	Region XII	TCGTTTCCAGTCGTCAC	1,822	21
VVA1634 F	Region XII	TGACACCCAACCTAGACCAC		21
VVA1634R	Region XII	ATTGATGCCAACCTGAG	1,364	21
VVA1636F	Region XII	TGTCCACGACTTGAACACG		21
VVA1637R	Region XII, flanking region	AACATCAACCAGCGAGTCGAAC	1,547	21
VVA1612bF	Region XII, flanking region	TGTGGAGAGCGGCAAGATCAAG		21
VVA1637R	Region XII, flanking region	AACATCAACCAGCGAGTCGAAC	1,200	21
Vvu16S51-F[a]	16S rRNA gene	CAAGTCGAGCGGCAGCA		25
Vvu16S221-R[a]	16S rRNA gene	TCCTGACGCGAGAGGCC	171	25
Vvu16SA-P[a] (2091859)	16S rRNA gene type A allele	6-FAM-TGATAGCTTCGGCTCAA-MGBNFQ	Probe	25
Vvu16SB-P[a] (2091860)	16S rRNA gene type B allele	VIC-CCCGTAGGCATCATGC-MGBNFQ	Probe	25
Vv-*pilF*-F[b]	Type IV pilus assembly protein *pilF* gene	CGATTGGTAGGCAATAGAC		28
Vv-*pilF*-R[b]	Type IV pilus assembly protein *pilF* gene	GCAACTCAACCTCAAGACG	917	28
Vv-*pilF*-F2[b]	Type IV pilus assembly protein *pilF* gene	AGGCCAAGCACAAAAAGATCC		This study
Vv-*pilF*-R2[b]	Type IV pilus assembly protein *pilF* gene	TTCACCAGCGCCACATTACC		This study
vllY-F	Hemolysin *vllY* gene	TATGTGGGATGGCATTTCGAG		44
vllY-R	Hemolysin *vllY* gene	CAACATCCGACTTCGCTAGGC	237	44
hlyIII-F	Hemolysin III *hlyIII* gene	CCCAACAAGGCTATCGACCAA		44
hlyIII-R	Hemolysin III *hlyIII* gene	GGTCACACACGGGTTAGGCAT	318	44
nanA-F	Sialic acid catabolism cluster	TKATCGCCGCTCCYCATACA		This study
nanA-R	Sialic acid catabolism cluster	GCAACGCCACCGTATTCAAC	745	45
Mann Hemo F	Hemolysin upstream of mannitol operon	ACATTTGACGGATGAGCAG	357; 1,821	49
Mann Hemo R	Hemolysin upstream of mannitol operon	TCCCAGACAAACAGATGATG	357	49
Mann TRAP F	TRAP-type mannitol transport system	CGCTGAAGAAATGTCAAACG	361	49
Mann TRAP R	TRAP-type mannitol transport system	ACGCATTTTCAACCCTTT	361; 1,821	49
Man IIA F	Mannitol fermentation operon	GATGTTGGTGAACAACTTCTCTGC		49
Man IIA R	Mannitol fermentation operon	TCTGAAGCCTGTTGGATGCC	243	49
VVA0081F	Ribosomal protein S12 methylthiotransferase	CGTTTACGCCAAACCAAACC		This study, 21
VVA0081R	Ribosomal protein S12 methylthiotransferase	ACCAATAGCTCTTTCACCCC	637	This study, 21
VVA0303F	Phospholipase/hemolysin	CACCAACACATCACAAAACG		This study, 21
VVA0303R	Phospholipase/hemolysin	GTTGAACTCGACGATCTTCG	661	This study, 21
VVA0320F	Zn hydrolase	GTGTAAGGCACCACTTCAAG		This study, 21
VVA0320R	Zn hydrolase	GCAAAAGCTGGGCAAAAAC	723	This study, 21
VVA0331F	RTX toxin	GCCGTTAACATTGACGATCC		This study, 21
VVA0331R	RTX toxin	CGCAAGGCTGCTTAAATCTTC	756	This study, 21
VV0316F	YJ-like *nab1* allele	GGCCACCCCTTCAATTGAG		47
VV0316R	YJ-like *nab1* allele	GTCGCATACACAACCGTGG	435	47
VV0312F	YJ-like *nab2* allele	CGACGAAGCACTGGCGTTTAA A		47
VV0312R	YJ-like *nab2* allele	GCTCGAGCATCTCCCAATACT	986	47
VV10803F	CM-like *nab1* allele	TTATCGGCGACAAGGTGA		47
VV10803R	CM-like *nab1* allele	ATCCATTACATAGGCAAATATG	346	47
VV10808F	CM-like *nab2* allele	TATTCGTTTAGCCAAACAGTTGA		47
VV10808R	CM-like *nab2* allele	CCACTTCATCCCAACGCGTT	902	47

[a] Used for real-time PCR.
[b] Used for gene sequencing.
[c] 6-FAM, 6-carboxyfluorescein; MGBNFQ, minor groove-binding nonfluorescent quencher.

incubated at 37°C and 200 rpm for 2 to 4 h. After reaching the late logarithmic phase at an OD_{600} of 1.6, cells were harvested by centrifugation (5.000 × *g*, 10 min, 4°C), washed twice, and resuspended in the harvested volume of ice-cold phosphate-buffered saline (PBS). Culture supernatants were filtered (0.2 μm), and 500 μl of filtered culture supernatants or 1:10-diluted cells in PBS was mixed with the same volume of 4% human erythrocyte suspensions in PBS (blood donation service of the German Red Cross, Berlin-Dahlem, Germany) and incubated for 2 h at 37°C on a tilting shaker. Preparations were centrifuged at 2,500 rpm and 4°C for 10 min. Release of hemoglobin into the supernatant was determined by measuring the absorbance at 570 nm. Erythrocyte suspensions were incubated with 500 μl of 2% Triton X-100 in PBS or BHIB and with sterile PBS or

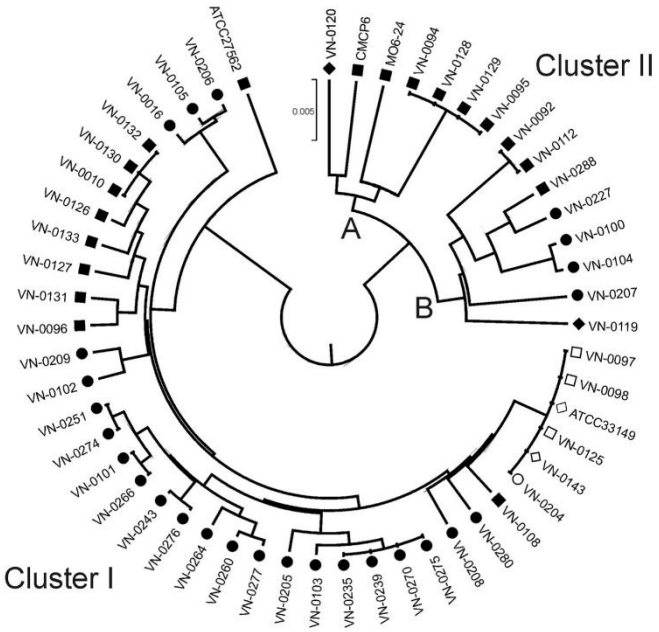

FIG 1 Population structure of *Vibrio vulnificus* isolates from the Baltic Sea region determined using concatenated MLST sequences of 10 housekeeping genes (MEGA version 5.1). Clinical BT1 reference strains ATCC 27562, CMCP6, and MO6-24 and one biotype 2 strain from a Japanese eel (ATCC 33149) were included for comparison. The evolutionary history was inferred using the unweighted-pair group method using average linkages (UPGMA), and evolutionary distances were computed using the maximum-composite-likelihood method. ■, human; ●, environmental (seawater); ◆, seafood (mussel); □, human (BT2); ○, environmental (BT2); ◇, diseased eel (BT2).

BHIB to determine the maximum and spontaneous hemoglobin release, respectively. Hemolytic activity (percent hemolysis) was calculated with the following equation: hemolysis (%) = 100 × (A_{570} sample − A_{570} spontaneous)/(A_{570} maximum − A_{570} spontaneous). Selected supernatants were heat treated at 56°C for 30 min for control purposes and showed complete loss of hemolytic activity. All experiments were performed three times. Hemolytic activity was rated positive for values of >20% in Table 5. For Fig. 2, strains were judged as hemolytic when supernatants or cells (or both) were positive.

Serum resistance. A colorimetric serum sensitivity assay as described by Moll et al. with slight modifications was employed, which allows high-throughput screening of bacterial resistance to human serum (40, 41). Bacterial cells were transferred from sheep blood (SB) agar plates to wells of a sterile 96-well plate (Brand, Wertheim, Germany) containing 0.2 ml LB broth per well and incubated overnight at 37°C. A microplate replicator (Boekel Scientific, Feasterville, PA) was used to transfer bacteria from the overnight cultures to a new 96-well plate containing 0.1 ml LB broth. Plates were incubated for 5 h at 37°C, and bacteria were transferred to new 96-well plates containing 100 μl peptone glucose broth (1% glucose, 0.0075% bromthymol blue, 1% peptone, 0.5% NaCl, pH 7.4) with different concentrations of human serum (0, 10, 20, 40, 60, and 80% pooled serum obtained from healthy volunteers). Plates were incubated at 37°C for 24 h, and serum resistance was determined by examining the color change from blue to yellow, indicating bacterial growth. Experiments were performed at least three times. Strains of *Escherichia coli* K-12 and *E. coli* K-12(pKT107) carrying the pKT107 serum resistance plasmid (40) were used as negative and positive controls, respectively. Isolates that showed growth in the presence of 60 to 80% human serum were classified as serum resistant. Isolates that grew only in the presence of 20 to 40% and 0 to 10% human serum were classified as intermediate resistant and sensitive, respectively. For Fig. 2 (binary data) all strains that showed strong resistance (growth in 60 to 80% human serum) were rated resistant.

Grouping of isolates based on virulence-associated traits. Similarity patterns based on virulence-associated phenotypic and genotypic traits were determined by cluster analysis by complete linkage using simple matching of binary data (BioNumerics version 6.6.4; Applied Maths, Sint-Martens-Latem, Belgium).

Nucleotide sequence accession number. The *pilF* sequence of the ATCC 33149 has been deposited in GenBank (accession number HF568870).

RESULTS

Multilocus sequence typing. All isolates were successfully subtyped at 10 loci using multilocus sequence typing as described by Bisharat et al. (19, 34), with the exception of the two genome-sequenced reference strains, MO6-24 and CMCP6. Sequence data were concatenated in the order of loci used to define the allelic profile to produce a sequence (4,326 bp in length) for each strain. To evaluate genetic relationships among strains, phylogenetic clustering based on concatenated sequence information of all 10 gene fragments was investigated with the minimum evolution (ME) algorithm using MEGA v 5.1 (Fig. 1). The resulting dendrogram showed a clear separation into two divergent clusters, I and II, which is in accordance with other studies (19, 21, 42). Most of

TABLE 2 Analysis of *V. vulnificus* DNA sequence polymorphisms using the DnaSP program

Genes	No. of:			Nucleotide diversity (pi)[a]	Avg no. of nucleotide differences (k)[a]	Mutation rate (θG)[a]
	Sites	Mutations (Eta)[a]	Segregating polymorphic sites[a]			
All	4,326	71.19 (308)	67.96 (294)	0.0038 (0.01644)	16.41 (71.1118)	15.62 (67.59)
Chromosome 1	2,301	61.28 (141)	59.54 (137)	0.0061 (0.01410)	14.10 (32.4493)	13.45 (30.94)
Chromosome 2	2,025	82.47 (167)	77.53 (157)	0.0094 (0.01909)	19.09 (38.6625)	18.09 (36.65)
All (cluster 1 isolates)	4,326	38.6 (167)	37.22 (161)	0.0018 (0.0079)	9.02 (39.03)	7.96 (34.44)
All (cluster 2 isolates)	4,326	43.9 (190)	43.23 (187)	0.0036 (0.01576)	15.76 (68.19)	14.25 (61.23)

[a] Data are normalized for number of sites and expressed per 1,000 bases. Raw values are shown in parentheses.

the environmental strains from the Baltic Sea region belonging to biotype 1 (22 of 28) fall into cluster I, which also contains some clinical strains and all biotype 2 strains. Interestingly, cluster II is further split into two branches, A and B. Branch A contains mainly older human isolates from the Baltic Sea (Denmark, 1994) and human isolates from overseas (United States and Asia), while all but one of the newer human isolates from Germany investigated in our study fall into branch IIB. Thirty-five different STs were obtained among the 49 Baltic Sea isolates, indicating a high degree of genotypic diversity. Comparison of MLST data to data for 188 sequence types in the PUBMLST database revealed that 27 new STs had been identified. The DnaSP program was used to estimate DNA sequence variation parameters in concatenated sequences (Table 2). Nucleotide diversity and mutation rates were higher (2-fold) for cluster II isolates (pi = 0.0036; θG = 14.25) than for cluster I isolates (pi = 0.0018; θG = 7.96), as already observed by Bisharat et al. (19). Generally, nucleotide diversities and mutation rates were higher for chromosome 1 alleles than for chromosome 2 alleles. The phi test for the concatenated sequences of the 49 isolates gave a *P* value of 0, indicating statistically significant evidence for recombination. As determined by the phi test for individual loci, three of the 10 MLST loci showed statistically significant evidence for recombination (*glp*, *pyrC*, and *metG*). When these three loci were analyzed separately for cluster I and cluster II isolates, significant evidence for recombination was indicated only for cluster II isolates. Lack of evidence for recombination was detected in the remaining seven loci (Table 3). Split decomposition was used to visualize the relationship of the isolates and to visualize the impact of recombination in each locus (see Fig. S1 in the supplemental material).

Intact-cell MALDI-TOF MS. We recently evaluated matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) as a rapid tool to identify *Vibrio* at the species level (39). Here we investigated the potential of MALDI-TOF MS for discrimination of major lineages of *Vibrio vulnificus* revealed by MLST and biotyping. Following routine species identification using MALDI-TOF MS, spectra were carefully inspected for sets of subcluster- or branch-identifying biomarker ions. To further analyze the respective biomarker ions, we made use of the steadily growing genome sequence databases and applied theoretical masses of biomarker proteins derived from translated gene sequences as references (Table 4). Based on our subset of isolates, biotype 2 strains could be discriminated from biotype 1 strains using a biomarker ion at *m/z* 6,196 that corresponds to ribosomal protein 32 (RL32). In contrast, biotype 1 strains displayed a peak for RL32 at *m/z* 6,168, corresponding to a K-to-R exchange in the respective protein sequence. Similarly, putative biomarker ions indicative of cluster II strains (C-type strains), e.g., ribosomal protein L25 (*m/z* 10,276), integration host factor subunit alpha (IHFA) (*m/z* 10,705), and sigma 54 modulation protein (*m/z* 11,052) could be identified. To further discriminate branch IIA from IIB, possible branch-identifying biomarker ions were observed at, among others, *m/z* 10,607 (integration host factor subunit beta [IHFB]), *m/z* 9,477 (RS17), *m/z* 11,100 (RL24), and *m/z* 8,070 (RL31) The discriminative value of these putative biomarkers has to be further evaluated using extended sets of isolates from different sources.

Detection of virulence-associated genes by PCR. Detection of virulence genes and typing of polymorphic DNA sequences were carried out via PCR using published primers or sequencing of PCR products (Table 1). Types were designated either clinical (C) or environmental (E) in accordance to published nomenclature based on PCR assays targeting sequence polymorphisms in the 16S rRNA (A→E; B→C) and *vcg* genes and by sequencing the *pilF* gene (25, 26, 28). In case of the 16S rRNA gene, all strains of cluster II possessed allele B (only VN-0207 is of type AB) and all cluster I

TABLE 3 Level of recombination using the pairwise homoplasy test (PHI) implemented in Splits Tree software

Gene(s)	Statistically significant evidence for recombination	*P* value
All	Yes	0
Chromosome 1	Yes	0
Chromosome 2	Yes	2.62E−12
dtdS (chromosome 2)	No	0.7863
glp (chromosome 1)	Yes	2.13E−5
Cluster I	No	0.05173
Cluster II	Yes	2.82E−7
gyrB (chromosome 1)	No	0.5056
lysA (chromosome 2)	No	1.439E−6
mdh (chromosome 1)	No	0.2934
metG (chromosome 1)	Yes	2.83E−4
Cluster I	No	0.01959
Cluster II	Yes	0.01595
pntA (chromosome 2)	No	0.9509
pyrC (chromosome 2)	Yes	0.02787
Cluster I	No	1
Cluster II	Yes	0.08041
tmaA (chromosome 2)	No	0.9051
purM (chromosome 1)	No	0.7035

TABLE 4 Selected subcluster- or branch-identifying biomarkers observed by MALDI-TOF mass spectrometric analysis of *V. vulnificus* isolates from different MLST lineages

	Group-specific peaks			Peaks present in multiple groups			
Tentative protein identity	Exptl mass (avg *m/z*)	Predicted avg mass [$(M + H)^+$], Da	Subcluster/branch specificity	Exptl mass (avg *m/z*)	Predicted avg mass [$(M + H)^+$], Da	Presence in cluster/branch	Amino acid exchange
50S ribosomal protein L32	6,196	6,195.9 (Met loss)	BT 2 isolates	6,168	6,167.9 (Met loss)	Most BT 1 isolates	K→R
Integration host factor subunit alpha	10,705	10,705.2 (Met loss)	Cluster II isolates	10,806	NA[a]	Most cluster I isolates	NA
Putative sigma 54 modulation protein	11,052	11,052.5	Most cluster II isolates	11,067	11,066.5	Most cluster I isolates	T→S
Integration host factor subunit beta	10,607	10,607.1	Cluster IIA isolates	10,592	10,593.0	Cluster IIB and cluster I isolates	V→I
30S ribosomal protein S17	9,477	9,477.1 (Met loss)	Cluster IIA isolates	9,420	NA	Cluster IIB and many cluster I isolates	NA
50S ribosomal protein L24	11,100	11,100.9 (Met loss)	Cluster IIA isolates	11,086	NA	Cluster IIB and most cluster I isolates	NA
NA	7,393	NA	Cluster IIA isolates	7,365	NA	Cluster IIB and most cluster I isolates	NA
50S ribosomal protein L31	8,070	8,070.2	Cluster IIB isolates	8,084	8,084.2	Most cluster IIA and cluster I isolates	E→D

[a] NA, not assigned.

strains were type A, with the exceptions of ATCC 27562 and VN-0102, which possessed both alleles (type AB). The *vcg* type C allele was found exclusively in cluster IIA. Analysis of the 16S rRNA and *vcg* gene also revealed that 10 (53%) and 13 (68%) of the 19 clinical BT1 isolates were of the respective E type. While 10 (53%) of the clinical BT1 isolates showed *pilF* type E, all cluster II strains and only three environmental strains of cluster I possessed *pilF* type C (Table 5).

Five PCR assays described by Cohen et al. were performed to check for the presence of the 33-kb pathogenicity region XII (PRXII) (21): four PCR assays amplify parts of the genes encoding two putative chondroitinase AC lyases (genes VVA1613 and VVA1636), a putative arylsulfatase A (gene VVA1634), and a putative methyl-accepting chemotaxis protein (gene VVA1625), whereas another PCR proves complete absence of the region by targeting the flanking regions of PRXII (genes VVA1612 and VVA1637). Strains were considered positive for PRXII when at least three of the four genes on PRXII could be amplified and no amplicon was detected with PCR primers VVA1612F and VVA1637R. Eighty-four percent (16 of 19) of all BT1 clinical strains were positive for PRXII, whereas only 32% (9 of 28) of the BT1 environmental strains contained this region. Interestingly, the presence of the pathogenicity region XII was proven in all cluster II strains by detecting all four genes of PRXII, with the exception of VN-0227, which showed no amplification of VVA1625. In contrast, BT1 strains of cluster I (only 10 out of 32 positive, including 7 clinical isolates) contained a different PRXII in which one gene (VVA1634) could not be detected with the primers used (VN-0010, VN-0016, VN-0096, VN-0105, VN-0126, VN-0130, VN-0131, VN-0132, VN-0133, and VN-0206).

Furthermore, oligonucleotide primers for three potential virulence genes located in a 53-kb region of chromosome II (21) were designed and applied in PCR assays: open reading frames (ORFs) VVA0303, encoding a putative thermolabile hemolysin, VVA0320, encoding a zinc-dependent hydrolase, and VVA0331, encoding a 489-kDa RTX-like protein (43). These results are not displayed in Table 5 because all strains were positive for VVA0303 and VVA0320 and only strains VN-0016, VN-0105, VN-0120, VN-0206, and VN-0207 were negative for VVA0331. Another suspected pathogenicity region (21) spanning VVA0080 to VVA0186 was investigated by targeting ORF VVA0081, encoding a ribosomal protein S12 methylthiotransferase. As all strains were positive for VVA0081 with the exception of strain VN-0119, the results are also omitted from Table 5. Similar results were obtained for the hemolysin genes *hlyIII* and *vllY* (44), as each strain possessed both genes.

The strains were further examined for the presence of the virulence-associated gene *nanA* of the sialic acid catabolism region (45, 46), which could be detected in 12 of 19 (63%) clinical and in only 5 of 28 (18%) environmental BT1 isolates. All cluster II strains were *nanA* positive except three cluster IIB strains, while most (84%) of the BT1 cluster I strains were *nanA* negative. Furthermore, allele types of the *nab1* and *nab2* genes were determined, which are responsible for biosynthesis of nonulosonic acids (NulOs) such as sialic acids (47). Sixty-eight percent (13 of 19) of the clinical BT1 isolates possessed CM-like alleles of the *nab1* and *nab2* genes, and one strain was not typeable. Nearly half of the environmental isolates (46%) also showed one of these genotypes. Six out of seven cluster IIA isolates possessed CM-like alleles of the *nab1* and *nab2* genes, whereas this was the case for only three of the cluster IIB isolates.

Mannitol fermentation. Mannitol fermentation has recently been correlated with clinical genotypes (*vcg* type C and 16S rRNA type B), and mannitol transport and fermentation genes were found to be predominantly present in the C genotype (48, 49). Of the 47 biotype 1 isolates in our study, mannitol fermentation was observed in 15 of 19 (79%) isolates from human cases, but in only 5 of 28 (18%) isolates from environmental sources (Table 5). Interestingly, the five mannitol-positive environmental isolates were also positive for pathogenicity region XII. The ability to ferment mannitol was in complete accordance with the presence of the mannitol fermentation operon, which was determined by PCR

TABLE 5 Genotypic and phenotypic characterization of *V. vulnificus* strains used in this study

Strain	Biotype	MLST cluster	Mannitol fermentation[e]	Genotyping					Sequence typing		Human serum resistance[f]	Hemolytic activity in:	
				16S rRNA type	*vcg* type	PRXII	*nanA*	*nab1* and *nab2*	MLST type	*pilF* type		Supernatant	Cells
VN-0010	1	I	+	A	E	+[a]	−	CM-like	43	E	Res	+	+
VN-0016	1	I	+	A	E	+[a]	−	YJ-like	103	C	Res	+	+
VN-0092	1	IIB	+	B	E	+	+	YJ-like	118	C	Res	+	+
VN-0094	1	IIA	+	B	C	+	+	CM-like	110	C	Res	+	+
VN-0095	1	IIA	+	B	C	+	+	CM-like	110	C	Res	+	+
VN-0096	1	I	+	A	E	+[a]	+	YJ-like	111	E	Int	+	−
VN-0097	2	I	−	A	E	−	−	YJ-like	112	C[c]	Sen	+	+
VN-0098	2	I	−	A	E	−	−	YJ-like	112	C[c]	Int	+	+
ATCC 33149	2	I	−	A	E	−	−	YJ-like	112	C[c]	Sen	+	+
VN-0100	1	IIB	−	B	E	+	+	CM-like	128	C	Res	−	−
VN-0101	1	I	−	A	E	−	−	YJ-like	113	E	Int	+	+
VN-0102	1	I	−	AB	E	−	−	−	114	E	Int	−	+
VN-0103	1	I	−	A	E	−	−	CM-like	100	E	Res	−	+
VN-0104	1	IIB	−	B	E	+	+	CM-like	115	C	Res	−	−
VN-0105	1	I	+	A	E	+[a]	+	YJ-like	116	E	Res	+	+
VN-0108	1	I	−	A	E	−	+	YJ-like	117	E	Res	+	−
VN-0112	1	IIB	+	B	E	+	+	YJ-like	118	C	Res	+	+
VN-0119	1	IIB	+	B	E	+	−	YJ-like	130	C	Int	−	−
VN-0120	1	IIA	+	B	C	+	+	−	119	C	Sen	+	+
ATCC 27562	1	I	−	AB	E	−	−	−	3	E	Res	+	+
VN-0125	2	I	−	A	E	−	−	YJ-like	112	C[c]	Sen	+	+
VN-0126	1	I	+	A	E	+[a]	−	CM-like	120	E	Res	+	+
VN-0127	1	I	−	A	E	−	+	YJ-like	121	E	Res	+	+
VN-0128	1	IIA	+	B	C	+	+	CM-like	110	C	Res	−	+
VN-0129	1	IIA	+	B	C	+	+	CM-like	110	C	Res	−	+
VN-0130	1	I	+	A	E	+[a]	−	CM-like	43	E	Res	+	+
VN-0131	1	I	+	A	E	+[a]	+	CM-like	122	E	Res	−	+
VN-0132	1	I	+	A	E	+[a]	−	CM-like	43	E	Res	+	+
VN-0133	1	I	+	A	E	+[a]	−	CM-like	131	E	Res	+	+
VN-0143	2	I	−	A	E	−	−	YJ-like	112	C[c]	Int	+	+
VN-0204	2	I	−	A	E	−	−	YJ-like	112	C[c]	Int	+	+
VN-0205	1	I	−	A	E	−	−	CM-like	65	E	Sen	−	+
VN-0206	1	I	+	A	E	+[a]	−	YJ-like	132	E	Sen	+	+
VN-0207	1	IIB	−	AB	E	+	+	YJ-like	123	C	Res	−	−
VN-0208	1	I	−	A	E	−	−	YJ-like	105	C	Res	+	+
VN-0209	1	I	−	A	E	−	−	−	124	C	Int	−	+
VN-0227	1	IIB	−	B	E	+[b]	−	−	125	C	Int	−	−
VN-0235	1	I	−	A	E	−	−	CM-like	133	E	Res	+	+
VN-0239	1	I	−	A	E	−	−	CM-like	133	E	Res	+	+
VN-0243	1	I	−	A	E	−	−	YJ-like	107	E	Int	+	+
VN-0251	1	I	−	A	E	−	−	YJ-like	44	E	Sen	+	+
VN-0260	1	I	−	A	E	−	−	YJ-like	126	E	Int	−	−
VN-0264	1	I	−	A	E	−	−	YJ-like	108	E	Res	−	+
VN-0266	1	I	−	A	E	−	−	YJ-like	113	E	Sen	+	+
VN-0270	1	I	−	A	E	−	−	CM-like	133	E	Res	−	+
VN-0274	1	I	−	A	E	−	−	YJ-like	44	E	Int	+	+
VN-0275	1	I	−	A	E	−	−	CM-like	133	E	Res	+	+
VN-0276	1	I	−	A	E	−	−	YJ-like	134	E	Int	+	+
VN-0277	1	I	−	A	E	−	−	YJ-like	127	E	Res	−	−
VN-0280	1	I	−	A	E	−	−	−	109	E	Res	+	+
MO6-24	1	IIA	+	B[d]	C[d]	+	+	CM-like	NA[g]	C[d]	Res	+	+
CMCP6	1	IIA	+	B[d]	C[d]	+	+	CM-like	NA	C[d]	Res	+	+
VN-0288	1	IIB	−	B	E	+	−	CM-like	135	C	Res	+	−

[a] PCR positive for all tested genes of PRXII except VVA1634.
[b] PCR positive for all tested genes of PRXII except VVA1625.
[c] Slightly different *pilF* type C in biotype 2 strains (see the text).
[d] Sequence deduced from published genome.
[e] Mannitol fermentation tested biochemically and by presence of mannitol fermentation operon (PCR).
[f] Res, resistant; Int, intermediate; Sen, sensitive.
[g] NA, not assigned.

analysis of the gene encoding the IIA domain of the mannitol phosphotransferase system. All isolates were also tested for the presence and orientation of two genes that are immediately upstream of the mannitol fermentation operon in the three published *V. vulnificus* genomes, a putative hemolysin gene (Mann Hemo) and a TRAP-type mannitol transport gene (Mann TRAP), as described by Froelich and Oliver (49). Although these two genes could be detected in each of the strains, their orientation being identical to the published genomes was a specific feature of the *vcg* type C strains of cluster IIA.

Cytotoxicity against Caco-2 cells. To investigate the virulence of *V. vulnificus* isolates to human intestinal epithelial cells, Caco-2 cells were exposed to supernatants of *V. vulnificus* liquid cultures. Supernatants of all tested strains showed strong cytopathic effects on Caco-2 cells, inducing either cell lysis, cell rounding, or other morphological changes (data not shown). Most of the strains induced lysis of 80 to 100% of the cells, which was determined by LDH activity released into the medium. A few strains (VN-0010, VN-0102, VN-0103, VN-0105, VN-0108, and VN-0125) caused no lysis according to the LDH measurement but induced strong morphological changes of the cells, which were detected by microscopic examination.

Hemolysis assay. Of the 47 biotype 1 isolates in our study, significant hemolytic activity of culture supernatants (>20%) was observed in 15 of 19 (79%) isolates from human cases and 15 out of 28 (54%) isolates from environmental sources. Three isolates from clinical cases that displayed no hemolytic activity in culture supernatant were positive when viable cells were used in the assay. Seven of the 28 (25%) BT1 environmental strains (including all five cluster IIB strains) showed no hemolytic activity when supernatants or cells were tested (Table 5; see Fig. S2 in the supplemental material). Heat inactivation of culture supernatants of 13 randomly chosen isolates (VN-0010, VN-0095, VN-0096, VN-0101, VN-0108, VN-0112, VN-0120, VN-0126, VN-0204, VN-0260, VN-0280, ATCC 33149, and MO6-24) completely abolished hemolytic activity.

Human serum resistance. Of the 47 biotype 1 isolates in our study, strong resistance to human serum (growth in 60 to 80% human serum) was observed in 18 of 19 (95%) isolates from human cases. One clinical isolate (VN-0096) showed intermediate serum resistance (growth in 20 to 40% human serum). Fourteen of the 28 environmental isolates (50%) also displayed strong serum resistance, nine (32%) displayed intermediate serum resistance, and five (18%) isolates were classified as serum sensitive (growth in 0 to 10% human serum). All results are included in Table 5.

Biotype 2 isolates. For comparison we included a subset of isolates of biotype 2. Strains of this biotype are regarded as eel pathogens but have also been obtained from human infections. We studied four Danish isolates from 1994 (three clinical strains from fisherman after handling eels [VN-0097, VN-0098, and VN-0125] and one environmental isolate [VN-0204]), one isolate from a diseased eel isolated in Sweden (VN-0143), and the reference strain ATCC 33149 from a diseased eel in Japan. These isolates turned out to be phylogenetically highly related (all ST 112) despite their different origins. All biotype 2 isolates were E type with respect to the *vcg* and 16S rRNA genes, were negative for *nanA* and pathogenicity region XII, were positive for *hlyIII*, *vllY*, VVA0303, VVA0320, and VVA0331, and possessed YJ-like alleles of the *nab1* and *nab2* genes. The isolates were not able to ferment mannitol and showed no or only intermediate resistance to human serum but displayed comparably high hemolytic activities. A *pilF* gene variant of the C type that was identical in all biotype 2 strains was found by sequencing the complete *pilF* gene.

DISCUSSION

This study was carried out as the public awareness of *Vibrio* infections has increased in Germany due to sporadic deaths caused by contact with contaminated Baltic Sea water. Climate warming is thought to drive bacterial waterborne infectious diseases, and the seasonal environmental temperature changes observed in the Baltic Sea region correlate with the emergence of *Vibrio* infections (6, 7, 15). Forecasts indicate a further increase of infections, as the effects of climate warming on surface temperatures is more pronounced at higher latitudes (6–8, 50). MLST grouping of *Vibrio vulnificus* isolates revealed the existence of two major phylogenetic clusters (19, 21). Though the MLST clusters I and II do not show a clear distinction between clinical and environmental strains, it was found that in cluster II, clinical strains of biotype 1 are overrepresented (19). When applying the MLST typing scheme to a diverse selection of isolates from the Baltic Sea region (Fig. 1), a comparable topology was obtained regarding two phylogenetic lineages. Cluster I contained Baltic Sea biotype 2 strains (three clinical and two environmental) which possessed the same sequence type (ST) as the reference strain from Japan (ATCC 33149). Of the biotype 1 strains, nine clinical isolates and 22 environmental isolates were grouped into cluster I, while seven clinical and six environmental strains belonged to cluster II. The phylogenetic tree analyses indicated that cluster II splits into two subclusters, branches IIA and IIB. Most of the investigated clinical Baltic Sea strains were isolated before 2000, and only four isolates (VN-0092, VN-0108, VN-0112, and VN-0288) were from recent years (2010 and 2011). It is remarkable that the latter isolates do not cluster with the older strains. Three of the recently isolated strains are grouped in branch IIB, and one strain (VN-0108) belongs to cluster I; however, it is clearly separated from all other clinical biotype 1 strains of cluster I. As VN-0108 was isolated from a patient suffering from a coinfection with a *Vibrio cholerae* non-O1, O139 strain, its pathogenic potential is unclear and will not be discussed in detail. The MLST analyses of the clinical strains from the early 1990s revealed that they are separated in branch IIA and in cluster I; however, all strains showed closely related sequence types within the respective group.

Due to its relatedness to the C-type strains of cluster IIA from Asian and American sources, which have been reported to possess higher virulence potential (19–21, 47) and to be more likely to cause lethal systemic infection with more severe indicators of virulence (31), branch IIB could represent a currently clinically relevant clonal lineage confined to the Baltic Sea and perhaps European coasts. Only four more STs of strains isolated in European countries that fall into this branch, which was formerly designated branch D of cluster II by Bisharat et al. (19), were present in the PUBMLST database (two clinical isolates [ST57, Spain, 2001; ST61, Sweden, 1997] and two environmental isolates [ST14, Denmark, 1996; ST45, Germany, 1995]). Therefore, we focused on the comparison of branch IIB strains to the remaining strains using a panel of virulence-related genotypic and phenotypic traits. Cluster analysis based on these data displayed in a binary table (1/0) resulted in a grouping of the Baltic Sea isolates in two clusters which largely correspond to MLST clusters I and II (Fig. 2). The

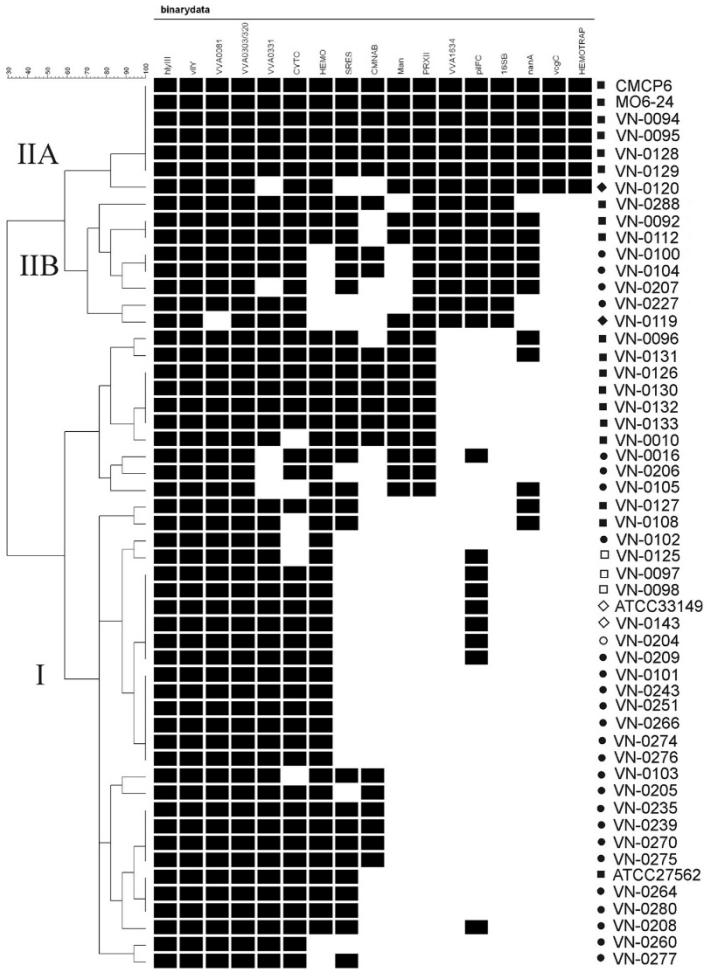

FIG 2 Grouping of isolates based on virulence-associated traits. Similarity patterns were determined by cluster analysis by complete linkage using simple matching of binary data (BioNumerics version 6.6.4; Applied Maths, Sint-Martens-Latem, Belgium). IIA, IIB, and I, cluster designation taken from MLST analysis. ■, human; ●, environmental (seawater); ◆, seafood (mussel); □, human (BT2); ○, environmental (BT2); ◇, diseased eel (BT2).

two genome-sequenced C-type strains CMCP6 and MO6-24 grouped with cluster IIA Baltic Sea strains, which are clearly separated from cluster IIB strains. Most of the clinical strains of cluster I form a separate branch immediately below the IIB strains. The majority of the environmental strains together with the biotype 2 strains are displayed in the bottom part of the dendrogram.

Two genetic traits, gene VVA1634 of pathogenicity region XII (PRXII) and the 16S rRNA type B, were present exclusively in cluster II strains and could be used to discriminate the two clusters. In agreement with Cohen et al. (21), PRXII was not restricted to cluster II. Interestingly, in all 10 BT1 isolates of cluster I with PRXII (7 clinical strains), gene VVA1634, encoding a putative arylsulfatase, was either missing or altered and could not be detected in these isolates with the PCR assay. PRXII showed a good correlation with the isolation source of BT1 strains, as 84% of clinical isolates and only 32% of environmental isolates possessed this region, supporting the potential role of PRXII-encoded gene products mediating sulfate reduction, oligopeptide transport, and chondroitinase or aryl sulfatase activities for pathogenicity of *V. vulnificus* (20, 21, 29).

Differentiation of MLST clusters IIA and IIB is possible, as all cluster IIA strains possessed the clinical associated *vcg* type C, whereas the newly described Baltic Sea isolates belonging to cluster IIB possessed the environmental *vcg* type E, irrespective of their

isolation source. Furthermore, the genetic arrangement of the putative hemolysin and TRAP transporter genes (HEMOTRAP), similar to the published genomes (49), was also present exclusively in cluster IIA. All cluster II strains possessed *pilF* type C, which has previously been correlated with pathogenicity (1, 28) and was only rarely found in cluster I BT1 strains. In contrast to the study of Roig et al. (28), in which nearly all clinical isolates possessed the *pilF* type C allele, more than half of the clinical strains in our strain collection possessed *pilF* type E. Altogether, our data on the distribution of type C and E alleles of the *pilF*, 16S rRNA, and *vcg* genes among environmental and clinical isolates from the Baltic Sea region support the opinion of Thiaville et al. that E-type strains can be pathogenic and should not be classified as avirulent *per se* (31).

The mannitol fermentation operon and the *nanA* gene were present in most cluster II strains but could also be detected in some cluster I strains. A correlation with MLST cluster II (designated lineage 1) was already described for *nanA* (45). Both markers showed a significant correlation with the isolation source and may be relevant for virulence, since they can promote a selective advantage by enabling the use of alternative carbon and nitrogen sources (48, 51). The *nanA* gene may be of particular importance, as it is a component of the sialic acid catabolism (SAC) cluster, whose gene products confer the ability of enteropathogens or commensals to metabolize sialic acid components of mucins in the human intestine (52), and was already found to be essential for pathogenicity of *V. vulnificus* (46).

Whereas in MLST clusters I and IIB almost equal percentages of CM-like and "not typeable" alleles of the *nab1* and *nab2* genes versus YJ-like alleles were found, all cluster IIA isolates possessed CM-like or "not typeable" alleles. According to Lewis et al. (47) the latter two alleles are associated with high or intermediate NulO expression, while YJ-like alleles are associated with lower expression. In addition, they demonstrated a higher proportion of CM-like alleles in clinical lineage I isolates (corresponding to cluster IIA) and speculated that NulO expression contributed to the virulence of these strains by participating in host-pathogen interactions (47). PCR analyses of several other suspected virulence genes (VVA0081, VVA0303, VVA0320, VVA0331, *hlyIII*, and *vllY*) revealed that they are too frequently present to be useful for a discrimination of strains, as was already shown for *hlyIII* and *vllY* by Wong et al. (44).

Cytotoxic and hemolytic properties were exhibited by most of the strains irrespective of their origin, as described by others (53, 54). However, it is also worth mentioning that all clinical isolates showed hemolytic activity in testing of supernatants or intact cells or both. Serum resistance was found in 95% of the clinical strains irrespective of their phylogenetic origin, but 50% of the environmental strains also grew in 60 to 80% serum. This is in accordance with the results reported by Roig et al., who observed serum resistance in more than 90% of clinical isolates and ca. 40% of nonclinical isolates and postulated serum resistance as a prerequisite for pathogenicity (28).

Altogether, cluster IIA constitutes a rather homogenous lineage, since all strains were able to ferment mannitol, showed hemolytic activity, possessed the *pilF* C, 16S rRNA B, and *vcg* C alleles, and were positive for PRXII and the *nanA* gene. Furthermore, most of the strains showed strong serum resistance and the CM-like alleles of the *nab1* and *nab2* genes. In contrast, cluster IIB strains showed a higher genetic diversity with a less defined pattern of virulence-associated traits. MLST lineages correlate with some virulence-associated genotypes; however, clinical strains are distributed in all lineages.

Epidemiological surveillance and risk assessment of potential virulent *Vibrio vulnificus* strains require rapid and effective analytical tools, as bacteria of this species are widespread along the German Baltic Sea coastline and raise to significant counts ($>10^5$ CFU $\cdot$ liter^{-1}) in hot summers (55). As the MLST scheme is laborious, requiring the sequence analysis of 10 conserved genes, we evaluated intact-cell MALDI-TOF MS (ICMS) analysis, which allows rapid species identification of *Vibrio* spp. (39) but could also have the potential for discrimination of bacteria below the species level. This approach revealed potentially discriminating biomarker sets for biotype 2 strains as well as for the identification of the cluster II and cluster IIB isolates, which will be further evaluated using extended subsets of isolates. Likewise, the 16S rRNA B and *vcg* C alleles could serve as a marker for cluster II and specifically cluster IIA, respectively. Gene polymorphisms are valuable markers to recognize strains belonging to MLST clusters which include a high proportion of clinical strains. For assessment of potential pathogenicity, the presence/absence of pathogenicity region XII, the mannitol fermentation operon, or *nanA* can be studied quickly by simple PCR assays. As traditional biochemical typing is still performed by routine laboratories, mannitol fermentation could also be used for risk assessment of potentially pathogenic strains (48). These rapid tests may be complemented by human serum resistance assays that allow easy and high-throughput screening of large *V. vulnificus* strain collections.

The clustering based on the binary data suggests that the probability for strains to be highly virulent is lower for strains clustering at the bottom of the diagram (Fig. 2). Presumably, strains of cluster I which test negatively for growth in 60 to 80% human serum, pathogenicity region XII, mannitol fermentation, or *nanA* are likely to possess less pathogenic potential. On the other hand, environmental isolates that are phylogenetically related to clinical strains and already possess a number of virulence-associated traits might become virulent, e.g., by horizontal transfer of additional virulence genes or adaptations in response to selective forces favoring the evolution of pathogenic clones.

Further studies will focus on the investigation of differences concerning the virulence potential of environmental and clinical cluster II strains. Genome sequencing of selected cluster IIB strains is currently in process, and further investigations will include knockout strategies with selected genes, for example, components of pathogenicity region XII, to compare mutants and wild-type strains in animal models.

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2.3 Publication 2

Multiplex PCR for Detection of Virulence Markers of *Vibrio vulnificus*

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2.4 Publication 3

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Article

Virulence Profiles of *Vibrio vulnificus* in German Coastal Waters, a Comparison of North Sea and Baltic Sea Isolates

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Abstract: *Vibrio vulnificus* is a halophilic bacterium of coastal environments known for sporadically causing severe foodborne or wound infections. Global warming is expected to lead to a rising occurrence of *V. vulnificus* and an increasing incidence of human infections in Northern Europe. So far, infections in Germany were exclusively documented for the Baltic Sea coast, while no cases from the North Sea region have been reported. Regional variations in the prevalence of infections may be influenced by differences in the pathogenicity of *V. vulnificus* populations in both areas. This study aimed to compare the distribution of virulence-associated traits and genotypes among 101 *V. vulnificus* isolates from the Baltic Sea and North Sea in order to assess their pathogenicity potential. Furthermore, genetic relationships were examined by multilocus sequence typing (MLST). A high diversity of MLST sequences (74 sequence types) and differences regarding the presence of six potential pathogenicity markers were observed in the *V. vulnificus* populations of both areas. Strains with genotypes and markers associated with pathogenicity are not restricted to a particular geographic region. This indicates that lack of reported cases in the North Sea region is not caused by the absence of potentially pathogenic strains.

Keywords: multilocus sequence typing; virulence-associated traits; genotypes; pathogenicity potential; vibrio infection; public health risk; global warming

1. Introduction

1.1. Background

Vibrio vulnificus belongs to the family of *Vibrionaceae* and is ubiquitously found in coastal, estuarine, and brackish environments in the water column, in sediments, as well as in or associated to fish, bivalve mollusks, crustaceans, and plankton [1,2]. The species causes foodborne infections resulting either in mild gastroenteritis with diarrhea, vomiting and abdominal pain or life-threatening primary septicemia, with mortality rates of 61% [3]. *V. vulnificus* can also enter the body through preexisting wounds exposed to seawater or through skin lesions incurred by handling of seafood or by fishing accidents. Due to the high multiplication rate of the pathogen, wound infections may quickly progress to necrotizing fasciitis and even secondary septicemia with mortality rates of 17% [3]. Regular alcohol abuse and liver diseases such as hemochromatosis, hepatitis, and cirrhosis are among

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the major risk factors for developing severe *V. vulnificus* infections, probably due to elevated serum iron levels in these patients [3,4].

In the United States, 43% of *V. vulnificus* infections manifest as primary septicemia and 45% as wound infections [3], whereby the incidence of wound infections is rising [5]. In contrast, foodborne infections are of minor significance in Germany, as reported cases occurred exclusively after contact to seawater [6]. Despite the high number of individuals with predisposing diseases (e.g., 36 million persons in the U.S.) and the great abundance of *V. vulnificus* in seawater and seafood, infections are only sporadically observed (about 100 reported cases annually in the U.S.) [7]. This indicates that so far unknown host factors may be essential in allowing infections in only a few people, and/or that only a small proportion of the *V. vulnificus* population is able to cause human infection [4]. Consequently, identification of putative virulence factors and pathogenicity markers are major targets of *V. vulnificus* research worldwide.

Several studies on multilocus sequence typing (MLST) of clinical and environmental *V. vulnificus* isolates showed a subdivision into two major lineages or clusters. Generally, most isolates within MLST cluster I were of environmental origin, while the majority of strains within cluster II were clinical isolates, thus implying a higher virulence potential for cluster II strains [8–11]. Molecular analyses revealed two alleles of the 16S rRNA gene (type A, type B) and of the so called "virulence correlated gene" of unknown function (*vcg*; type E and type C). 16S rRNA-type B and *vcg*-type C are correlated with increased mouse virulence and with the clinical origin of isolates [12–15]. Recent studies showed a high association of additional genes with clinical isolates from the Baltic Sea region and northeastern USA. These included the 33-kb genomic region XII, a mannitol fermentation operon, and the *nanA* gene encoding a *N*-acetylneuraminate lyase, the key enzyme for sialic acid catabolism [10,11,16]. This implies that these potential virulence markers should be additionally addressed when characterizing *V. vulnificus* pathogenicity.

V. vulnificus occurrence shows seasonal and regional variations with high temperature and low salinity as the major factors supporting growth [17,18]. The greater abundance of *V. vulnificus* in seawater and seafood in the summer months is reflected by increased incidences of human infection [19]. Especially in Northern Europe, occasional human infections have been linked to climate anomalies with most of the cases following heat waves, as documented for the years 2003, 2006 and 2010 [6,20–23]. Thus, impacts of climate change, such as rising sea surface temperatures and an increasing frequency of heat waves are assumed to favor proliferation and distribution of the pathogen in coastal areas at higher latitudes. This in turn may lead to higher incidences of *V. vulnificus* illness in Northern Europe [20–23]. Especially the Baltic Sea represents a high risk area, as it is a low salinity intercontinental sea and one of the fastest warming marine ecosystems on Earth [20]. So far, *V. vulnificus* infections in Germany have been documented for the Baltic Sea region, while no cases have been reported from the North Sea coast [6].

1.2. Aim of the Study

In view of climate projections and the demographic change, investigations on the pathogenicity potential of *V. vulnificus* isolates present in the North Sea and Baltic Sea are highly demanded as both regions represent popular tourist destinations. Variations in the prevalence of infections in both geographical regions may be influenced by a differing pathogenic potential of *V. vulnificus* populations. This study aimed to examine the genetic relationship of *V. vulnificus* isolates from both regions by MLST as well as the distribution of virulence-associated traits and genotypes to assess their pathogenic potential.

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2. Experimental Section

2.1. Bacterial Strains

Multilocus sequence typing (MLST) and characterization of virulence-associated traits were conducted on a total of 101 *V. vulnificus* strains, including 50 strains originating from the North Sea and 51 strains recovered from the Baltic Sea. Strains were exclusively isolated from water ($n = 56$) and sediment samples ($n = 45$) collected between 2010 and 2012 (between May and October) by local health authorities. Sampling sites were located along the German Baltic Sea and North Sea coastline, as well as within the estuaries of the rivers Ems and Weser. Detailed information on the location and classification of the sampling sites is given in Figure 1 and Supplementary Table S1. The isolates derived from strain collections gathered within the German research programs KLIWAS and VibrioNet [6,18,23–26]. To achieve a diverse collection, isolates were chosen from each available sampling site and date. Strains were obtained from nine sampling sites in the North Sea on 13 different sampling dates and from 18 sampling sites in the Baltic Sea on 16 different sampling dates.

All isolates and detailed sampling information are listed in Supplementary Table S2. Species identities of all strains were confirmed by a species-specific *toxR* polymerase chain reaction (PCR) amplification as described previously [27]. In parallel, strains were identified by a matrix assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry analysis using the MALDI Biotyper System (Bruker Daltonic, Bremen, Germany) and the direct transfer method [28]. Bacterial strains were grown overnight on LB (Lysogeny broth) agar at 37 °C. Single colonies were transferred to a stainless steel target and overlaid with matrix solution (10 mg/mL α-cyano-4-hydroxycinnamic acid in acetonitrile, water, and trifluoroacetic acid, 50:47.5:2.5, *v*/*v*).

Figure 1. Geographical location of sampling sites along the North Sea and Baltic Sea coastline. For detailed information on sampling sites, refer to Supplementary Table S1. Grey dots indicate sampling sites at which strains of MLST cluster II have been isolated at least once. White dots represent sampling sites at which all isolated strains belonged to MLST cluster I. Sampling Sites 3, 5 and 6 are located in estuaries, rivers are not indicated in the map.

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2.2. Biotyping and Mannitol Fermentation

Strains were characterized by a PCR described by Sanjuán *et al.* [29] allowing simultaneous amplification of target genes specific for *V. vulnificus*, biotype 2 and serovar E. Strains were further tested for the ability to cleave indole from tryptophan and to ferment sorbitol. *V. vulnificus* strains were streaked onto LB agar plates and incubated at 37 °C for 24 h. For indole reaction, 5 mL DEV-tryptophan-broth (0.1% tryptophan, 1% peptone, 0.5% NaCl, pH 7.2, Merck KGaA, Darmstadt, Germany) were inoculated with a single colony and incubated at 37 °C for 24 h. Generated indole was detected by dropping 100 µL Kovac's reagent (Merck KGaA, Darmstadt, Germany) on top of the culture. A positive indole reaction resulted in the formation of a red colored upper phase. For sorbitol fermentation, 5 mL sorbitol fermentation broth (1% sorbitol, 0.0075% bromothymol blue, 1% peptone, 0.5% NaCl, pH 7.4) were inoculated, incubated at 37 °C, and examined for fermentation after 24 h and five days indicated by a color change from blue/green to yellow. *V. vulnificus* strains were tested for mannitol fermentation analogously to the sorbitol fermentation test with 1% mannitol, as described previously [11].

2.3. Multilocus Sequence Typing

MLST analysis was performed based on ten housekeeping genes (*glp*, *gyrB*, *mdh*, *metG*, *purM*, *dtdS*, *lysA*, *pntA*, *pyrC*, *tnaA*) as described by Bisharat *et al.* [8,9] using primers and protocols published on the *V. vulnificus* Multilocus Sequence Typing website [30]. Electropherograms were assembled and trimmed using SeqMan Pro (v12; DNASTAR Lasergene, Madison, WI, USA) and Accelrys Gene (v2.5, Accelrys Inc., San Diego, CA, USA). Allele sequences at each locus and corresponding allelic profiles were compared to the PubMLST database. New allele sequences and allelic profiles (sequence types, STs) were submitted to the PubMLST database. A list of all strains with corresponding allelic profiles is provided in Supplementary Table S3. To elucidate clonal relationships, allelic profiles were analyzed with the PHYLOViZ software using the goeBURST algorithm [31]. Related strains with allelic profiles differing in one of ten loci (single locus variants, SLV) were assigned to a distinct clonal complex. The PHYLOViZ software was used to generate a full Minimum Spanning Tree (MST) displaying the relationships between STs of the whole dataset. Allele sequences were concatenated in the order of loci used to define the allelic profile to generate a 4326 bp concatemer for each strain. A phylogenetic tree was constructed in MEGA ver. 6 [32] based on the alignment of concatenated allele sequences using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) [33]. Distances were computed using the Maximum Composite Likelihood method [34] and the reliability of the tree was assessed by bootstrap analysis with 1000 replicates. Sequences from clinical and environmental biotype 1 isolates from the Baltic Sea region from our previous study [11] were included in the analysis for comparison. The same procedure was performed on concatemers of 1299 bp obtained by concatenating the sequences of three housekeeping genes in the order *gyrB*, *dtdS* and *pyrC*.

2.4. PCR Analyses

The RTP Bacteria DNA Kit (STRATEC Biomedical AG, Birkenfeld, Germany) was used for extraction of genomic DNA. PCR amplifications were carried out on a Mastercycler EP Gradient (Eppendorf, Hamburg, Germany) in a volume of 25 µL with 1× PCR-buffer (2 mM $MgCl_2$), 0.2 mM of each dNTP, 0.2 µM of each primer, and 1.5 U DreamTaq DNA Polymerase (Thermo Fisher Scientific Biosciences GmbH, St. Leon-Rot, Germany). Real-Time PCR was performed using a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Primers were synthesized by Metabion International AG (Martinsried, Germany). PCR products were purified using the MSB® Spin PCRapace Kit (STRATEC Biomedical AG, Berlin, Germany) and sequenced on both strands through sequencing service (Eurofins MWG GmbH, Ebersberg, Germany). Genotyping of the *vcg* and 16S rRNA genes was performed by conventional PCR amplification and Real-Time

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PCR, respectively [13,14]. In addition, all *V. vulnificus* isolates were tested for the presence of the gene *nanA* belonging to the sialic acid catabolism cluster and the gene *manIIA* of a mannitol fermentation operon by PCR amplification using published primer pairs [35,36]. The presence of genomic region XII was determined by amplification of the 5′ flanking region together with parts of the first gene VVA1613 of region XII encoding a chondroitinase AC lyase (VVA1612F and VVA1613R, 2257 bp). A second PCR assay, targeting the flanking regions, generates an amplicon only when region XII is completely absent (VVA1612bF and VVA1637R, 1200 bp). All strains were further tested for presence of VVA1634 of region XII, encoding an arylsulfatase A (VVA1634 F and VVA1634R, 1364 bp). Strains that contained region XII but were negative for VVA1634 were further tested with an additional primer pair (VVA1633a_F and VVA1635c_R, 2483 bp) designed based on published genome sequences (MO6-24/O, CMCP6 and YJ016) to amplify the whole arylsulfatase A gene. Generated amplicons of two strains (VN-10 and VN-16, characterized in Bier *et al.* [11]) were selected for further sequence analysis using a set of eleven primers. The sequences of the arylsulfatase A gene of VN-0010 (accession number LN879390) and VN-0016 (accession number LN879391) have been deposited in GenBank. All primer pairs, target genes, corresponding annealing temperatures, and amplicon sizes are listed in Supplementary Table S4.

2.5. Serum Resistance

A colorimetric assay that allows high-throughput screening of bacterial resistance to human serum was employed as previously described [11]. Experiments were performed in triplicate. Isolates that showed growth in the presence of 60%–80% human serum were classified as serum resistant. Isolates that only grew in the presence of 20%–40% or 0%–10% human serum were classified as intermediate resistant or susceptible, respectively.

2.6. Statistical Analyses

Descriptive statistics were used to compare the distribution of virulence-associated traits and genotypes among *V. vulnificus* isolates from the North Sea and Baltic Sea. Chi-square test for independence was applied with two-by-two contingency tables to test if observed differences regarding the geographical origin were statistically significant (p-values $\leqslant 0.05$).

3. Results and Discussion

3.1. Multilocus Sequence Typing

Multilocus sequence typing (MLST) was performed on ten housekeeping genes according to Bisharat *et al.* [8,9] and revealed high genetic diversity among environmental *V. vulnificus* isolates from German coastal waters. Among the 101 environmental *V. vulnificus* isolates examined in this study, a total of 74 different sequence types (STs) were identified, including 65 new STs with 127 newly identified allele sequences. The majority of STs (73%) were present only once in the strain collection. Fourteen STs (19%) were represented by two isolates and only six STs (ST128, ST219, ST226, ST244, ST269 and ST268) by three to four isolates. None of the STs represented by more than one isolate showed geographical dispersion over both investigated areas. The ratio between the number of different STs (Table 1) and the number of strains was comparable between the North Sea (0.76) and the Baltic Sea (0.71), indicating a similar degree of genetic diversity.

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Table 1. Distribution of MLST clusters and virulence-associated traits and genotypes among *V. vulnificus* isolates from the Baltic Sea and the North Sea.

Geographical Region (Number of Strains)	MLST Cluster (%)			*vcg*-Type (%)		16S rRNA-Type (%)			*nanA* (%)	Region XII (%)	Mannitol Fermentation (%)	Serum Resistance (%)			Risk Group (%) [a]		No. of Different Virulence Profiles (N)	No. of Strains (N)
–	I	IIA	IIB	C	E	A	AB	B	–	–	–	R	I	S	1	2	–	–
Total (n = 101)	79	6	15	6	94	79	14	7	47	37	40	79	14	7	42	59	17	74
Baltic Sea (n = 51)	71	0	29	0	100	71	27	2	24	35	14	76	18	6	61	39	8	36
North Sea (n = 50)	88	12	0	12	88	88	0	12	70	38	66	82	10	8	22	78	14	38

MLST, multilocus sequence typing; R, resistant; I, intermediate resistant; S, susceptible; ST, sequence type. [a] Risk Group 2 comprising strains with two or more pathogenicity markers, Risk Group 1 comprising strains without or with one pathogenicity marker.

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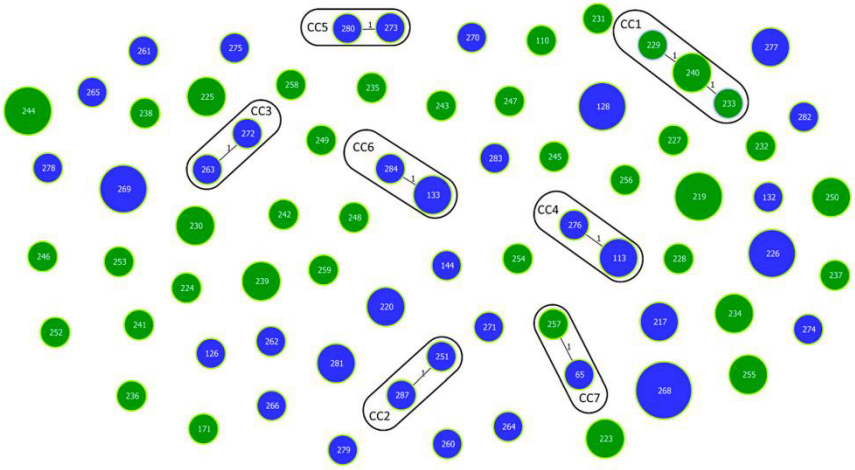
The high genetic diversity is also demonstrated by the high number of different STs found among isolates originating from the same sampling site on the same date. For example, five different STs were observed among six strains recovered on the same date at a beach in Binz (Sampling Site 26).

Investigation of clonal relationships using the goeBURST algorithm revealed seven clonal complexes defined at the single locus variant (SLV) level, including one triplet (ST229-ST233-ST240 with ST240 as the predicted founder) and six doublets (ST251-ST287, ST272-ST263, ST113-ST276, ST273-ST280, ST133-ST284 and ST65-257) (Figure 2A). When double and triple locus variants (DLV and TLV) were considered in the analysis, two previously observed doublets were expanded (ST273-ST280-ST269-ST126-ST270-ST265 and ST133-ST284-ST268) and two additional doublets (ST239-ST258 and ST132-ST277) were assigned (Figure 2B). The remaining 50 STs were singletons that differ in four to nine loci from other STs. The Minimum Spanning Tree drawn using an extension of the goeBURST rules shows that most of these singletons (62%) differ in six or seven loci from other STs in the tree (Figure 2B). Of the nine clonal complexes identified at the TLV level, only one doublet is associated with isolates from both seas (ST65-257, CC7), whereas all other groups consisted of strains recovered from either the North Sea or the Baltic Sea (Figure 2B).



(A)

Figure 2. *Cont.*

For a larger version of Figure 2A, see p.141.

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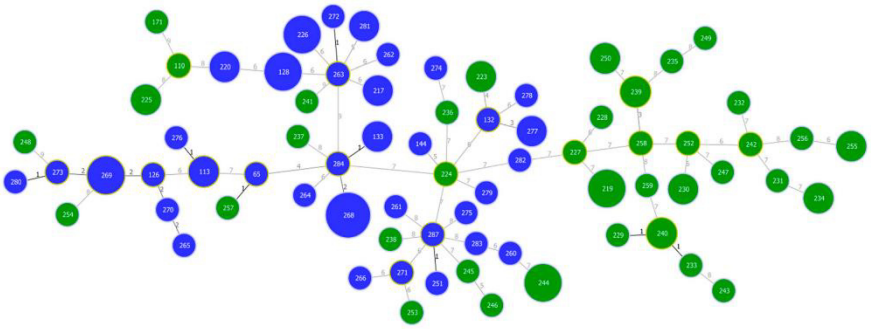

(**B**)

Figure 2. Population structure of *V. vulnificus* biotype 1 isolates from the North Sea and Baltic Sea obtained by goeBURST analysis based on MLST allelic profiles. Each sequence type (ST) is displayed as a circle with a size proportional to the number of isolates by which it is represented. The different colors indicate the geographical origin: North Sea (green) and Baltic Sea (blue). Single locus variants (SLVs) are connected via black lines. Light green halos around the circles indicate the respective founder of the group. (**A**) Population snapshot based on MLST allelic profiles. Clonal complexes (CC1–CC7) formed at the SLV level are highlighted by black edging; (**B**) Full Minimum Spanning Tree based on MLST allelic profiles. The number of different alleles between two STs is shown next to the connection lines.

As analysis of clonal relationships using allelic profiles does not consider the degree of heterogeneity at the nucleotide sequence level, an UPGMA tree was constructed based on the alignment of concatenated allele sequences to further evaluate genetic relationships among the strains (Figure 3).

Isolates from the Baltic Sea region characterized in our previous study [11] were included to increase the dataset and to identify strains with a close relationship to clinical strains. All isolates were divided into two major clusters I and II, as previously reported for MLST of *V. vulnificus* [8–11,16,37]. The majority of environmental isolates from the Baltic Sea (71%) and the North Sea (88%) investigated in this study fall into the "environmental" cluster I (Table 1). Twelve per cent of the North Sea isolates belonged to the "clinical" cluster II [8–11,16,37]. The proportion of cluster II isolates was more than twice as high among isolates from the Baltic Sea (29%), which was statistically significant ($\chi^2 = 6.941$, degrees of freedom (df) = 1, $p < 0.05$). Additionally, further separation of cluster II into two branches A and B was observed, concordantly with our previous analysis of clinical and environmental isolates from the Baltic Sea region [11]. Interestingly, all cluster II isolates of the North Sea fall into branch A, while those of the Baltic Sea were found in branch B (Figure 3). Although in this study, cluster IIA strains were exclusively obtained from the North Sea, our previous investigations had shown their existence in the Baltic Sea [11].

For a larger version of Figure 2B, see p.142.

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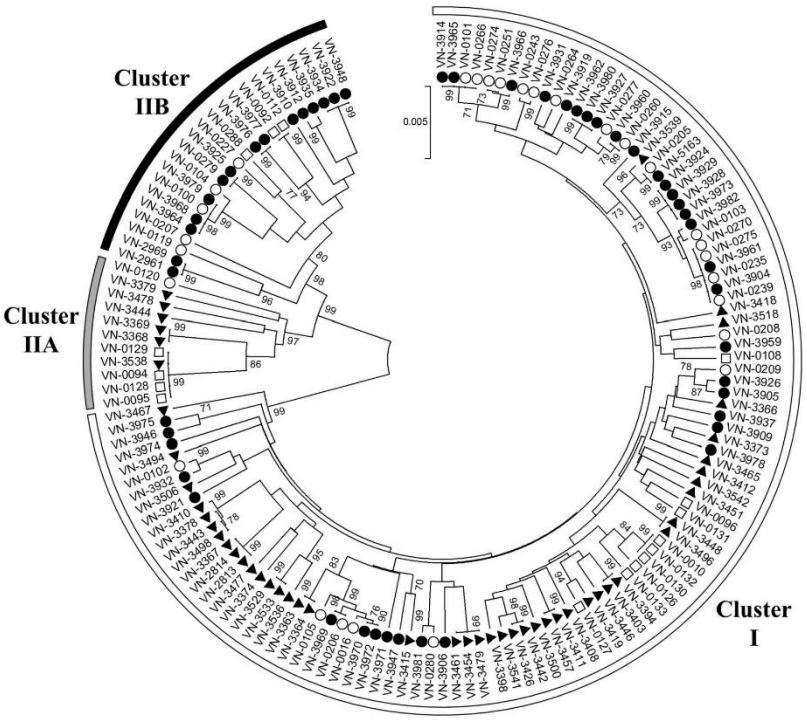

Figure 3. Population structure of *V. vulnificus* biotype 1 isolates from the North Sea (▲) and Baltic Sea (●) based on concatenated MLST sequences of ten housekeeping genes. Bootstrap values above 70% are shown next to the branches. Semicircles around the tree highlight the association of strains to MLST cluster I (white), IIA (grey), and IIB (black). Sequences from clinical (▫) and environmental (○) Baltic Sea isolates from a previous study [11] were included for comparison.

Cluster IIB strains were obtained from sampling sites distributed along the whole Baltic Sea coast (Figure 1) and accounted for a significant proportion of the *V. vulnificus* Baltic Sea isolates (29%). While none of the North Sea isolates investigated in this study fell into this branch, a cluster IIB strain originating from the Netherlands (strain no. 478867 and ST138 isolated in 1998 from fish/eel) is stored in the PubMLST database, indicating that cluster IIB also occurs in the North Sea. All cluster IIB strains listed in the PubMLST database (n = 19) were isolated in Europe. Most of them (n = 17) originate from the Baltic Sea region, thus indicating that cluster IIB strains represent a characteristic part of the *V. vulnificus* population in the Baltic Sea.

Interestingly, some environmental isolates are highly related to clinical strains and may represent a public health risk. For example, the North Sea isolate VN-3538 within cluster IIA shows the same sequence type found in four clinical strains (VN-0094, VN-0095, VN-0128 and VN-0129) from the Baltic Sea region. These five strains cluster together with two additional isolates from the North Sea (VN-3369 and VN-3368) indicating a close relationship. Another subcluster is found within cluster IIB. The Baltic Sea isolates VN-03977 and VN-03976 form a well supported group together with the clinical isolates VN-0092 and VN-0112. Within cluster I, one clinical isolate (VN-0127) and one North Sea isolate (VN-3419) form a group.

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A dendrogram with a comparable topology regarding the two major clusters and subclusters was obtained using the concatenated sequences of only three loci (*gyrB*, *dtdS* and *pyrC*) (Supplementary Figure S1). Consequently, MLST analysis based on these three loci seems to be sufficient for rapid identification of cluster I and II, respectively.

3.2. Distribution of Virulence-Associated Traits and Genotypes in MLST Clusters

All 101 *V. vulnificus* isolates were classified as biotype 1 based on multiplex PCR analysis and biochemical characteristics (positive indole reaction, negative for sorbitol fermentation). The isolates were examined for the ability to grow in human serum and to ferment mannitol (phenotypically and genotypically), as well as for the presence of the virulence-associated *nanA* gene and genomic region XII. In addition, all strains were typed based on the *vcg* and 16S rRNA genes to differentiate clinical associated C-type strains (16S rRNA-type B, *vcg*-type C) from environmental associated E-type strains (16S rRNA-type A, *vcg*-type E). All results are displayed in detail in supplementary Table S5. In addition, Figure 4 illustrates the distribution of virulence-associated traits and genotypes among the different MLST clusters and geographical areas.

The investigations revealed cluster-specific distributions of the 16S rRNA and *vcg* genotypes in the strain collection. MLST cluster I (n = 80) comprised all A/E-type isolates observed in this study and one A/C-type isolate (VN-3477). All cluster IIA strains (n = 6) were 16S rRNA-type B and *vcg*-type C, with the exception of one B/E-type isolate, which is in accordance with previous reports [10,11]. All strains of MLST cluster IIB (n = 15) were of the *vcgE*-type and characterized by possessing the 16S rRNA-type B allele, either alone (type B, 7%) or in combination with the type A allele (type AB, 93%). The presence of both 16S rRNA alleles in one strain has already been described before, as multiple copies of rRNA loci exist per genome [10,11,14]. In all AB-type isolates of this study, amplification of the type B allele reached the threshold at an earlier cycle compared to that of the type A allele. Thus, the type B allele seems to be present in a higher copy number in these isolates. The 16S rRNA-type B is regarded as an indicator for strain virulence, as the majority of clinical isolates were found to be type B (76%) [14]. In addition, a considerable proportion of clinical isolates has been described to possess both alleles (15%) [14], and 16S rRNA-type B as well as type AB strains are associated with a higher ability to cause lethal systemic infections in mice [15].

Overall, these results confirm correlations found in other studies [10,11] between MLST clusters and genotypes of the *vcg* and 16S rRNA genes, with type B/C being indicative for MLST cluster IIA and type AB/E or B/E being indicative for MLST cluster IIB. However, exceptions regarding the *vcg*-type observed in this and other studies [10] imply that these correlations are not absolute.

In our previous study [11] all cluster IIA isolates were of the B/C-type, resistant to human serum, and were positive for *nanA*, region XII, and mannitol fermentation. In contrast, cluster IIA strains of this study showed a higher variability of virulence-associated traits. All cluster IIA strains were able to ferment mannitol. This characteristic was also observed in 40% and 13% of cluster I and IIB isolates, respectively. The majority of isolates belonging to cluster IIA (83%) and IIB (73%) were positive for the gene *nanA*, while this was only the case for 39% of cluster I isolates. All cluster IIB and the majority of cluster IIA (83%) and cluster I (75%) isolates showed growth in the presence of 60%–80% human serum. 100% of cluster IIB and 50% of cluster IIA isolates contained region XII. 24% of cluster I isolates also showed presence of region XII, whereby cluster-specific differences were observed regarding the arylsulfatase A gene VVA1634. Overall, MLST cluster II showed a higher prevalence of the clinical associated region XII, *nanA* and the mannitol fermentation operon, confirming observations of other studies [10,11,36].

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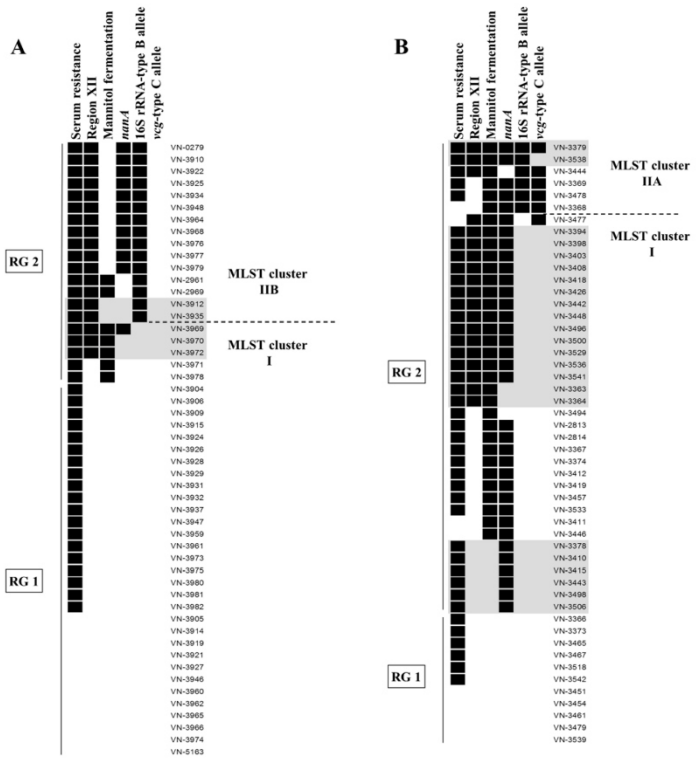

Figure 4. Combined results of MLST analysis and the investigation of virulence-associated traits and genotypes among *V. vulnificus* isolates from the Baltic Sea (**A**) and the North Sea (**B**). Presence of a pathogenicity marker is indicated by a black box. Strains rated as resistant (growth in 60%–80% human serum) are displayed as positive for serum resistance. Virulence profiles that have already been found in clinical isolates from the Baltic Sea region [11] are highlighted in grey. Risk Group 2 (RG2) comprises strains with two or more pathogenicity markers, while strains without or with one pathogenicity marker were assigned to Risk Group 1 (RG1) (see text). The figure was created using BioNumerics v7.5 (Applied Maths, Sint-Martens-Latem, Belgium).

The 33-kb genomic region XII has been implicated to contribute to virulence of *V. vulnificus*, as this region has predominantly been found in clinical isolates and in strains possessing the clinical genotype (16S rRNA-type B and *vcg*-type C) [10,11]. Region XII encompasses 22 open reading frames, including two chondroitinases and an arylsulfatase A gene cluster that may contribute to pathogenesis either directly or by simply mediating a selective advantage within the human host [10,38]. For detection and characterization of region XII, three PCR assays were conducted. In one PCR we targeted the 5′ flanking sequence and the first gene of region XII encoding a chondroitinase AC lyase (VVA1613). Amplification of this region is in complete accordance with the presence of region XII [10,11]. A second PCR, targeting the 5′ and 3′ flanking sequences confirmed absence of the whole region XII in all VVA1613-negative strains.

A third PCR assay targeted the gene VVA1634 of region XII encoding an arylsulfatase A. As previously observed [11], this gene could only be detected in region XII-positive strains of MLST cluster II with the used primers. In contrast, no amplicon was generated in region XII containing

For a larger version of Figure 4, see p.143.

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strains of MLST cluster I (n = 17). To elucidate if VVA1634 is absent or if possible single nucleotide polymorphisms (SNPs) prevent amplification in these strains, we designed primers to amplify the whole arylsulfatase A gene. In 14 cases a product with the expected size of 2.5 kb could be generated, indicating that the gene is present. Sequencing of the amplicon in selected strains and comparison with the two genome sequenced reference strains of MLST cluster II (CMCP6 and MO6-24/O) revealed cluster-specific gene polymorphisms, including both primer binding sites of VVA1634F and VVA1634R. The absence of the gene in the remaining three strains cannot be excluded, however, it seems likely that more SNPs may have prevented amplification in these strains. In total, 71 SNPs were found in VVA1634 between strains of MLST cluster I and II resulting in eighteen amino acid exchanges. These cluster specific variations should be considered when examining the role of this arylsulfatase A for pathogenesis of *V. vulnificus*.

3.3. Distribution of Virulence-Associated Traits and Genotypes among Isolates from the North Sea and the Baltic Sea

Data analysis revealed distinct differences in the distribution of virulence-associated traits and genotypes among the North Sea and Baltic Sea isolates (Figure 4). *Vcg*-type C strains were exclusively found among the North Sea isolates, and the 16S rRNA-type B allele was observed in a greater proportion of Baltic Sea isolates (29% type AB or B) compared to the North Sea isolates (12%) (Table 1). These observations match the geographical distribution pattern of MLST cluster IIA and IIB isolates described above.

The majority of the North Sea isolates were positive for the *nanA* gene (70%) and for mannitol fermentation (66%) while this was only the case in 24% and 14% of the Baltic Sea isolates, respectively (Table 1). These differences proved to be statistically significant (χ^2 = 25.54; df = 1, $p < 0.001$ for *nanA* and χ^2 = 32.72; df = 1, $p < 0.001$ for mannitol fermentation). Region XII was evenly distributed in 35% and 38% of the North Sea and Baltic Sea isolates, respectively. In addition, the proportions of isolates showing intermediate resistance and resistance to human serum were similar among the North Sea and Baltic Sea isolates (Table 1).

In this study, a total of seventeen different combinations (virulence profiles, VPs) of the six investigated virulence-associated traits and genotypes were identified among the 101 environmental *V. vulnificus* isolates (Table 2). Six VPs were already observed in clinical isolates [11]. The North Sea isolates showed a higher variability regarding the different combinations of virulence-associated traits, with fourteen different VPs compared to eight VPs found among the Baltic Sea isolates (Table 1). Nine VPs were only found among the North Sea isolates, three VPs were specifically detected among the Baltic Sea isolates, and five VPs were identified in isolates from both seas (Table 2). Isolates sharing the same virulence profile belonged to the same MLST cluster (Table 2). In total, the observed differences in the geographical distribution of virulence-associated traits and genes, as well as of distinct VPs show that the populations of the North Sea and the Baltic Sea vary widely.

3.4. Assessment of the Pathogenicity Potential of Environmental Strains

Several MLST analyses conducted on *V. vulnificus* demonstrated that the majority of strains of MLST cluster II are of clinical origin [8–11,16,37]. This cluster is also highly correlated with the clinical associated alleles of the *vcg* and 16S rRNA genes (*vcg*-type C, 16S rRNA-type B) that are often used to predict strain virulence [12,13,39]. In addition, the majority of cluster II strains are positive for the *nanA* gene, region XII, and mannitol fermentation which are traits associated with clinical origin [10,11,16,36]. Environmental isolates of cluster II are therefore assumed to have a high potential to cause infection and were categorized into Risk Group 2 in this study (Table 2).

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Table 2. Virulence profiles and corresponding number of *V. vulnificus* strains with respect to geographical origin and MLST cluster.

Risk Group [a]	Virulence Profile	No. of Isolates (Geographical Origin)	MLST Cluster
Risk Group 1	–	17 (12 BS, 5 NS)	I
	Res	25 (19 BS, 6 NS)	I
Risk Group 2	Man-*nanA*	2 (2 NS)	I
	Region XII-Man-*nanA*-vcgC	1 (1 NS)	I
	Res-Man	3 (2 BS, 1 NS)	I
	Res-Man-*nanA*	8 (8 NS)	I
	Res-*nanA* [b]	6 (6 NS [c])	I
	Res-Region XII-Man [b]	4 (2 BS, 2 NS)	I
	Res-Region XII-Man-*nanA* [b]	14 (1 BS, 13 NS)	I
	Man-*nanA*-16S_B-vcgC	1 (1 NS)	IIA
	Res-Man-*nanA*-16S_B-vcgC	2 (2 NS)	IIA
	Res-Region XII-16S_B [b]	2 (2 BS)	IIB
	Res-Region XII-Man-16S_B	2 (2 BS)	IIB
	Res-Region XII-Man-16S_B-vcgC	1 (1 NS)	IIA
	Res-Region XII-Man-*nanA*-16S_B [b]	1 (1 NS [c])	IIA
	Res-Region XII-Man-*nanA*-16S_B-vcgC [b]	1 (1 NS [c])	IIA
	Res-Region XII-*nanA*-16S_B	11 (11 BS)	IIB

MLST, multilocus sequence typing; Res, growth in 60%–80% human serum; Man, mannitol fermentation; 16S_B, presence of 16S rRNA-type B allele in type B or type AB; BS, Baltic Sea; NS, North Sea. [a] Risk Group 2 comprising strains with two or more pathogenicity markers, Risk Group 1 comprising strains without or with one pathogenicity marker. [b] profile already found in clinical isolates from the Baltic Sea region [11]. [c] profile exclusively found in the North Sea in this study, but previously observed among clinical or environmental isolates from the Baltic Sea region [11].

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In our previous study, we observed that the majority of clinical biotype 1 isolates from cases in the Baltic Sea region (59%) belonged to the "environmental" MLST cluster I and were of the A/E-genotype. However, these clinical isolates were characterized by the presence of the *nanA* gene, region XII, or the ability to ferment mannitol. Therefore, environmental strains of MLST cluster I possessing at least one of these pathogenicity markers were also assigned to Risk Group 2. Isolates that were negative for all tested virulence-associated traits or that showed only serum resistance were merged in Risk Group 1, as they possess the lowest probability to cause human infection.

Among the Baltic Sea isolates, the majority of strains belonged to Risk Group 1 (61%). In contrast, the majority of the North Sea isolates were assigned to Risk Group 2 (78%). Only a small proportion of strains (22%) was likely to be less pathogenic as no or only one single pathogenicity marker was present (Table 1, Figure 4). Remarkably, this significant difference (χ^2 = 18.947, df = 1, $p < 0.001$) is in contradiction with the observed prevalence of infection in both geographical areas. All cases of *V. vulnificus* infection in Germany were documented for the Baltic Sea region, while no cases have been reported from the North Sea coast so far. One explanation for this discrepancy could be the relatively rare occurrence and lower abundance of *V. vulnificus* in the North Sea, where the presence of *V. vulnificus* is restricted to low-salinity environments in the river estuaries of Weser and Ems [18,23]. In contrast, *V. vulnificus* has repeatedly been found at bathing sites along the whole German Baltic Sea coast of Mecklenburg-Western Pomerania, where it represents the most abundant potentially pathogenic *Vibrio* species together with *V. cholerae* non-O1/non-O139 [23,40].

Apart from differences in occurrence and abundance of potential pathogenic *V. vulnificus* in the two regions, more aspects have to be taken into account when reasons for the lack of cases in the North Sea region are sought for. Missing information on infections may be a result of underreporting and lack of public perception for this pathogen in the North Sea region. Furthermore, data on recreational activities in relation to water and weather conditions (temperature, salinity, *etc.*) are of great relevance, as infections are the result of exposition to the pathogen. In this study, only molecular characteristics of *V. vulnificus* isolates from the two areas were investigated.

The results of our study reveal that a high proportion of North Sea isolates could be of clinical significance (78%). This finding is of concern, as an increase in the occurrence of *V. vulnificus* in the North Sea is expected due to climate change. Multiple linear regression models based on the presence and abundance of *V. vulnificus* at the German North Sea coast between 2009 and 2011 estimate an increase of up to 30% for the occurrence of *V. vulnificus* by the end of 2099 [23]. Furthermore, an expansion of low-salinity habitats after heavy rainfall events would support a further dissemination of *V. vulnificus* along the German North Sea coast [23].

4. Conclusions

The aim of this study was to compare MLST profiles and virulence-associated traits of *V. vulnificus* strains isolated from the German North Sea and Baltic Sea coasts and to assess their pathogenicity potential. Climate change will favor growth conditions of mesophilic vibrios in the next decades in these two seas and *V. vulnificus* is expected to benefit greatly from rising sea surface temperatures [20,21]. As the two German coastlines are popular recreational areas visited by millions of tourists every year, a risk assessment for strains of this potentially dangerous species is heavily in demand. Isolates of *V. vulnificus* from the German North Sea coastline have not been investigated in detail before and studies addressing the pathogenicity potential of *V. vulnificus* from the Baltic Sea are scarce [11]. In this study a high genetic diversity was observed by MLST among North Sea and Baltic Sea isolates. Combination of MLST data and presence of virulence-associated traits suggested grouping of strains unlikely to cause human infection in Risk Group 1. Analysis of the *V. vulnificus* populations of the two areas revealed that the proportion of strains with two or more pathogenicity markers (Risk Group 2) was higher in the North Sea than in the Baltic Sea. Although the occurrence of *V. vulnificus* in the North Sea is lower than in the Baltic Sea and restricted to the river estuaries [18,23],

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this study clearly demonstrates that increased awareness for this pathogen by both the public health sector and local medical staff is required for the North Sea region.

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2.5 Publication 4

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Survey on antimicrobial resistance patterns in *Vibrio vulnificus* and *Vibrio cholerae* non-O1/non-O139 in Germany reveals carbapenemase-producing *Vibrio cholerae* in coastal waters

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An increase in the occurrence of potentially pathogenic *Vibrio* species is expected for waters in Northern Europe as a consequence of global warming. In this context, a higher incidence of *Vibrio* infections is predicted for the future and forecasts suggest that people visiting and living at the Baltic Sea are at particular risk. This study aimed to investigate antimicrobial resistance patterns among *Vibrio vulnificus* and *Vibrio cholerae* non-O1/non-O139 isolates that could pose a public health risk. Antimicrobial susceptibility of 141 *V. vulnificus* and 184 *V. cholerae* non-O1/non-O139 strains isolated from German coastal waters (Baltic Sea and North Sea) as well as from patients and retail seafood was assessed by broth microdilution and disk diffusion. Both species were susceptible to most of the agents tested (12 subclasses) and no multidrug-resistance was observed. Among *V. vulnificus* isolates, non-susceptibility was exclusively found toward aminoglycosides. In case of *V. cholerae*, a noticeable proportion of strains was non-susceptible to aminopenicillins and aminoglycosides. In addition, resistance toward carbapenems, quinolones, and folate pathway inhibitors was sporadically observed. Biochemical testing indicated the production of carbapenemases with unusual substrate specificity in four environmental *V. cholerae* strains. Most antimicrobial agents recommended for treatment of *V. vulnificus* and *V. cholerae* non-O1/non-O139 infections were found to be effective *in vitro*. However, the occurrence of putative carbapenemase producing *V. cholerae* in German coastal waters is of concern and highlights the need for systematic monitoring of antimicrobial susceptibility in potentially pathogenic *Vibrio* spp. in Europe.

Keywords: antimicrobial resistance pattern, Baltic Sea, North Sea, carbapenemase, disk diffusion, broth microdilution

INTRODUCTION

The family *Vibrionaceae* within the class of Gammaproteobacteria comprises eight genera of Gram-negative, facultative anaerobic, straight, or curved rods that are mostly oxidase-positive, halophilic, and motile (Farmer and Janda, 2004). Members of this family are ubiquitously distributed in

aquatic ecosystems worldwide. They can be found as free-living bacteria and as commensals of aquatic organisms and play an important role in nutrient cycling of natural aquatic habitats. Due to their metabolic diversity and their adaptive abilities to changing environmental conditions, a seasonal, and geographical variability of total *Vibrio* populations is observed in response to climatic influences and seawater circulations (Mansergh and Zehr, 2014).

Among the *Vibrionaceae*, a number of important human pathogenic bacteria have been identified that can cause gastrointestinal infections, wound infections or septicemia. *Vibrio cholerae*, *Vibrio vulnificus*, and *Vibrio parahaemolyticus* are considered as the most clinically relevant human pathogens within the genus *Vibrio* (Daniels and Shafaie, 2000). The latter species is widely disseminated in estuarine, marine, and coastal so surroundings and the leading cause of human intestinal infections after consumption of raw and undercooked seafood (Letchumanan et al., 2014). *V. cholerae* and *V. vulnificus* are also part of the microbial community in coastal or estuarine aquatic ecosystems with moderate salinities (Thompson and Polz, 2006).

V. cholerae is a well-known human pathogen consisting of more than 200 serogroups (Kaper et al., 1995; Lutz et al., 2013). Toxigenic *V. cholerae* of the O1 or O139 serogroup are the causative agents of cholera, an endemic disease in many Asian and African countries with symptoms of severe watery diarrhea, vomiting, and dehydration. All other serogroups designated as *V. cholerae* non-O1/non-O139 have also been linked to sporadically occurring human infections ranging from extraintestinal wound or ear infections (Huhulescu et al., 2007) to relatively mild or sometimes severe gastroenteritis (Tobin-D'Angelo et al., 2008), whereby smaller diarrheal outbreaks were also reported (Luo et al., 2013). Additionally, rarely occurring bacteremia has been described with mortality rates up to 61.5% (Petsaris et al., 2010). Since 2000, around 40 cases of *V. cholerae* non-O1/non-O139 infections in the United States have been reported to the CDC annually[1]. *V. cholerae* infections contracted in Germany were mainly ear or wound infections caused by non-toxigenic non-O1/non-O139 strains that were acquired through contact to seawater (Huehn et al., 2014). Due to the rare occurrence of *V. cholerae* non-O1/non-O139 infections, there are no official recommendations on antibiotic therapy (Petsaris et al., 2010). However, in case of bacteremia an early administration of antibiotic therapy can prevent a fatal outcome. Several case studies on *V. cholerae* non-O1/non-O139 bacteremia and wound infection exist, where fluoroquinolones and third-generation cephalosporins have been used (Huhulescu et al., 2007; Petsaris et al., 2010). But also treatment with ampicillin or last-line carbapenems has been described (Feghali and Adib, 2011; Lu et al., 2014).

V. vulnificus is known as a highly virulent pathogen. Although infections occur only sporadically, they can rapidly progress to septicemia, especially in persons with predisposing risk factors (e.g., immunocompromising conditions or chronic liver diseases resulting in elevated serum iron levels; Oliver, 2006). Foodborne infections can either result in a relatively mild gastroenteritis or in primary septicemia with mortality rates of 61% (Shapiro et al., 1998; Oliver, 2006). A second infection route for *V. vulnificus* is through open wounds exposed to seawater. Due to the high multiplication rate of the pathogen, wound infections may quickly progress to necrotizing fasciitis, which often makes surgical debridement or amputation necessary (Daniels and Shafaie, 2000). Delayed treatment promotes progression to secondary septicemia with mortality rates about seventeen per cent (Shapiro et al., 1998; Daniels and Shafaie, 2000). Surgical interventions should be considered early to prevent a fatal outcome as poor blood perfusion in necrotic tissue can impede the achievement of effective concentrations of antimicrobial agents (Chen et al., 2012). However, to avoid septicemia and a further distribution of the pathogen additional antibiotic therapy is indispensable and should be administered as early as possible. Due to the fast progression of *V. vulnificus* infections, the presence of antimicrobial resistance preventing an effective therapy can be fatal for the patient. A combination of a tetracycline with a third-generation cephalosporin or single-agent therapy with fluoroquinolones is recommended by the CDC[2], while trimethoprim-sulfamethoxazole in combination with an aminoglycoside is proposed for the treatment of pregnant women and children.

In the U.S., 95% of all seafood-related deaths can be attributed to *V. vulnificus*, whereas infections in Germany were almost exclusively wound infections occurring after contact to seawater (Oliver, 2006; Huehn et al., 2014). So far, *Vibrio* infections in Germany occur only sporadically but incidences peaked after extreme heatwaves (Huehn et al., 2014). Due to impacts of climate change, a rise in the occurrence of *V. vulnificus* and *V. cholerae* is predicted for European waters (Baker-Austin et al., 2012). Changing demography is expected to further contribute to higher incidences of *Vibrio* infections (Baker-Austin et al., 2012). In view of these forecasts and the potential severity of infections, an investigation on antimicrobial susceptibility of *Vibrio* spp. is demanded to provide guidance for medical intervention, but also for epidemiological purposes. For this reason, our study aimed to assess antimicrobial resistance prevalence among *V. vulnificus* and *V. cholerae* non-O1/non-O139 posing a public health risk for the population. Environmental isolates were obtained from German coastal and estuarine waters of the open North Sea and the intracontinental Baltic Sea. In addition, we also included isolates from clinical sources and retail seafood for comparison and to give a more comprehensive overview of antimicrobial resistance patterns of these two species in Germany. To our knowledge, this is the first study examining antimicrobial susceptibility of *V. vulnificus* and *V. cholerae* non-O1/non-O139 in Northern Europe on a large scale.

MATERIALS AND METHODS

Bacterial Strains

The strains used in this study are summarized in **Table 1** and listed in detail in Supplementary Tables S1, S2. Antimicrobial susceptibilities were determined for a total of 325 bacterial

[1] http://www.cdc.gov/cholera/non-01-0139-infections.html

[2] http://emergency.cdc.gov/disasters/vibriovulnificus.asp

TABLE 1 | Origin and source of *V. cholerae* non-O1/non-O139 (*n* = 184) and *V. vulnificus* (*n* = 141) strains included in the study.

Origin	Geographical origin	Source	Source code	No. of strains
V. cholerae				
Environmental (E) (2009–2014; *n* = 131)	Baltic Sea (BS) (*n* = 79)	Seawater (sw)	E-BS-sw	54
		Sediment (sd)	E-BS-sd	4
		Seawater/sediment (sw/sd)	E-BS-sw/sd	21
	North Sea (NS) (*n* = 52)	Bivalve mollusks (bm)	E-NS-bm	26
		Seawater (sw)	E-NS-sw	12
		Seawater/sediment (sw/sd)	E-NS-sw/sd	14
Clinical (C) (1995–2012; *n* = 18)	Travel-associated (ta) (*n* = 7)	Extraintestinal (ext)	C-ta-ext	1
		Intestinal (int)	C-ta-int	6
	Germany/Austria (G/A) (*n* = 11)	Extraintestinal (ext)	C-G/A-ext	9
		Intestinal (int)	C-G/A-int	2
Retail (R) (2008–2014; *n* = 35)	Germany (G) (*n* = 35)	Bivalve mollusks (bm)	R-G-bm	2
		Crustacean (cr)	R-G-cr	26
		Fish (fi)	R-G-fi	7
V. vulnificus				
Environmental (E) (2004–2012; *n* = 122)	Baltic Sea (BS) (*n* = 70)	Seawater (sw)	E-BS-sw	46
		Sediment (sd)	E-BS-sd	24
	North Sea (NS) (*n* = 52)	Seawater (sw)	E-NS-sw	29
		Sediment (sd)	E-NS-sd	21
		Bivalve mollusks (bm)	E-NS-bm	2
Clinical (C) (1994–2011; *n* = 19)	Denmark (D)	Extraintestinal (ext)	C-D-ext	14
	Germany (G)	Extraintestinal (ext)	C-G-ext	5

strains, including 141 isolates of *V. vulnificus* (19 clinical, 122 environmental) and 184 isolates of *V. cholerae* non-O1/non-O139 (18 clinical, 131 environmental, 35 retail). The majority of environmental strains were isolated by health authorities during the German research programs KLIWAS[3] and VibrioNet[4] between 2004 and 2014. Water and sediment samples were mostly collected at bathing sites along the Baltic Sea and North Sea coastline as well as within the estuaries of the rivers Ems and Weser (Böer et al., 2012). Environmental isolates from bivalve mollusks were obtained from coastal areas of the North Sea. Isolates from retail samples were collected by health authorities of Germany and sent to the National Reference Laboratory for Monitoring Bacteriological Contamination of Bivalve Mollusks of the Federal Institute for Risk Assessment (BfR), Germany. Clinical *V. vulnificus* and *V. cholerae* non-O1/non-O139 isolates were characterized in previous studies (Bier et al., 2013; Schirmeister et al., 2014).

DNA Extraction

DNA extraction was performed with two methods that are equally applicable for *Vibrio* species. Genomic DNA of *V. vulnificus* isolates was extracted from 1 ml of an overnight culture using the RTP Bacteria DNA Kit according to the manufacturer's protocol (STRATEC Biomedical AG, Birkenfeld, Germany).

Genomic DNA of *V. cholerae* strains was extracted using a boiling method: 1.5 ml of an overnight culture were centrifuged at 14,000 g for 4 min. The cell pellet was suspended in 300 µl TE buffer (10 mM Tris-HCl, 0.5 mM EDTA, pH 8), boiled for 10 min at 95°C, and subsequently cooled on ice. After centrifugation at 14,000 g for 2 min, a 200 µl aliquot of the supernatant was transferred to a new sterile tube. DNA preparations were stored at −20°C.

Species Confirmation

Species confirmation of all *V. vulnificus* and *V. cholerae* strains was carried out by species-specific *toxR* PCR amplification as previously described (Bauer and Rørvik, 2007) and in parallel by MALDI-TOF MS analysis. MALDI-TOF MS analysis was performed using a Microflex LT system mass spectrometer (Bruker Daltonik, Bremen, Germany) following the manufacturer's settings. MALDI spectra were obtained by the direct transfer method according to the manufacturer's protocol as previously described (Schirmeister et al., 2014).

Characterization of *V. cholerae* Isolates

V. cholerae isolates were characterized and subtyped via multiplex PCR targeting *rfb* sequences specific for O1 and O139 serogroups, *toxR,* and *ctxA*. PCR amplification was performed in a final volume of 25 µl with 1x PCR buffer (3 mM $MgCl_2$), 0.2 mM of each deoxynucleoside triphosphate, 0.5 µM of O1 *rfb* primers, 0.125 µM of O139 *rfb, toxR,* and *ctxA* primers, 1.5 U DreamTaq DNA polymerase (Thermo Fisher Scientific Biosciences GmbH, St. Leon-Rot, Germany), and 2 µl of genomic DNA. After an initial denaturation step at 94°C for 4 min, the cycling conditions

[3]http://www.kliwas.de/KLIWAS/EN/03_ResearchTasks/03_vh3/04_304/304_node.html
[4]http://www.vibrionet.de/

were the following: 30 cycles at 94°C for 30 s, 59°C for 30 s, 72°C for 30 s, followed by a final extension step at 72°C for 5 min.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility to the following 13 antimicrobial agents was determined by broth microdilution according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2012a) using custom-defined microtiter plates (EUMVS2, Trek Diagnostic Systems, East Grinstead, United Kingdom): ampicillin, ceftazidime, cefotaxime, chloramphenicol, ciprofloxacin, colistin, florfenicol, gentamicin, kanamycin, nalidixic acid, streptomycin, tetracycline, and trimethoprim. Test ranges are shown in Supplementary Table S3. Additionally, all isolates were tested for their susceptibility to amoxicillin-clavulanic acid (20/10 μg), cefepime (30 μg), imipenem (10 μg), levofloxacin (5 μg), meropenem (10 μg), and sulfamethoxazole-trimethoprim (23.75/1.25 μg) by the disk diffusion method, according to the guidelines of the CLSI using commercially available disks (Oxoid GmbH, Wesel, Germany; CLSI, 2012b).

Strains showing non-susceptibility to imipenem (zone diameter ≤ 19) were tested against an additional panel of β-lactams by broth microdilution (imipenem, ertapenem, cefepime, cefoxitin, temocillin; EUVSEC2, Trek Diagnostic Systems) or disk diffusion (aztreonam, 30 μg).

Following the guidelines of the CLSI, tests were performed with Mueller-Hinton agar and cation-adjusted Mueller-Hinton broth without supplementation of additional sodium chloride (CLSI, 2010a). *Escherichia coli* ATCC 25922 was used for quality assurance. Minimal inhibitory concentration (MIC) values and inhibition zone diameters of all strains are listed in Supplementary Tables S1, S2. Results were interpreted using the criteria summarized in Supplementary Table S3. In general, results were interpreted according to CLSI clinical breakpoints specific for *Vibrio* spp. (CLSI, 2010a), which derived from breakpoints for *Enterobacteriaceae* (CLSI, 2010b). In cases where CLSI breakpoints for *Vibrio* spp. were obsolete or not available, the latest CLSI breakpoints for *Enterobacteriaceae* were used: document M100-S25 for aztreonam, cefepime, ertapenem, gentamicin, kanamycin, imipenem, meropenem, nalidixic acid, and trimethoprim (CLSI, 2015); document Vet01-S2 for florfenicol (CLSI, 2013). Other interpretive criteria were used for colistin (EUCAST clinical breakpoints for *Enterobacteriaceae*[5] ; EUCAST, 2015), temocillin (BSAC interpretive criteria for systemic infections; Andrews, 2009), and streptomycin (based on different studies of *Vibrio* spp. and *E. coli*; National Food Institute, 2013; Shaw et al., 2014), as no CLSI breakpoints were available.

Molecular Investigation of Resistance Determinants

PCR amplification was conducted to detect specific antimicrobial resistance determinants depending on the phenotype found. Non-susceptible isolates were screened for genes mediating resistance to streptomycin (*aadA1, aadA2,* and *strA/B*) and β-lactams (bla_{PSE-1}, $bla_{OXA-1-like}$, $bla_{TEM-1-like}$) that are widespread in *Enterobacteriaceae* and other Gram-negative bacteria. Specifically imipenem-resistant strains (zone diameter ≤ 19) were tested for the presence of several carbapenemase and AmpC β-lactamase encoding genes. Presence of class 1 integrons was investigated by PCR amplification of the corresponding integrase gene *intI1* in all β-lactam and streptomycin non-susceptible strains.

Standard PCR reactions were performed using a Mastercycler EP gradient (Eppendorf, Hamburg, Germany) in a volume of 25 μl with 1x PCR buffer (2 mM $MgCl_2$), 0.2 mM of each deoxynucleoside triphosphate (dNTP), 0.2 μM of each primer, 1.5 U DreamTaq DNA polymerase, and 2 μl of genomic DNA. After an initial denaturation step at 94°C for 4 min, the cycling conditions were the following: 30 cycles of denaturation at 94°C for 30 s, primer annealing for 30 s, and extension at 72 °C for 1 min per kb, followed by a final extension step at 72°C for 10 min.

All primer pairs, target genes, corresponding annealing temperatures, and amplicon sizes are listed in Supplementary Table S4. Enterobacterial strains carrying class 1 integrons and investigated resistance determinants (*aadA1*, *aadA2,* bla_{PSE-1}, bla_{OXA-1}, bla_{TEM-1}, bla_{NDM-1}, bla_{IMP-1}, bla_{VIM-2}, bla_{KPC-3}, bla_{OXA-48}, bla_{ACC-1}, bla_{CMY-1}, bla_{CMY-2}, bla_{DHA-1}, bla_{ACT-1}, bla_{FOX-1}, *strA*, and *strB*), as well as one *V. cholerae* non-O1/non-O139 isolate carrying a class 1 integron with the *aadA1* gene were used as positive controls. Susceptible *V. cholerae* and *V. vulnificus* strains were included as negative controls.

All streptomycin resistant *V. cholerae* (VN-3469, VN-5095, VN-10191, and VN-10192) and *V. vulnificus* (VN-0098, VN-0100, VN-0125, VN-0129) isolates were further examined for mutations in the *rpsL* gene encoding ribosomal protein S12. Streptomycin susceptible (*V. cholerae*: VN-0298, VN-2997, VN-3955, VN-4226, and *V. vulnificus*: VN-0096, VN-0274, VN-3368) and intermediate resistant strains (*V. cholerae*: VN-3944, VN-4261, and *V. vulnificus*: VN-3418, VN-3981, VN-10121) were included as controls. For specific amplification and sequencing of the whole *rpsL* gene in *V. cholerae* and *V. vulnificus*, two primer pairs Vc-rpsL-F/Vc-rpsL-R and Vv-rpsL-F/Vv-rpsL-R were designed based on published genome sequences (*V. cholerae* strains NIH41, N16961, O395, and *V. vulnificus* strains CMCP6, YJ016, MO6-24/O). Purification of PCR products was performed using the MSB® Spin PCRapace Kit (STRATEC Biomedical AG, Berlin, Germany). Sequencing was conducted on both strands through sequencing service (Eurofins MWG GmbH, Ebersberg, Germany). Electropherograms were assembled and trimmed using SeqMan Pro (v12; DNASTAR Lasergene, Madison, Wisconsin). Sequences were analyzed and compared to the sequences of reference and control strains using Accelrys Gene (v2.5, Accelrys Inc., San Diego, California).

Test for Carbapenemase Activity: Carba NP Test II/Blue-Carba Test

Strains non-susceptible to imipenem (zone diameter ≤ 19) were grown overnight at 37°C on chromID™ CARBA (bioMérieux, Nürtingen, Germany). Bacterial colonies were subsequently tested for carbapenemase activity with the improved Carba NP test II (Dortet et al., 2014) using 12 g/L imipenem/cilastatin

[5]http://www.eucast.org

(Zienam®, MSD SHARP, and DOHME GMBH, Haar, Germany) and two calibrated loops (10 µl) as bacterial inoculum to increase enzyme quantity. The Blue-CARBA test was performed as described (Pires et al., 2013) and in addition analogously to the Carba NP test II with two loops of bacterial colonies in 200 µl of the test solution in microcentrifuge tubes and supplementation of tazobactam or EDTA to inhibit class A or metallo-carbapenemases, respectively. Two *E. coli* strains positive for NDM-1 and KPC-2, respectively, as well as a KPC-3-positive *Klebsiella pneumoniae* strain served as positive controls.

Statistical Analyses

Descriptive statistics were used to analyze resistance prevalence to different antimicrobial agents (**Table 2**). Chi-square test for independence was applied with 2 × 2 contingency tables to test if observed differences displayed in **Table 3** were statistically significant (P-values ≤ 0.05). MIC_{50} and MIC_{90} were defined as the concentration at which growth of 50 and 90% of the isolates was inhibited, respectively.

RESULTS

Antimicrobial Susceptibility of Clinical and Environmental *V. vulnificus* Isolates

Fifty-seven per cent of all examined *V. vulnificus* isolates showed susceptibility to all antimicrobial agents tested (with the exception of colistin). All 141 *V. vulnificus* isolates, regardless of their origin were susceptible to quinolones, fluoroquinolones, phenicols, tetracyclines, folate pathway inhibitors, aminopenicillins with or without β-lactamase inhibitors, carbapenems, and third- and fourth- generation cephalosporins. The clinically relevant agents cefotaxime, ceftazidime, and tetracycline were among the most effective antimicrobial agents *in vitro* as they showed MIC_{90} values identical to the lowest concentration tested (**Table 4**). Non-susceptibility was exclusively observed toward aminoglycosides with 40 and 3% of all strains showing intermediate resistance (MIC 32 mg/L) and resistance (MIC 64 mg/L) to streptomycin, respectively. One clinical isolate was intermediate resistant to kanamycin (MIC 32 mg/L) while all strains were susceptible to gentamicin. The percentage of non-susceptible strains with respect to different origins is shown in **Table 3**. No significant difference was observed between clinical ($n = 19$) and environmental ($n = 122$) isolates, nor was there a significant difference between isolates from the Baltic Sea and the North Sea ($p > 0.05$, χ^2). None of the examined gene determinants encoding streptomycin resistance (*aadA1, aadA2*, *strA/B*), nor class 1 integrons were detected in streptomycin non-susceptible *V. vulnificus* isolates. Sequence analysis of the *rpsL* gene revealed that all four streptomycin resistant *V. vulnificus* isolates (VN-0098, VN-0100, VN-0125, VN-0129) as well as two susceptible (VN-0274, VN-3368) and two intermediate resistant isolates (VN-3918, VN-10121) carried one silent point mutation A-291-T compared to the three reference strains (CMCP6, YJ016, and MO6-24/O). An additional silent mutation C-351-T within the *rpsL* gene was observed in strain VN-0100.

Antimicrobial Susceptibility of *V. cholerae* non-O1/non-O139 Isolated from Clinical, Environmental and Seafood Samples

All 184 isolates investigated in this study were confirmed to be non-toxigenic *V. cholerae* non-O1/non-O139 isolates. The

TABLE 3 | Overall resistance occurrence in *V. cholerae* and *V. vulnificus* isolates with respect to different origins.

	Retail	Clinical	Environmental	North Sea	Baltic Sea
V. cholerae	($n = 35$)	($n = 18$)	($n = 131$)	($n = 52$)	($n = 79$)
Strains susceptible to all antimicrobial agents	27 (77%)	10 (56%)	86 (66%)	28 (54%)	58 (73%)
Non-susceptible strains	8 (23%)	8 (44%)	45 (34%)	24(46%)	21 (27%)
V. vulnificus		($n = 19$)	($n = 122$)	($n = 52$)	($n = 70$)
Strains susceptible to all antimicrobial agents		8 (42%)	72 (59%)	28 (54%)	44 (63%)
Non-susceptible strains		11 (58%)	50 (41 %)	24 (46 %)	26 (37 %)

TABLE 2 | Susceptibility vs. resistance occurrence (%) found among *V. cholerae* non-O1/non-O139 isolates from different origins[a].

Antimicrobial agent	Total ($n = 184$)			Retail ($n = 35$)			Clinical ($n = 18$)			Environmental ($n = 131$)			North Sea ($n = 52$)			Baltic Sea ($n = 79$)		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
Amoxicillin/clavulanic acid	98	2	0	100	0	0	100	0	0	97	3	0	94	6	0	99	1	0
Ampicillin	89	0	11	89	0	11	83	0	17	90	0	10	85	0	15	94	0	6
Imipenem	97	1	2	100	0	0	100	0	0	95	2	3	94	0	6	96	3	1
Meropenem	98	2	<1	100	0	0	100	0	0	97	2	1	94	4	2	99	1	0
Nalidixic acid	99	0	1	100	0	0	89	0	11	100	0	0	100	0	0	100	0	0
Streptomycin	78	20	2	86	11	3	83	17	0	75	23	2	65	29	6	81	19	0
Trimethoprim	99	0	1	97	0	3	100	0	0	100	0	0	100	0	0	100	0	0

S, susceptible; I, intermediate resistant; R, resistant.

[a]All strains were susceptible to ceftazidime, chloramphenicol, ciprofloxacin, cefotaxime, cefepime, florfenicol, gentamicin, kanamycin, levofloxacin, trimethoprim/sulfamethoxazole and tetracycline.

TABLE 4 | Antimicrobial MIC distributions for the *V. cholerae* and *V. vulnificus* isolates tested.

Antimicrobial agent	Test range (mg/L)	Breakpoints[a] (mg/L)			MIC (mg/L) distribution for *V. vulnificus* ($n = 141$)			MIC (mg/L) distribution for *V. cholerae* ($n = 184$)		
		S	*I*	*R*	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range
Ampicillin	0.5–32	≤8	16	≥32	1	2	≤0.5–2	2	8	≤0.5–>32
Cefotaxime	0.06–4	≤1	2	≥4	≤0.06	≤0.06	≤0.06–0.12	≤0.06	≤0.06	≤0.06–0.12
Ceftazidime	0.25–16	≤4	8	≥16	≤0.25	≤0.25	≤0.25–0.5	≤0.25	≤0.25	≤0.25–0.5
Chloramphenicol	2–64	≤8	16	≥32	≤2	≤2	≤2	≤2	≤2	≤2
Ciprofloxacin	0.008–8	≤1	2	≥4	0.015	0.03	≤0.008–0.06	≤0.008	≤0.008	≤0.008–0.5
Florfenicol	2–64	≤4	8	≥16	≤2	≤2	≤2	≤2	≤2	≤2
Gentamicin	0.25–32	≤4	8	≥16	2	2	0.5–4	1	2	≤0.25–4
Kanamycin	4–128	≤16	32	≥64	8	16	≤4–32	≤4	8	≤4–16
Nalidixic acid	4–64	≤16		≥32	≤4	≤4	≤4–8	≤4	≤4	≤4–>64
Streptomycin	2–128	≤16	32	≥64	16	32	4–64	16	32	8–64
Tetracycline	1–64	≤4	8	≥16	≤1	≤1	≤1–2	≤1	≤1	≤1
Trimethoprim	0.5–32	≤8		≥16	1	1	≤0.5–4	≤0.5	1	≤0.5–>32

S, susceptible; I, intermediate resistant; R, resistant.

[a]*Criteria used for interpretation and corresponding references are given in Supplementary Table S3.*

majority of isolates (67%) were susceptible to all antimicrobial agents tested (with the exception of colistin). Eighteen per cent of the strains showed intermediate resistance to one or two antimicrobial agents (mostly to streptomycin) and the remaining strains (15%) showed full resistance to at least one antimicrobial agent. None of the *V. cholerae* isolates showed multidrug-resistance, defined as resistance to three or more classes of antimicrobial agents (Chen et al., 2010). Resistance profiles are shown in Supplementary Table S2, while resistance occurrence is given in **Table 2.**

As observed among *V. vulnificus*, all *V. cholerae* strains were susceptible to ciprofloxacin, chloramphenicol, florfenicol, cefotaxime, sulfamethoxazole-trimethoprim, levofloxacin, ceftazidime, cefepime, gentamicin, kanamycin, and tetracycline. Additionally, 98% of the isolates were susceptible to amoxicillin/clavulanic acid. The most effective clinically relevant agents *in vitro* were ciprofloxacin, cefotaxime, ceftazidime, and tetracycline as they showed MIC_{90} values identical to the lowest concentration tested (**Table 4**).

Similar to the *V. vulnificus* isolates, a small proportion of *V. cholerae* strains showed resistance to streptomycin (2%), while 20% of the strains were intermediate resistant. In contrast to *V. vulnificus,* the most frequent antimicrobial resistance found among all *V. cholerae* isolates was resistance to ampicillin (11%). Resistance to nalidixic acid and trimethoprim was rarely observed in two clinical isolates and in one isolate from seafood. Non-susceptibility to the carbapenems imipenem and meropenem was observed in 5 and 3% of the environmental isolates, respectively.

Clinical strains showed the highest percentage of non-susceptible strains (44%), followed by environmental strains (34%) and by strains isolated from retail seafood (23%) (**Table 3**). However, statistical analysis revealed that the observed differences to environmental isolates are not significant ($p > 0.05$, χ^2). Comparison between the geographical origin of environmental strains revealed that the percentages of strains non-susceptible to streptomycin, ampicillin, amoxicillin/clavulanic acid, meropenem, and imipenem were higher in the North Sea compared to the Baltic Sea (**Table 2**). The higher occurrence of non-susceptible *V. cholerae* strains in the North Sea (**Table 3**) was statistically significant ($\chi 2 = 5.327$, d.f. $= 1$, $p < 0.05$).

Analysis of MIC distributions revealed that susceptibilities of the two *Vibrio* species were rather similar (**Table 4**). Differences were exclusively observed for kanamycin and ciprofloxacin, where MIC_{90} values of *V. cholerae* were two and four times lower and in case of ampicillin four times higher than those for *V. vulnificus*.

Susceptibility to colistin was excluded from any statistical analysis and tables, since *V. vulnificus* and *V. cholerae* possess an intrinsic resistance to colistin, which is used for selective growth on cellobiose-polymyxin B-colistin agar (Massad and Oliver, 1987). However, five *V. cholerae* strains were highly susceptible to colistin (MICs ≤ 2 mg/L) and would therefore fail to grow on this selective agar.

Neither class 1 integrons, nor gene determinants encoding streptomycin resistance (*aadA1, aadA2, strA/B*) were detected among non-susceptible *V. cholerae* isolates. Sequence analysis of the *rpsL* gene revealed three silent point mutations C-198-T, A-251-T, and G-360-A in two streptomycin resistant *V. cholerae* isolates (VN-10191, VN-10192) as well as within a susceptible isolate (VN-4226). In addition, PCR amplifications to detect β-lactamase genes (bla_{PSE-1}, $bla_{OXA-1-like}$, $bla_{TEM-1-like}$) were negative in all tested *V. cholerae* isolates.

Examination of Carbapenem Non-susceptible *V. cholerae* Isolates

Among the 131 environmental *V. cholerae* non-O1/non-O139 isolates analyzed, resistance to the carbapenem imipenem (zone diameter ≤ 19) was observed in four strains (VN-2808, VN-2825,

VN-2923, and VN-2997). These strains additionally showed resistance to ampicillin, intermediate resistance to amoxicillin-clavulanic acid, as well as intermediate or full resistance to meropenem (**Table 5**). In contrast, they were susceptible to the third- and fourth-generation cephalosporins ceftazidime, cefotaxime, and cefepime. Further characterization revealed resistance to aztreonam and ertapenem, while the strains were susceptible to temocillin and intermediate resistant to cefoxitin. The four strains grew on chromID™ CARBA agar, while growth was inhibited on chromID™ OXA-48 (bioMérieux GmbH, Nürtingen, Germany), indicating the expression of carbapenem-hydrolyzing enzymes other than OXA-48 type carbapenemases. To further investigate the presence of carbapenemases, Blue-CARBA and Carba NP II tests were conducted on these strains. Imipenem-hydrolyzing activity was detected in intact cells and crude cell extracts of each of the four strains. This activity was inhibited by tazobactam but not by EDTA, suggesting the presence of Ambler class A carbapenemases rather than class B metallo-carbapenemases or class D OXA-carbapenemases (Dortet et al., 2012).

PCRs to identify genes encoding Ambler class A carbapenemases were performed. However, no products with expected sizes were observed using primers for detection of NMC-A, SME 1-3, IMI 1-3, or KPC 1-5. In the case of NMC-A, SME, and IMI, a general failure of PCR amplification cannot be excluded as no positive control strains were available.

In addition, the strains were negative for PCR-amplification of specific genes encoding Ambler class B metallo-carbapenemases (VIM 1-2, IMP, NDM-1), class D carbapenemase (OXA-48), and AmpC-β-lactamases (MOX 1-2, CMY 1-11, LAT 1-4, BIL-1, DHA 1-2, ACC, MIR-1T, ACT-1, FOX 1-5b) as well as of class 1 integrons.

DISCUSSION

Prevalence of Antimicrobial Resistance in *V. vulnificus* and *V. cholerae* non-O1/non-O139

In this study, *V. vulnificus* isolates from German coastal waters as well as of clinical origin were susceptible to quinolones, fluoroquinolones, phenicols, tetracyclines, folate pathway inhibitors, aminopenicillins with or without β-lactamase inhibitors, carbapenems, and third- and fourth-generation

TABLE 5 | β-lactam MIC values and inhibition zone diameters found in the putative carbapenemase-producers and in carbapenem susceptible isolates selected as negative controls.

	Antimicrobial agent	Strain ID of *V. cholerae* isolates									
		Breakpoints[d]			Putative carbapenemase-producers				Carbapenem susceptible controls		
		S	I	R	VN-02997	VN-02825	VN-02923	VN-02808	VN-10145	VN-00301	VN-00161
MIC values (mg/L)[a]	Ampicillin	≤8	16	≥32	>32	>32	>32	>32	2	2	2
	Cefepime	≤8	16	≥32	0.5	0.25	0.5	0.25	0.12	≤0.06	≤0.06
	Cefoxitin	≤8	16	≥32	16	16	16	16	4	4	4
	Cefotaxime	≤1	2	≥4	0.12	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06
	Ceftazidime	≤4	8	≥16	0.5	0.5	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25
	Ertapenem	≤0.5	1	≥2	2	>2	>2	2	0.12	0.03	0.06
	Imipenem	≤1	2	≥4	16	>16	>16	16	1	0.5	1
	Temocillin	≤8	-	>8	4	4	4	4	4	1	1
Inhibition zone diameter (mm)[b]	Amoxicillin/clavulanic acid	≥18	14–17	≤13	14	13.5	14	15	23	28	18
	Aztreonam	≥21	18–20	≤17	12	15	17	16	NA	NA	29
	Cefepime	≥25	19–24	≤18	28	26	28	28	34	40	30
	Ceftazidime	≥21	18–20	≤17	28	26	28	28	NA	NA	31
	Imipenem	≥23	20–22	≤19	14	15	16	15	30	36	26
	Meropenem	≥23	20–22	≤19	20	20	20	19	34	36	28
β-lactam resistance phenotype[c]					(AMC)-AMP-ATM-ETP-(FOX)-IPM-(MEM)	(AMC)-AMP-ATM-ETP-(FOX)-IPM-(MEM)	(AMC)-AMP-ATM-ETP-(FOX)-IPM-(MEM)	(AMC)-AMP-ATM-ETP-(FOX)-IPM-MEM	–	–	–

MIC, minimal inhibitory concentration; S, susceptible; I, intermediate resistant; R, resistant; NA, not assessed; AMC, amoxicillin/clavulanic acid; AMP, ampicillin; ATM, aztreonam; ETP, ertapenem; FOX, cefoxitin; IPM, imipenem; MEM, meropenem.

[a] *MIC values obtained by broth microdilution.*

[b] *Inhibition zone diameter obtained by disk diffusion.*

[c] *Results against non-β-lactams are not displayed; intermediate resistance is shown in brackets.*

[d] *Criteria used for interpretation with corresponding references are given in Supplementary Table S3.*

cephalosporins. Non-susceptibility was exclusively observed toward aminoglycosides; predominantly streptomycin and sporadically kanamycin. Similar observations were made by Han et al. (2007), who reported total susceptibility with comparable MIC_{90} values to chloramphenicol, ampicillin, ceftazidime, cefotaxime, ciprofloxacin, gentamicin, and tetracycline among *V. vulnificus* isolates from oysters of the Louisiana Gulf coast, USA (Han et al., 2007). In a recent study of *V. vulnificus* isolates from the Chesapeake Bay, USA (Shaw et al., 2014), the highest percentage of resistance was also observed against streptomycin. However, a large percentage of intermediate resistant strains to chloramphenicol (78%) and sporadically non-susceptibility to β-lactams was also reported in that study (Shaw et al., 2014). Compared to our study, higher percentages of non-susceptible strains to ampicillin, tetracycline, nalidixic acid, trimethoprim, and especially to the aminoglycosides streptomycin and gentamicin were observed in a study of 151 *V. vulnificus* isolates from South Carolina, USA, while resistance to chloramphenicol and meropenem was under one per cent (Baker-Austin et al., 2009).

Among *V. cholerae* non-O1/non-O139 isolated from clinical, environmental, and seafood samples in Germany, no multidrug-resistance was observed and the majority (67%) of isolates were susceptible to all antimicrobial agents tested. Full resistance was most frequently found toward ampicillin (11%) and streptomycin (2%). In addition, a considerable proportion of isolates showed intermediate resistance to streptomycin (20%). Resistance to nalidixic acid and trimethoprim was only sporadically found in isolates from clinical and seafood samples, respectively. While numerous studies on antimicrobial resistance of toxigenic *V. cholerae* O1, O139 strains have been published, data on *V. cholerae* non-O1/non-O139 are less frequent. A recent study showed similar antimicrobial resistance patterns among environmental *V. cholerae* non-O1/non-O139 isolates from the Chesapeake Bay, USA (Ceccarelli et al., 2015). No multidrug-resistant isolates were detected and resistance to β-lactams was found in some isolates. In one large-scale study from India on antimicrobial susceptibility of *V. cholerae* non-O1/non-O139 isolates, the highest percentage of resistant strains was also seen for ampicillin (88%) and streptomycin (85%) though with a considerably higher frequency (Kumar et al., 2009). In contrast to our study, Kumar et al. (2009) reported a high prevalence of multidrug resistance and only a small percentage of strains were susceptible to all ten antimicrobial agents tested (12%).

Several PCR analyses were performed to reveal the underlying molecular mechanisms responsible for the observed non-susceptibility to streptomycin, ampicillin, and imipenem.

Resistance to streptomycin is often mediated by enzymatic inactivation through adenylation by aminoglycoside (3″) adenyltransferases (*aadA* genes) or through phosphorylation by aminoglycoside phosphotransferases (*strA*/*strB* genes; Shaw et al., 1993; Tsai et al., 2014). However, none of the *Vibrio* isolates was positive for amplification of *aadA1*, *aadA2*, or *strA*/*B* genes that are commonly found in *Enterobacteriaceae* and that have already been identified in *V. cholerae* (Hochhut et al., 2001; Sá et al., 2010; Yu et al., 2012). Ribosomal alterations resulting from mutations in the *rpsL* gene encoding ribosomal protein S12 can be another cause of streptomycin resistance (Shaw et al., 1993; Tsai et al., 2014). However, amino acid sequences of ribosomal protein S12 were identical in all investigated strains, irrespective of the streptomycin resistance phenotype. Observed single nucleotide polymorphisms within the *rpsL* gene of some resistant as well as of some susceptible isolates were silent mutations. This indicates that other streptomycin inactivating enzymes or other resistance mechanisms, such as decreased permeability or other ribosomal alterations (e.g., mutations in the *rrs* gene encoding the 16S ribosomal RNA) may be responsible for the observed phenotype (Shaw et al., 1993; Tsai et al., 2014).

Likewise, all β-lactam non-susceptible *Vibrio* isolates were negative for amplification of bla_{PSE-1}, $bla_{OXA-1-like}$, and $bla_{TEM-1-like}$ genes encoding common β-lactamases in *Enterobacteriaceae* and other Gram-negative bacteria. This suggests that resistance may be encoded by other β-lactamase genes which are known to show a high diversity in Gram-negative bacteria. β-lactam resistance may also be mediated by other mechanisms such as reduced permeability, increased efflux, or target alterations (e.g., reduced affinity or increased amount of penicillin-binding protein (PBP); Foster, 1983).

Carbapenemase Producing *V. cholerae*

Antimicrobial susceptibility patterns as well as growth patterns on different selective media indicated the presence of a β-lactamase with carbapenem hydrolyzing activity in four environmental *V. cholerae* non-O1/non-O139 isolates from the Baltic Sea and the North Sea. The expression of carbapenemases was confirmed by positive Blue-CARBA and positive Carba NP II tests, which specifically detect imipenem hydrolyzing activity (Dortet et al., 2012; Pires et al., 2013). Inhibition of carbapenemase activity by tazobactam but not by EDTA suggested the presence of Ambler class A carbapenemases rather than class B metallo-carbapenemases or class D OXA-carbapenemases (Dortet et al., 2012). However, we found no evidence for the presence of specific genes encoding Ambler class A carbapenemases.

The four strains showed an exceptional resistance profile: They were non-susceptible to aminopenicillins, carbapenems, cefoxitin, and aztreonam and were only slightly inhibited by the β-lactamase inhibitor clavulanic acid. However, they were fully susceptible to third- and fourth-generation cephalosporins as well as to temocillin. The observed resistance to aminopenicillins coupled with susceptibility to extended spectrum cephalosporins may indicate the presence of an OXA-type carbapenemase (Ambler class D) reviewed by Walther-Rasmussen and Høiby (2006). However, with some exceptions, e.g., OXA-23, these enzymes are generally not inhibited by tazobactam, as seen for the four strains in the Carba NP II and Blue-CARBA tests and generally don't mediate resistance to aztreonam (Walther-Rasmussen and Høiby, 2006).

So far, the identity of the enzyme responsible for imipenem hydrolyzing activity in the four strains remains unclear, as none of the examined carbapenemase and AmpC-β-lactamase genes could be detected. It cannot be excluded that in addition to a carbapenem hydrolyzing enzyme other resistance mechanisms,

such as reduced affinity of PBPs, porin alterations resulting in decreased membrane permeability or active efflux systems, either alone or in combination may also contribute to the observed phenotype (Walther-Rasmussen and Høiby, 2006; Queenan and Bush, 2007; Nordmann et al., 2012). Carbapenem-resistant *V. cholerae* have already been reported in other studies. NDM-1 carbapenemase was detected in a *V. cholerae* O1 El Tor Ogawa strain isolated from a 2-year old child (Mandal et al., 2012) as well as in *V. cholerae* isolated from seepage water in India (Walsh et al., 2011). Furthermore, increasing resistance to carbapenems was recently described among *V. cholerae* O1 or O139 strains isolated between 1986 and 2012 in southwest China (Gu et al., 2014).

CONCLUDING REMARKS

In this study, antimicrobial agents recommended as first choice agents for the treatment of *V. vulnificus* and *V. cholerae* non-O1/non-O139 infections such as fluoroquinolones, tetracyclines, and extended spectrum cephalosporins were found to be effective *in vitro* against both species. However, the administration of aminopenicillins, carbapenems, or aminoglycosides for treatment of *V. cholerae* non-O1/non-O139 infections, which has been reported in few studies (Daniels and Shafaie, 2000; Feghali and Adib, 2011; Lu et al., 2014) should be considered carefully, as non-susceptibility was most frequently observed against ampicillin and streptomycin and sporadically to carbapenems. For *V. vulnificus*, non-susceptibility was exclusively observed to the aminoglycosides streptomycin and kanamycin. However, gentamicin was effective against both species and could be an aminoglycoside of choice for the treatment of children and pregnant woman, as was also suggested by others (Shaw et al., 2014).

We report the detection of carbapenemase producing *V. cholerae* from different locations of the German coast line (North Sea and Baltic Sea) representing an environmental reservoir of carbapenem resistance. An entry into the sea resulting from sanitary pollution or human recreational activities cannot be excluded, but seems not likely as vibrios are indigenous bacteria of the marine environment and not intestinal commensals of humans or terrestrial animals. The strains displaying carbapenemase activity showed resistance to an unusual pattern of β-lactams. Therefore, characterization of the underlying genetic background is necessary to identify the responsible genes e.g., using whole genome sequencing as the most promising approach. Further investigations on the mobility as well as on the location of encoding genes are also needed, since location on mobile genetic elements would imply a higher risk for interspecies spread. Carbapenems are last line antimicrobial agents for treatment of multidrug-resistant Gram-negative bacteria and are of high therapeutic value (Nordmann et al., 2012). The occurrence of putative carbapenemase producing *V. cholerae* in the North and Baltic Sea is therefore of great concern and highlights the need for systematic monitoring of antimicrobial susceptibility in potentially pathogenic *Vibrio* spp. in Europe.

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SUPPLEMENTARY MATERIAL

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

2.6 Publication 5

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PVv3, a New Shuttle Vector for Gene Expression in *Vibrio vulnificus*

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An efficient electroporation procedure for *Vibrio vulnificus* was designed using the new cloning vector pVv3 (3,107 bp). Transformation efficiencies up to 2 × 10^6 transformants per μg DNA were achieved. The vector stably replicated in both *V. vulnificus* and *Escherichia coli* and was also successfully introduced into *Vibrio parahaemolyticus* and *Vibrio cholerae*. To demonstrate the suitability of the vector for molecular cloning, the green fluorescent protein (GFP) gene and the *vvhBA* hemolysin operon were inserted into the vector and functionally expressed in *Vibrio* and *E. coli*.

Vibrio vulnificus is an estuarine bacterium of coastal waters worldwide (1). The species is a potent pathogen that can cause severe wound infections with lethal outcome (2). Moreover, *V. vulnificus* is also responsible for deaths caused by consumption of contaminated seafood (3). In the United States, it is a leading cause of seafood-related deaths (4). Immunocompromised people and persons with underlying diseases (e.g., liver disease) are at especially high risk. Unfortunately, there is still insufficient information available to discriminate virulent strains from environmental strains, presumably with lower pathogenicity. Even though virulence factors, e.g., capsular polysaccharide (5) and RTX toxins (6), have been identified, the full spectrum of factors responsible for the high virulence of the species still has to be elucidated. Molecular genetic analyses are still hampered by the lack of fast and efficient transformation protocols for *V. vulnificus*. A periplasmic nuclease (Vvn) was demonstrated to be involved in preventing the uptake of foreign DNA by transformation (7). While attempts to electroporate standard cloning vectors (e.g., pBR322) failed, some large broad-host-range plasmids could be successfully introduced by electroporation, albeit at very low efficiencies (up to 2.4 × 10^2 transformants/μg) (8). Furthermore, large quantities of DNA (up to 100 μg/ml of buffer) had to be applied. It is not yet clear

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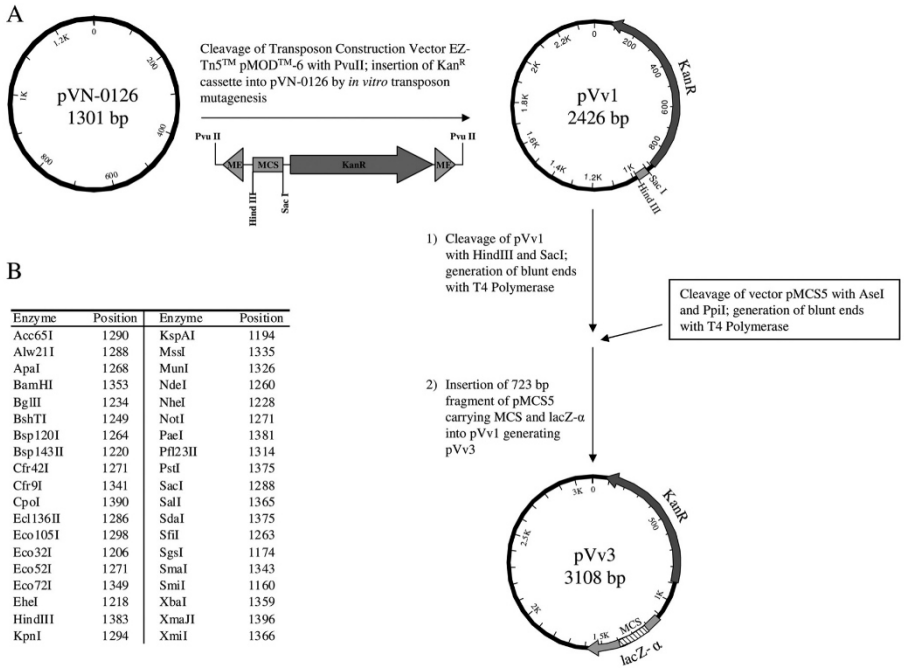
TABLE 1 Strains, plasmids, and primers used in this study

Name	Description	Reference(s)
Strains		
VN-0126	Biotype 1 (diseased human, Denmark, 1994)	12, 13
VN-0101	Biotype 1 (seawater, Germany, 2010)	12
VN-0130	Biotype 1 (diseased human, Denmark, 1994)	12, 13
VN-0227	Biotype 1 (seawater, Germany, 2004)	12
CMCP6	Biotype 1 (diseased human, South Korea)	23
E. coli Genehogs	Laboratory K-12 strain	Life Technologies, Darmstadt, Germany
Plasmids		
pVN-0126	Cryptic plasmid of *V. vulnificus* VN-0126	13
pVv1	pVN-0126 containing the Kan^r cassette of vector EZ-Tn5TM pMODTM-6	This study
pVv3	pVv1 containing the multiple-cloning site, *lacZ*α, and the T7 promoter of vector pMCS5	This study
EZ-Tn5 pMOD-6	Transposon mutagenesis vector	Epicentre Biotechnology, Madison, WI
pMCS5	Cloning vector	MoBiTec, Göttingen, Germany
pRSET-EmGFP	Plasmid containing the emerald green fluorescent protein gene	Life Technologies, Darmstadt, Germany
Primers		
*vvhBA*_F_Bam	GAGGATCCTCTGTTGATTTTGTGATAATGAGCCAA	This study
*vvhBA*_F2_Bam	TAGGATCCCTTACTCGTAATGAGGAATCTATGC	This study
*vvhBA*_R_Hind	GCTAAGCTTAACAGAGCCTAGAGTTTGACT	This study
*gfp*_F_Eco32I	TCGATATCATGGTGAGCAAGGGCGAGGAG	This study
*gfp*_R_Hind	CTTAAGCTTTTACTTGTACAGCTCGTCCAT	This study
Vv-PI-ori1_F	CAGGACCCAGACTTTACACACAATC	This study
Vv-PI-ori1_R	GGAATCAAGATGCTTTGGACAGG	This study
Vv-PI-ori1_F	CAGGACCCAGACTTTACACACAATC	This study
Vv-PI-ori2_R	ACTTCGCTATTTCACACCTGACG	This study
Vv-PI-ori2_F	TTCGCCCTGTCCAAAGCATC	This study
Vv-PI-ori2_R	ACTTCGCTATTTCACACCTGACG	This study

A



B

Enzyme	Position	Enzyme	Position
Acc65I	1290	KspAI	1194
AIw21I	1288	MssI	1335
ApaI	1268	MunI	1326
BamHI	1353	NdeI	1260
BglII	1234	NheI	1228
BshTI	1249	NotI	1271
Bsp120I	1264	PaeI	1381
Bsp143II	1220	Pfl23II	1314
Cfr42I	1271	PstI	1375
Cfr9I	1341	SacI	1288
CpoI	1390	SalI	1365
Ecl136II	1286	SdaI	1375
Eco105I	1298	SfiI	1263
Eco32I	1206	SgsI	1174
Eco52I	1271	SmaI	1343
Eco72I	1349	SmiI	1160
EheI	1218	XbaI	1359
HindIII	1383	XmaJI	1396
KpnI	1294	XmiI	1366

FIG 1 Construction of the shuttle vector pVv3. (A) Steps of pVv3 construction. (B) Restriction enzymes that allow blue-white screening of transformants in suitable *E. coli* K-12 strains.

whether ColE1 replicons are not able to replicate in *V. vulnificus* or whether the antibiotic resistance genes of the vectors were not functional in the species. R factors were shown to be unstable in *Vibrio* (9). Due to the inefficient electroporation of *V. vulnificus*, the current methods of choice to introduce foreign DNA into the bacteria are conjugation and, particularly, chitin-based natural transformation, which was originally established for *Vibrio cholerae* (10). However, these techniques are laborious and time-consuming and also require rather large amounts of DNA. Moreover, in studies with *V. vulnificus*, only linearized plasmids could be introduced into the cells by chitin transformation (11).

The facts that electroporation of *V. vulnificus* is feasible and that to date no studies have been published describing the use of vectors derived from *Vibrio* plasmids inspired us to optimize this technique. Here, we report on a rapid and efficient transformation system that was designed for *V. vulnificus* but that can also be used for other *Vibrio* species. The basis of this system is the new shuttle vector pVv3, constructed from a small naturally occurring *V. vulnificus* plasmid that could be easily introduced into most *Vibrio* strains by electroporation.

MATERIALS AND METHODS

Bacteria, plasmids, and oligonucleotide primers. The bacterial strains, plasmids, and primers used in this study are listed in Table 1.

Construction of the shuttle vector pVv3. pVv3 is a derivative of a cryptic plasmid (pVN-0126) isolated fom *V. vulnificus* VN-0126 (12, 13) using the GeneJet Plasmid Miniprep Kit (Thermo Scientific, Hudson, NH). To introduce a selective marker into the *Vibrio* plasmid, the kanamycin resistance gene cassette and multiple-cloning site (MCS) of the vector EZ-Tn5 pMOD-6 (Epicentre Biotechnologies, Madison, WI) were isolated by PvuII cleavage and inserted into pVN-0126 by *in vitro* transposon mutagenesis (Fig. 1). Following this, the preparation was introduced into *Escherichia coli* strain Genehogs (Life Technologies, Darmstadt, Germany) by electroporation. After incubation overnight, a number of colonies appeared on LB agar containing 100 μg/ml kanamycin. Six transformants were investigated with respect to their plasmid contents. In all of them, the resistance gene had been inserted into pVN-0126 (data not shown). One of the recombinant plasmids (pVv1) was sequenced using primers supplied with the transposon mutagenesis kit. Further sequencing was performed by primer walking. In the final step of pVv3 construction, the short MCS of pVv1 was replaced by the *lacZ*α gene, polylinker, and T7 promoter of the vector pMCS5 (MoBiTec, Göttingen, Germany) (Fig. 1). By this modification, pVv3 obtained 60 single sites for restriction endonucleases, 38 of which are located within *lacZ*α. Thus, the vector allows identification of recombinant plasmids in *E. coli* by blue-white selection, which subsequently can be selected for transformation of *V. vulnificus*.

Transformation of *Vibrio* strains. *Vibrio* strains were electroporated according to the protocol optimized for strain VN-0101 (Table 1). Bacteria were grown in 30 ml LB broth at 37°C under agitation. At an optical density at 580 nm (OD_{580}) of ca. 0.8, cells were sedimented (10,000 × *g*; 15 min; 4°C) and resuspended in 30 ml of ice-cold 1 mM Tris-HCl buffer, pH 6, supplemented with 200 mM sucrose. After repeating the washing step twice, cells were resuspended in 150 μl electroporation buffer. Fifty microliters of the cell suspension was mixed with 200 to 400 ng plasmid DNA, and the preparation was incubated for 10 min on ice. Electroporation was performed at settings of 7.5 kV cm^{-1}, 25 μF, and 200 Ω. Thereafter, 950 μl of SOC medium (14) was added, and the bacteria were incu-

TABLE 2 Effects of growth phase (OD_{580}), DNA amount, electric field strength, pH, and sucrose concentration of the electroporation buffer on transformation efficiency of *V. vulnificus* strain VN-0101

Varied parameter[a]	Value	No. of transformants per μg DNA
OD_{580}[b]	0.2	6×10^3
	0.4	2×10^4
	0.6	3.5×10^4
	0.8	5.8×10^5
	1	2.7×10^4
DNA amt (ng)	5	2×10^5
	25	2.8×10^5
	50	1.1×10^5
	100	5.8×10^4
	500	2×10^4
	1,000	1×10^4
Electric field strength (kV cm^{-1})[c]	2.5	0
	5	2.9×10^4
	7.5	3.5×10^5
	10	1×10^5
	12.5	1.3×10^4
	15	1.1×10^3
Sucrose concn (mmol $liter^{-1}$)[c]	100	2×10^4
	150	8×10^5
	200	1×10^6
	300	4.8×10^5
	400	3.9×10^4
pH (1 mM Tris-HCl)[b]	5	1.7×10^5
	5.5	1.8×10^5
	6	2.6×10^5
	6.5	1.8×10^5
	7	1.6×10^5

[a] Standard parameters: OD_{580}, 0.8; 7.5 kV cm^{-1}; 400 mM sucrose; pH 5.5.
[b] Transformation was performed with 10 ng of pVv3 DNA.
[c] Transformation was performed with 100 ng of pVv3 DNA.

bated at 37°C for 1 h, followed by plating of aliquots (100 μl) on LB agar containing 100 μg/ml kanamycin.

Molecular cloning and expression of the *gfp* gene and *vvhBA* hemolysin operon in *Vibrio*. The coding sequence for the emerald green fluorescent protein was amplified by PCR using plasmid pRSET-EmGFP (Life Technologies, Darmstadt, Germany) as the template. To allow in-frame fusion with *lacZ* of pVv3, recognition sites for the restriction endonucleases EcoRV and HindIII were embedded in the forward and reverse primers, respectively (Table 1). Upon ligation and transformation of *E. coli* strain Genehogs, positive clones were identified by PCR, followed by isolation and sequence analysis of their plasmid DNAs. For molecular cloning of the *V. vulnificus vvhBA* hemolysin operon, two different PCR products were made using DNA of the sequenced strain CMCP6 (Table 1) as the template. The reverse primer *vvhBA*_R_Hind, deduced from the 3′ end of *vvhA* (15), was used in combination with one of two forward primers. While the forward primer *vvhBA*_F_Bam was complementary to a region upstream of the described *vvhBA* promoter (16) (PCR product, *vvh*-l), the forward primer *vvhBA*_F2_Bam bound close to the *vvhB* start codon (PCR product, *vvh*-s). Both forward primers and the reverse primer harbored a recognition site for BamHI and HindIII, respectively. *E. coli* Genehogs cells transformed by the ligation reactions were plated on sheep blood agar (SBA) containing kanamycin. After incubation for 24 h at 37°C, positive clones were identified by a hemolytic halo around the colonies. The recombinant plasmids of these transformants were isolated and analyzed by sequencing. Constructs containing either *gfp* (pVv3-*gfp*) or *vvhBA* (pVv3-*vvh*-l and pVv3-*vvh*-s) were introduced into *V. vulnificus* strains (Table 1) by electroporation (see above). For molecular cloning of the *vvhBA* operon, *V. vulnificus* strains that showed no hemolytic activity on SBA after 48 h of incubation were selected. Expression of *vvhBA* was monitored by plating transformants onto SBA supplemented with kanamycin. To study the expression of *gfp*, transformants were grown in LB medium containing kanamycin up to an OD_{580} of ca. 0.5. The bacteria were sedimented by centrifugation, washed, resuspended in phosphate-buffered saline (PBS), and mounted on an Ibidi chamber (Ibidi GmbH, Germany). Fluorescence microscopy was performed using a Zeiss Axio Observer Z1 microscope with a 63× objective and differential interference contrast (DIC). Functional *gfp* expression was visualized using a metal halide light source (HXP 120 C) and a filter set with excitation at 450 to 490 nm (470/40), emission at 500 to 550 nm (525/50), and a dichroic beam splitter at 495 nm. Images were acquired with an AxioCam MRm monochromatic camera, using exposure times of 500 ms to 1 s.

Nucleotide sequence accession number. The sequence of pVv3 has been submitted to GenBank and is available under accession number HG326273.

RESULTS AND DISCUSSION

Sequence analysis of pVv3. The cloning vector pVv3 was constructed on the basis of the cryptic *V. vulnificus* plasmid pVN-0126. pVv3 has a size of 3,107 bp, 1,301 bp of which derived from pVN-0126. The latter plasmid contains three short open reading frames (ORFs). BLASTX searches did not reveal any significant homologies of these ORFS (168 bp, 177 bp, and 201 bp) to other peptides or proteins, though a noncoding region of pVN-0126 with 34 gaps over a stretch of 372 bp showed significant nucleotide similarity (72%) to the *Photobacterium phosphoreum* plasmid pPH1 (accession number AY789019). Comparison of pVN-0126 to replication regions of *Vibrio* plasmids (17, 18) did not clearly indicate *cis*-acting genetic elements involved in replication. A putative DnaA binding site clustered with two DAM methylation sites, and an inverted repeat might be indicative of the *oriV* of the plasmid (data not shown). Screening of 72 *V. vulnificus* strains by multiplex PCR using three primer pairs (Table 1) derived from pVN-0126 gave only one positive result with strain VN-0130 (12), which harbored an identical plasmid confirmed by sequencing. Hence, pVN-0126-related plasmids are apparently rare in *V. vulnificus*.

The vector can be easily introduced into many *Vibrio* strains. To investigate pVv3 transformation of *V. vulnificus*, the plasmid was introduced into 12 clinical and 9 environmental biotype 1, as well as 5 biotype 2, *V. vulnificus* strains (BfR strain collection) by electroporation, initially under conditions reported for the transformation of *Vibrio parahaemolyticus* (19, 20). To optimize *V. vulnificus* electroporation, some parameters (the OD_{580} of the culture, the pH and sucrose concentration of the electroporation buffer, the DNA amount, and the electric field strength) were analyzed in detail. Most of these studies were carried out with the biotype 1 strain VN-0101. Based on the transformation efficien-

TABLE 3 Transformation frequencies of *Vibrio* strains

Species	No. of transformable strains/no. of tested strains	Range of transformation frequencies (transformants per μg DNA)
V. vulnificus	23[a]/26	5×10^1–1×10^6
V. parahaemolyticus	7/7	1×10^4–1×10^6
V. cholerae	8/9	5×10^1–1×10^4

[a] For two of the strains, the number of transformants could not be determined due to spontaneous resistance to kanamycin.

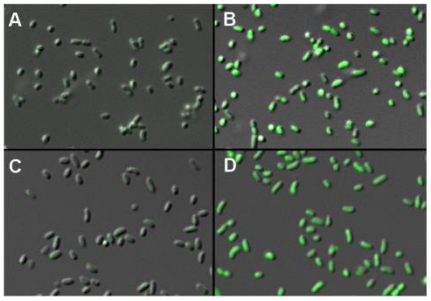


FIG 2 Overlay of DIC and fluorescence images demonstrating *gfp* expression from pVv3-*gfp* in *E. coli* Genehogs and *V. vulnificus* CMCP6 transformants. (A) *E. coli* Genehogs with pVv3. (B) *E. coli* Genehogs with pVv3-*gfp*. (C) *V. vulnificus* with pVv3. (D) *V. vulnificus* with pVv3-*gfp*.

cies obtained (Table 2), an optimized protocol for VN-0101 transformation was designed (see Materials and Methods). Notably, only 1 ng of plasmid DNA was sufficient to obtain transformants, while the highest efficiencies (2×10^6 transformants/μg DNA) were accomplished by electroporation of 10 to 25 ng of DNA. This is in strong contrast to results reported by McDougald et al. (8), who applied 100 μg DNA/ml for the electroporation of *V. vulnificus*. Interestingly, in our experiments, transformation efficiencies were slightly enhanced when the sucrose solution was prepared with distilled water (pH 5.5) instead of Tris-HCl. As the pH of water might vary in different laboratories, this issue should be examined individually. We also tested the minimal growth medium containing 0.4% glucose and 5 mM glycine betaine that improved *V. vulnificus* electroporation (8). However, these compounds did not yield higher transformation frequencies in our experiments. The electroporation procedure optimized for the strain VN-0101 was also applied to other *V. vulnificus*, *V. parahaemolyticus*, and *V. cholerae* strains, as these *Vibrio* species are most important for human infections and therefore are often used for functional analyses of, e.g., virulence genes. Table 3 shows that the vast majority of strains could be transformed by this protocol, although various efficiencies have been determined. The highest overall efficiencies were obtained with *V. parahaemolyticus*, yet it is possible that a modification of certain parameters would increase the transformation efficiencies of particular strains.

Stability and copy number of pVv3. To examine the stability of pVv3, the strains VN-0101 and Genehogs containing the vector were cultivated in LB broth for 5 days, equivalent to at least 50 generations. Thereafter, dilutions were plated on LB agar with or without kanamycin. While in *E. coli* no loss of pVv3 was observed, there was some reduction (10 to 50%) of plasmid-containing *Vibrio* cells. However, the reduction was very small and did not negatively reflect upon the usefulness of the vector. At the end of this experiment, the plasmid recovered from *Vibrio* and *E. coli* was sequenced again, demonstrating that no sequence alterations occurred. The copy number of pVv3 was estimated by isolating the plasmid, along with pBR329 as a reference, from *V. vulnificus* and *E. coli* and comparing the amounts of DNA in agarose gels. The vector pVv3 exhibited a significantly weaker band in agarose gels (data not shown), suggesting that its copy number is lower than 30, a value reported for pBR329 (21).

Green fluorescent protein (GFP) and the VvhA hemolysin are efficiently expressed in *Vibrio*. To test the applicability of pVv3 for molecular cloning and heterologous gene expression, the *gfp* gene and *vvhBA* operon were amplified by PCR and inserted into pVv3. All constructs were analyzed by functional studies in *E. coli* and *V. vulnificus*. As shown in Fig. 2, *gfp* was significantly expressed in both species, while the vector alone gave only background fluorescence. With the help of pVv3, *V. vulnificus* indicator strains can easily be constructed and used to study the behavior of the pathogen. It has been reported that a *V. vulnificus* strain

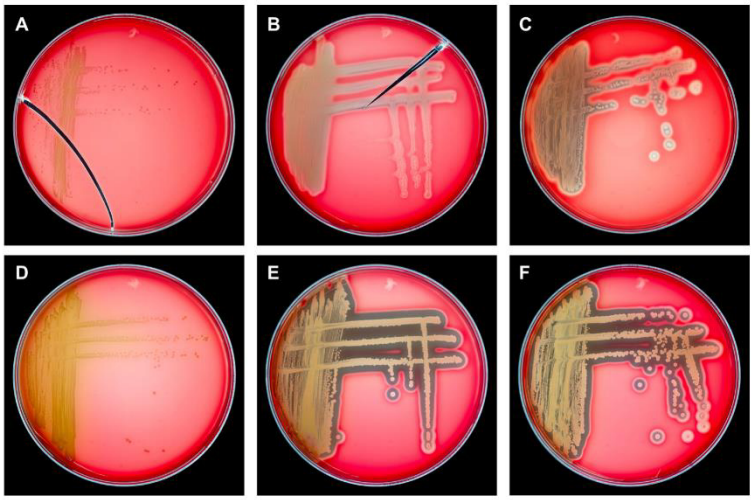


FIG 3 Hemolytic activity on sheep blood agar of *E. coli* Genehogs and *V. vulnificus* VN-227 transformants containing pVv3, pVv3-*Vvh*-l, or pVv3-*Vvh*-s. (A) *E. coli* Genehogs with pVv3. (B) *E. coli* Genehogs with pVv3-*Vvh*-s. (C) *E. coli* Genehogs with pVv3-*Vvh*-l. (D) *V. vulnificus* VN-227 with pVv3. (E) *V. vulnificus* VN-227 with pVv3-Vvh-s. (F) *V. vulnificus* VN-227 with pVv3-Vvh-l.

expressing *gfp* was in most cases similar to its wild-type counterpart in terms of growth and survival upon exposure to various biological stressors (22). To study the expression of *vvhBA* in *V. vulnificus*, environmental strains that did not show hemolysis after 48 h of incubation on sheep blood agar were selected for transformation. We determined the *vvhBA* sequence of one (VN-227) of these strains and found five amino acid exchanges within VvhA compared to the published VvhA sequence of *V. vulnificus* strain EDL174 hemolysin (reference 15 and data not shown). It is conceivable that these alterations account for the low hemolytic activity of strain VN-227. After introduction of pVv3-*vvh*-l or pVv3-*vvh*-s into the cells, transformants showed strong hemolytic activity even after 24 h of incubation. In contrast, the vector control exhibited no hemolysis (Fig. 3). Compared to *V. vulnificus*, *E. coli* transformants exhibited weaker hemolysis, which nevertheless was also clearly visible after 24 h of incubation. Most notably, both constructs conferred hemolytic activity, even though some *V. vulnificus* strains harboring the *vvhBA* operon, including its regulatory sequences (pVv3-*vvh*-l), showed slightly stronger hemolysis than strains in which *vvhBA* was fused to *lacZ* (pVv3-*vvh*-s). We also introduced the constructs into a weakly hemolytic *V. parahaemolyticus* strain, which similarly became hemolytic upon transformation (data not shown).

In conclusion, this new transformation system allows rapid molecular cloning in *Vibrio* and can be used for various applications, e.g., functional analyses of virulence genes or the complementation of mutations. Thus, *Vibrio* transformation now shares some benefits that hitherto were limited to other genera.

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3 General Discussion

3.1 Background

Since the first description of *V. vulnificus* as a new species in 1976 by the CDC (Hollis *et al*., 1976), numerous studies have investigated its occurrence and characteristics in many Asian and American countries (Strom and Paranjpye, 2000; Gulig *et al*., 2005; Oliver, 2005a, 2006, 2015; Jones and Oliver, 2009; Oliver, 2015). However, data on the occurrence of *V. vulnificus* in Europe and especially Germany was limited at the beginning of this work (Van Landuyt *et al*., 1985; Veenstra *et al*., 1993, 1994; Hoi *et al*., 1998; Arias *et al*., 1999, 2010; Barbieri *et al*., 1999; Bauer *et al*., 2006; Maugeri *et al*., 2006; Canigral *et al*., 2010; Collin and Rehnstam-Holm, 2011). Moreover, no information was available about virulence characteristics and phylogenetic relationships of *V. vulnificus* isolates from coastal waters and clinical cases in Germany.

The first case of *V. vulnificus* infection in Germany was reported from the Baltic Sea region in 1994 (Hoyer *et al*., 1995). In total, eleven confirmed cases were documented between 1994 and 2011 and additional six unconfirmed cases were reported in 2014. Despite their rare occurrence, infections with this fast-growing pathogen often demonstrate a devastating course of disease and can result in death (Shapiro *et al*., 1998; Frank *et al*., 2006). An increase in the occurrence of *V. vulnificus* in German and European coastal waters is expected due to impacts of climate change. Therefore, it is assumed that the incidence and clinical burden of *V. vulnificus* infections in Europe will rise in the future (Baker-Austin *et al*., 2010, 2012b). *V. vulnificus* infections in Germany are clearly associated with temperature anomalies, as most cases occurred during long heat waves resulting in unusually high seawater temperatures. The Baltic Sea will be particularly affected since it represents a low-salinity marine ecosystem with one of the highest warming rates of sea surface temperature (Baker-Austin *et al*., 2012b). Therefore, this intracontinental Sea offers *V. vulnificus* an attractive environment where it can flourish and proliferate to significant numbers when seawater temperature exceeds twenty degrees. Additionally, an improvement of growth conditions in the North Sea is expected due to decreasing salinity and increasing sea surface temperature and will favor a further dissemination of *V. vulnificus* in the next decades (Lowe *et al*., 2009; Baker-Austin *et al*., 2010; Brennholt *et al*., 2014). In view of these forecasts, the availability of reliable analytical tools to assess the occurrence and pathogenicity of *V. vulnificus* in German coastal waters is of great importance.

In 2003, after two severe cases of wound infection, local health authorities of Mecklenburg-Western Pomerania started a local sampling program to investigate the occurrence of *V. vulnificus* and other *Vibrio* spp. at bathing beaches along the Baltic Sea coastline (Hauk *et al*., 2010). Finally, the German research programs KLIWAS[5] and VibrioNet[6] were established in 2009 and 2010, respectively, to elucidate occurrence and virulence characteristics of *Vibrio* spp. in coastal waters of Germany. VibrioNet also included investigations on *Vibrio* prevalence in seafood and primary production as well as on epidemiology of *Vibrio* infections in Germany.

This dissertation was prepared within the research program VibrioNet, funded by the Federal Ministry of Education and Research. In this work, molecular and virulence characteristics as well as phylogenetic relationships of *V. vulnificus* isolates collected during both research programs were investigated. The following sections summarize the main results of the five publications included in this cumulative dissertation and provide a general discussion.

3.2 Genotypic and Phenotypic Characterization of Clinical and Environmental *Vibrio vulnificus* Isolates from the Baltic Sea Region

The first step of this work was the comprehensive characterization of *V. vulnificus* isolates from environmental samples and clinical cases from the Baltic Sea region representing the period from 1991 to 2011. Phenotypic and genotypic analyses were conducted to examine whether clinical strains obtained from diseased persons could be distinguished from environmental strains by specific virulence-associated traits. These investigations were combined with phylogenetic analyses using multilocus sequence typing to identify lineages with a higher pathogenicity potential.

3.2.1 Resistance to Human Serum

Resistance to the bactericidal effects of human serum plays a crucial role in *V. vulnificus* pathogenesis, since it seems to be a prerequisite for the progression of infection to septicemia (Johnson *et al*., 1984; Roig *et al*., 2010). In order to investigate the bacterial resistance to human serum, a method originally described by Moll *et al*. (1979) has been evaluated for the application in *V. vulnificus* research. This method proved to be a simple and straightforward tool that allows high throughput screening of large strain collections of *V. vulnificus*. Using this

[5] http://www.kliwas.de/KLIWAS/EN/03_ResearchTasks/03_vh3/04_304/304_node.html
[6] http://www.vibrionet.de/

method, not only the survival of the strains but also growth and metabolism at different concentrations of human serum were assessed. Resistance to human serum was widely distributed among the Baltic Sea biotype 1 isolates, with 94% of clinical but also 50% of environmental isolates showing strong serum resistance.

Resistance to human serum is associated with the expression of capsular polysaccharide (CPS), which protects against the bactericidal effects of human serum and prevents phagocytosis by macrophages (Johnson *et al*., 1984; Tamplin *et al*., 1985; Wright *et al.*, 1990). CPS expression, on the other hand, strongly depends on the growth phase and growth conditions (Wright *et al*., 1999). The serum susceptibility of few clinical strains may be a result of reduced CPS expression under the tested laboratory conditions. Conversely, the possibility exists that a susceptible strain from the environment might become resistant during the infection process in a host.

3.2.2 Hemolysis

Hemolytic activity is a strong virulence attribute of *V. vulnificus* and was examined using human erythrocytes (Publication 1). *V. vulnificus* expresses several enzymes with potential hemolytic activity. Among them, the hemolysin/cytolysin VvhA is secreted via the type II secretion system and its hemolytic activity can be detected in cell-free culture supernatants (Hwang *et al*., 2011). MARTX, on the other hand, acts in a contact-dependent manner and its hemolytic activity is cell-associated (Kim *et al*., 2008). In order to consider these different mechanisms, hemolytic activities of both, cell-free culture supernatants and of intact washed cell suspensions were examined.

The majority of all tested *V. vulnificus* strains (64%) showed hemolytic activity when culture supernatants as well as intact cells were tested. In 23% of the strains, hemolytic activity was observed in either the supernatant or the cell suspension and only 13% of all strains were rated as non-hemolytic in both assays.

There was no clear correlation between the hemolytic phenotype and the origin of the strains, as was also reported previously (Johnson and Calia, 1981). In addition, no association between variations in the hemolytic activity and the detection of several hemolysin genes was observed. The reason for this may be that *V. vulnificus* possesses numerous enzymes with potential hemolytic activity that could substitute each other's functions. Chen *et al.* (2003) identified three *rtxA* toxin genes (VVA1030/*rtxA1*, VVA0331, and VV1546) and several putative hemolysin genes (VVA0965/*vvhA*, VV0508, VV0601, VV0795, VV0914, VVA0118,

VVA1339, VV1495/*vllY*, VV2791/*hml*, VV3230/*hlyIII*, VVA0303/*vpl*) in the genome of *V. vulnificus* strain YJ016.

In this study, all strains were positive for the hemolysin genes *hlyIII* and *vllY*. Likewise, VVA0303 encoding a putative phospholipase with hemolytic activity was found in all investigated *V. vulnificus* strains. Five strains were negative for the RTXA toxin gene VVA0331, but four of them still showed strong hemolytic activity in intact cells as well as in cell-free supernatants. The detection of VVA0331 was not associated with a specific MLST cluster and did not correlate with a certain hemolytic phenotype. The exact function of the encoded RTX-like protein is unknown and its contribution to virulence appears questionable (Kim *et al*., 2008; Chou *et al*., 2009; Joseph, 2009).

Reduced hemolytic activity in some strains may be caused by the absence of other potential hemolysin or *rtxA* genes that were not investigated in this study. Examination of the cytolysin/hemolysin gene *vvhA* was not performed, as this gene is a generally accepted species marker present in all *V. vulnificus* strains (Nishibuchi, 2006). Similarly, the *rtxA1* gene is presumably present in all *V. vulnificus* isolates (Joseph, 2009; Kwak *et al*., 2011; Roig *et al*., 2011).

The concentration of VvhA and its hemolytic activity in the culture supernatant also depend on the production of the exoproteases VvpE and VvpM, which are known to degrade VvhA in the stationary growth phase (Lee *et al*., 2013). In this context it should be noted that all strains investigated in this study were positive for the *vvpE* gene (data not shown).

Differences in the hemolytic activity of the strains may also result from differential expression of the diverse hemolytic enzymes, or from sequence variations within the toxins. Kwak *et al.* (2011), for example, identified four different *rtxA1* gene variants with altered potency among 40 *V. vulnificus* strains. Sequence analysis of the *vvhA* gene in a non-hemolytic environmental strain (VN-0227) revealed five amino acid exchanges compared to the published VvhA sequence of *V. vulnificus* strain EDL174 (Publication 5), which could be responsible for the non-hemolytic phenotype. In this regard, the comparative sequence analysis of *vvhA* genes in environmental and clinical *V. vulnificus* isolates combined with functional expression studies could be a promising issue for further studies.

3.2.3 Cytotoxicity to Caco-2 Cells

To estimate the virulence potential of the strains, cytotoxicity assays using the human intestinal cell line Caco-2 were performed as alternative for animal experiments (Publication 1). Monolayers of Caco-2 cells were exposed to cell-free supernatants of *V. vulnificus* stationary phase cultures. Cytotoxicity was determined qualitatively by microscopic examination and quantitatively by measuring lactate-dehydrogenase (LDH) activity that is released upon cell lysis. Most of the strains induced lysis of 80 to 100% of the Caco-2 cells within two hours of incubation. Only six strains induced no detectable cell lysis according to the LDH measurement. In these cases however, microscopic examination revealed strong morphological changes of Caco-2 cells, e.g., cell rounding or elongation, which also resulted in complete destruction of the monolayer. There was no association of this phenotype with the origin of the respective strain or a certain phylogenetic lineage.

In conclusion, sterile culture filtrates of all tested *V. vulnificus* strains showed strong cytopathic effects on Caco-2 cells in a short period of time, inducing either cell lysis, cell rounding, or other morphological changes, which highlights the high virulence of this pathogen. In contrast, supernatants of some *V. parahaemolyticus* strains had no effects on the intestinal cells under the same conditions (data not shown). These results are in accordance with a previous study that described culture filtrates from all environmental and clinical *V. vulnificus* isolates to be cytotoxic for Chinese hamster ovary cells (Oliver *et al*., 1986).

Cytotoxicity of *V. vulnificus* to various cell lines is mediated by the cytolysin/hemolysin VvhA and MARTX (Gray and Kreger, 1985; Kim *et al*., 2008; Hor and Chen, 2013). MARTX has been described to induce cell rounding before lysis of HeLa cells (Kim *et al*., 2008). However, the cytopathic effects of cell-free supernatants observed in this study are not likely to be caused by MARTX since its activity is strongly dependent on contact between live bacterial cells and host cells (Kim *et al*., 2008). The observed cell rounding and detachment might also be caused by extracellular proteases, such as VvpE, whose expression is upshifted in the late stationary phase (Shin *et al*., 2005).

It should be noted that some strains showed large variations in this assay resulting in high standard deviations. Although strains were cultivated for a defined time, variations might result from different growth rates on different days, which in turn might lead to varying concentrations of cytotoxins and proteases in the supernatant. In order to reduce variations, some cultures were inoculated with a defined cell number and grown to an OD_{600} of 1.6 corresponding to the late exponential phase. However, cell-free supernatants obtained from

these cultures exhibited no cytotoxicity at all, even when incubation was extended to 24 hours (data not shown).

In another approach, intact cell suspensions of selected strains (eight clinical and seven environmental isolates belonging to different MLST clusters and exhibiting different virulence characteristics) were incubated with Caco-2 cells at different MOIs (multiplicity of infection) (Figure 2, unpublished results). This assay showed more reproducible results. However, clinical isolates showed no higher cytotoxicity compared to the environmental strains in these tests. Two clinical strains were non-cytotoxic even at MOI 100. As there was no clear association of cytotoxicity and the origin of strains, further experiments were not performed.

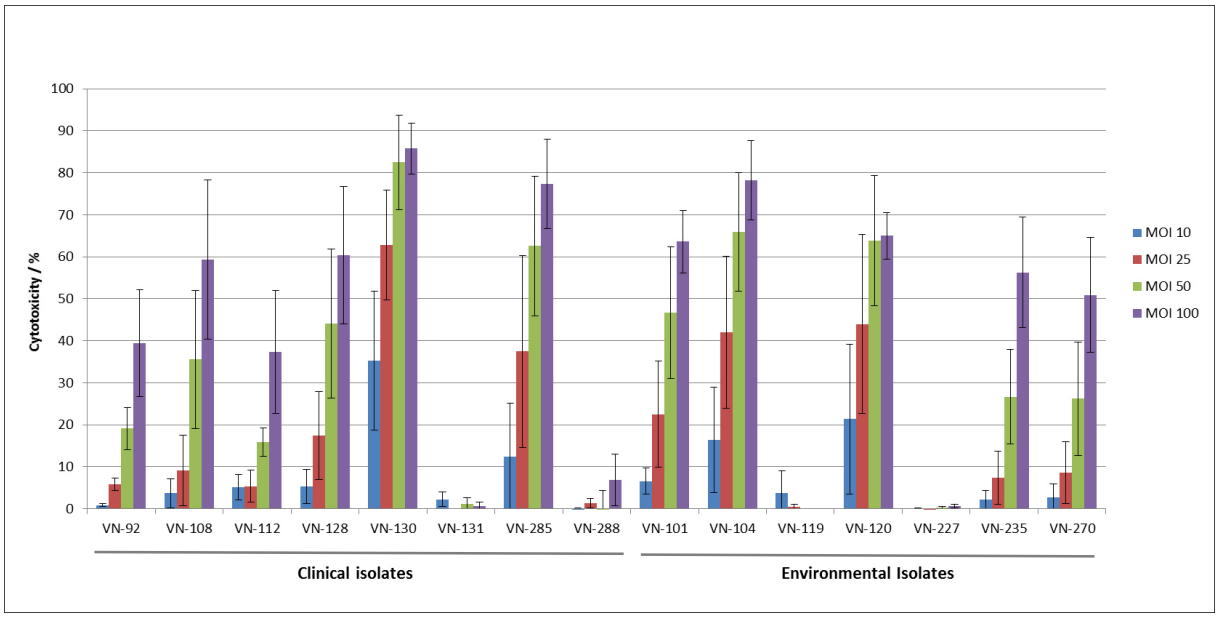

Figure 2. *In vitro* cytotoxicity of clinical (left) and environmental (right) *V. vulnificus* cells towards the human colon carcinoma cell line Caco-2. Bacteria were grown in Brain-Heart-Infusion broth, harvested in the mid-exponential growth phase (OD_{600} 0.8), and washed in phosphate-buffered saline. Monolayers of Caco-2 cells were exposed to bacterial cell suspensions in Dulbecco's modified Eagle medium (with 4.5 g/L glucose, without phenolred) at different multiplicities of infection (MOIs 10, 25, 50, and 100). After incubation for two hours at 37 °C and 5% CO_2, cytotoxicity was determined by measuring lactate dehydrogenase activity using the Cytotox96 kit (Promega). The means and standard deviations of three experiments are displayed.

Gulig *et al.* (2005) made similar observations for environmental and clinical *V. vulnificus* strains from the USA regarding the ability to induce apoptosis in murine J774 macrophages. Most of the strains induced apoptosis and neither relation to the origin of strains nor to virulence in mice was found. Additionally, they analyzed the same strain collection for cytotoxicity in various cell culture models and found a wide range of lysis, but again no correlation with mouse virulence (Gulig *et al.*, 2005).

3.2.4 Detection of Potential Virulence-Associated Genes

Clinical and environmental strains of the Baltic Sea region were further screened for the presence of genes and genomic regions that may be associated with pathogenicity. The strongest correlation with the clinical origin was observed for the putative pathogenicity region XII, a mannitol fermentation operon, and the *nanA* gene of the sialic acid catabolism region. These genes were found in most clinical but in only few environmental isolates confirming results of other studies (Cohen *et al*., 2007; Lubin *et al*., 2012). This indicates a potential role of the respective gene products (mediating sialic acid and mannitol catabolism, chondroitinase and aryl sulfatase activities, and sulfate reduction) for pathogenicity of *V. vulnificus*. While the *nanA* gene has already been proven to be essential for virulence of *V. vulnificus* (Jeong *et al*., 2009), the roles of mannitol fermentation and region XII during pathogenesis and their possible contribution to virulence have still to be elucidated.

PCR analyses of some potential virulence genes, e.g., several hemolysin genes (*hlyIII, vllY*, and VVA301), the metalloprotease gene *vvpE* (data not shown), the RTX toxin gene VVA331, and the gene *viuB* mediating siderophore utilization (data not shown) revealed that these are too frequently present to be useful for a discrimination of clinical and environmental *V. vulnificus* strains.

3.2.5 Genotyping of 16S rRNA, *vcg* and *pilF* Genes

In several studies, rapid methods have been developed to distinguish virulent from avirulent strains based on gene polymorphisms in specific genes. Among the most prominent genes are the 16S rRNA gene, the virulence-correlated gene (*vcg*), and the *pilF* gene (Rosche *et al*., 2005; Vickery *et al*., 2007; Roig *et al*., 2010; Baker-Austin *et al*., 2012a). In those studies, the clinical isolates possessed predominantly the 16S rRNA-type B, *vcg*-type C and *pilF*-type C alleles. However, the majority of clinical isolates from the Baltic Sea region exhibited the corresponding "environmental"-genotypes. These results are in accordance with other studies that reported E-type strains exhibiting high virulence in mice (Thiaville *et al*., 2011) and a relatively low prevalence of clinical-associated alleles in clinical isolates from northeastern USA (Reynaud *et al*., 2013) and Florida (Gordon, 2008). Consequently, strains possessing the "environmental"-genotype can be pathogenic and should not be classified as avirulent *per se*. This has already been discussed by Thiaville *et al.* (2011), who also observed environmental C-type strains that were attenuated in mice. Therefore, these gene polymorphisms cannot solely be used to detect potential pathogenic strains.

3.2.6 Multilocus Sequence Typing

Multilocus sequence typing (MLST) was performed to investigate phylogenetic relationships between clinical and environmental isolates from the Baltic region (Publication 1), as well as between environmental isolates from the North Sea and Baltic Sea (Publication 3). These analyses revealed a high genetic heterogeneity among the *V. vulnificus* isolates from Germany, as 109 different sequence types (STs) were identified among the 150 isolates examined in these studies.

In both studies, a separation into two major lineages was evident, which has also been observed in many other studies on *V. vulnificus* isolates from different sources and geographical regions (Bisharat *et al*., 2005, 2007b; Cohen *et al*., 2007; Sanjuan *et al*., 2011; Thiaville *et al*., 2011; Lubin *et al*., 2012; Reynaud *et al*., 2013). In accordance with the literature, Publication 1 showed that the majority of cluster I strains originated from environmental sources, while cluster II consisted mainly of clinical isolates. This suggests that *V. vulnificus* isolates belonging to this lineage may have a high pathogenicity potential and may be better adapted to the conditions in the human host. In fact, a higher ability to cause more severe skin infections and a higher incidence of lethal systemic infections in mice were already observed for MLST cluster II isolates compared to cluster I isolates (Thiaville *et al*., 2011).

Nonetheless, more than half of the examined clinical isolates from the Baltic Sea region were found in the "environmental" cluster I, indicating that some cluster I strains have the potential to cause severe human infections. This is also in line with the study of Thiaville *et al.* (2011), who identified highly virulent strains within MLST cluster I.

Interestingly, cluster II further split into two subclusters IIA and IIB. Cluster IIA contained the clinical reference strains from overseas (United States and Asia) and represents the typical "clinical" cluster II described in the literature (Bisharat *et al*., 2005, 2007b; Thiaville *et al*., 2011). Cluster IIB, on the other hand, seems to be a characteristic and widely distributed lineage within the German Baltic Sea that might be confined to European coasts (Publication 1 and 3). As this subcluster showed close relationship to cluster IIA and included recently isolated clinical strains, branch IIB could represent a clinically relevant clonal lineage within the Baltic Sea. While virulence of MLST cluster I and IIA strains has already been investigated in a mouse model (Thiaville *et al*., 2011), virulence of cluster IIB strains has still to be elucidated in a proper animal model.

As correlations between MLST clusters and specific virulence-associated traits as well as genotypes exist, cluster analysis based on all investigated phenotypic and genotypic characteristics divided *V. vulnificus* isolates into clusters that correspond to MLST

clusters/subclusters (Publication 1, Figure 2, p. 32). Genomic region XII, *nanA*, and the mannitol fermentation operon were present in the majority of MLST cluster II strains and may contribute to the higher virulence potential of this lineage (Cohen *et al*., 2007; Lubin *et al*., 2012; Reynaud *et al*., 2013). In accordance with the literature (Cohen *et al*., 2007; Thiaville *et al*., 2011), the 16S rRNA-type B and *vcg*-type C alleles were strongly associated with the "clinical" MLST cluster II and could serve to identify this virulent lineage. While the presence of 16S rRNA-type B allele was a strong indicator for both MLST subclusters IIA and IIB, the *vcg*-type C allele was exclusively present in MLST cluster IIA.

MLST is a rather time- and cost-intensive method. For cluster identification, sequence analysis of only three loci (*gyrB, dtdS*, and *pyrC*) seemed to be sufficient (Publication 3). Additionally, *vcg* and 16S rRNA-typing can be used to predict MLST clusters and subclusters.

3.2.7 Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry

The affiliation of a strain to a distinct MLST cluster may also be determined by intact-cell matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). Potential biomarker ions have been identified that might allow discrimination of major lineages as well as of different biotypes of *V. vulnificus* (Publication 1). A further evaluation of these biomarker ions using strains from various geographical regions would be a promising research project

One putative biotype-specific biomarker ion corresponded to ribosomal protein 32 (RL32). Sequence analysis revealed an amino acid exchange (K-to-R) in biotype 2 strains compared to biotype 1 strains. Since all biotype 2 strains investigated in Publication 1 were of the same sequence type and serovar E, I expanded sequence analyses to a more diverse selection of biotype 2 strains (19 different sequence types and three serovars: A, E, and I; unpublished results). In these investigations, the K-to-R exchange was specifically found in biotype 2 strains of serovar E, which is regarded as the zoonotic serovar of biotype 2 (Amaro and Biosca, 1996; Sanjuan *et al*., 2009). Presumably, MALDI-TOF MS and sequence analysis of ribosomal protein 32 (RL32) can be used for identification of biotype 2 serovar E that poses a risk for human infection.

3.2.8 Estimation of the Pathogenicity Potential of *Vibrio vulnificus*

In conclusion, cytotoxicity and hemolysis assays proved inappropriate for the discrimination of clinical and environmental strains under the tested laboratory conditions. Most *V. vulnificus* strains - irrespective of their origin - exerted strong hemolytic activity and profound cytopathic effects in a short period of time. In addition, these time-consuming analyses are not suitable for screening of large strain collections. However, they can be valuable tools for comparison of wildtype strains with isogenic mutants lacking potential virulence factors. The genotypic analyses proved to be more valuable for the discrimination of *V. vulnificus* isolates from the Baltic Sea region. The *nanA* gene, genomic region XII and the mannitol fermentation operon showed the strongest correlations with the clinical origin.

Because of the high occurrence of clinical isolates and putative pathogenicity markers within MLST cluster II as well as of the associated high virulence in mice (Thiaville *et al*., 2011), environmental *V. vulnificus* isolates belonging to this lineage were considered to possess a high potential to cause human infection. As mentioned before, more than half of the clinical Baltic Sea isolates grouped within the "environmental" MLST cluster I and exhibited the environmental genotype with respect to the 16S rRNA and *vcg* genes. Most of these clinical strains form a separate branch in both cluster analyses (Publication 1, Figure 1 and 2, p. 27, p. 32) and are characterized by possessing the *nanA* gene, region XII or the ability to ferment mannitol. The majority of the environmental strains within cluster I, on the other hand, showed only a low prevalence of these potential pathogenicity markers.

These results suggest, that environmental isolates of MLST cluster I, which are negative for serum resistance, genomic region XII, *nanA*, and mannitol fermentation are likely to possess less pathogenic potential. On the other hand, environmental isolates that are phylogenetically related to clinical strains and possess a great number of virulence characteristics can pose a risk for human infection.

For the assessment of potential pathogenicity, environmental isolates should be investigated for the presence of region XII and the *nanA* gene as well as for their ability to ferment mannitol. The latter can be tested biochemically or by PCR amplification of genes within the mannitol fermentation operon. Analysis of the 16S rRNA and *vcg* genes can be used to identify virulent strains of MLST cluster II. These analyses can be complemented by high-throughput screening of bacterial resistance to human serum as a prerequisite for pathogenicity.

3.3 Multiplex PCR for Simultaneous Detection of Putative Pathogenicity Markers of *Vibrio vulnificus*

So far, comparison of clinical and environmental *V. vulnificus* isolates revealed that no single virulence-associated trait or genotype can be used as a reliable marker for pathogenicity on its own. Therefore, the potential of environmental *V. vulnificus* isolates to cause infection should be estimated by testing a combination of the following genes: 16S rRNA-type B allele, *vcg*-type C allele, the *nanA* gene, genomic region XII and the mannitol fermentation operon. In order to simplify the analysis of large strain collections, a multiplex PCR protocol was developed that allowed simultaneous amplification of these five putative pathogenicity markers. An additional gene target, *toxR*, served as species marker and amplification control.

Eleven *V. vulnificus* strains with different virulence characteristics were used for optimization of the multiplex PCR. Different primer sequences, cycling conditions (annealing temperature, number of amplification cycles) as well as different concentrations of primers, $MgCl_2$, dNTPs, and Taq polymerase were tested to achieve reliable and reproducible amplification. The optimized multiplex PCR protocol was then validated using a strain collection consisting of 71 previously characterized *V. vulnificus* isolates as well as of other *Vibrionaceae* (n = 50) and non-*Vibrio* spp. (n = 11).

Multiplex PCR allowed robust amplification of four of the five pathogenicity markers with a high sensitivity and specificity and thus represents a cost and time-efficient method for screening of large strain collections. Only amplification of the 16S rRNA-type B allele was less sensitive (73%) compared to the Real-Time-PCR assay. However, amplification of this gene showed a specificity of 100%.

3.4 Prevalence of Virulence-Associated Characteristics among Environmental *Vibrio vulnificus* Isolates from the North Sea and Baltic Sea

Further investigations aimed to determine the prevalence of the most relevant potential pathogenicity markers in environmental strains from German coastal waters (101 isolates from 2010 to 2012). In this context, strains originating from the North Sea and the Baltic Sea were compared and phylogenetically analyzed, as both seas represent largely separated aquatic environments with diverging conditions, e.g., in salinity and temperature.

This study confirmed previous results from MLST analysis, such as the high genetic diversity of *V. vulnificus* isolates, the separation of isolates into MLST cluster I, IIA, and IIB, as well as correlations between MLST clusters and virulence-associated traits and genotypes.

Remarkably, MLST cluster IIB strains of this study originated exclusively from the Baltic Sea and accounted for a significant proportion of the *V. vulnificus* Baltic Sea isolates. In contrast, all cluster II isolates of the North Sea fell into branch A (Publication 3, Figure 3, p. 51), indicating that there are substantial differences between the *V. vulnificus* populations of both areas. This assumption is further supported by the observation of a different prevalence of virulence-associated traits and genotypes, as well as by the occurrence of characteristic virulence profiles. While similar proportions of strains in both areas exhibited region XII and resistance to human serum, the detection rate of *nanA* and mannitol fermentation was significantly higher among strains from the North Sea. Differences regarding the 16S rRNA-type B allele and *vcg*-type C allele in both areas result from the strong association of these genotypes with specific MLST clusters and match the geographical distribution pattern of MLST cluster IIA and IIB isolates. The prevalence of "clinical" MLST cluster II isolates was higher in the Baltic Sea. The detection rate of potential pathogenicity markers was very low among MLST cluster I isolates of Baltic Sea, whereas a high prevalence of these markers was found among cluster I isolates of the North Sea.

In conclusion, the majority of the Baltic Sea isolates (61%) were considered to possess a lower pathogenicity potential, while most of the North Sea isolates (78%) were regarded to pose a public health risk. So far, all reported *V. vulnificus* infections in Germany were associated with contact to the Baltic Sea, while no cases have been documented in the North Sea region. The data presented in this study indicate, however, that strains with a higher probability to cause infection are more prevalent in the North Sea.

3.5 Antimicrobial Resistance among *Vibrio vulnificus* Isolates from German Coastal Waters

Apart from the characterization of virulence-associated traits, the occurrence of antimicrobial resistance needs to be assessed to estimate the health risk emanating from *V. vulnificus* isolates in German coastal waters.

Although *V. vulnificus* infections occur only sporadically, the disease can be serious. The importance of proper and early treatment is emphasized by the fast progression of infections to septicemia, due to the strong multiplication of the pathogen. A mortality rate of 100% has been described for patients that did not receive antimicrobial therapy within the first 72 hours of the onset of illness (Klontz *et al.*, 1988). Therefore, after first signs of infection, antimicrobials should be administered to prevent a further dissemination of the pathogen in the body. However, due to poor blood perfusion, the achievement of effective

concentrations of antimicrobial agents can be hindered in necrotic tissue (Chen *et al*., 2012). Thus, surgical debridement and amputation of extremities should also be considered in an early phase of infection (Chen *et al*., 2012).

A retrospective analysis of cases in Taiwan revealed that the best antimicrobial therapy of necrotizing fasciitis caused by *V. vulnificus* is either the combination of a tetracycline (e.g., doxycycline and minocycline) with a third-generation cephalosporin (e.g., cefotaxime and ceftazidime) or single-agent therapy with fluoroquinolones (e.g., ciprofloxacin, moxifloxacin, and levofloxacin) (Chen *et al*., 2012), which is in accordance with the recommendations by the CDC[7]. Among others, the good tissue penetration ability is an argument for a treatment with fluoroquinolones (Chen *et al*., 2012). Another study reported a higher *in vivo* efficacy for the combination of ciprofloxacin and cefotaxime against *V. vulnificus* septicemia in mice compared to the cefotaxime-minocycline combination. In addition, ciprofloxacin showed a more effective inhibition of *rtxA1* transcription compared to minocycline or cefotaxime (Jang *et al*., 2014). Trimethoprim-sulfamethoxazole in combination with an aminoglycoside is proposed by the CDC[8] for the treatment of pregnant women and children as doxycycline and fluoroquinolones are contraindicated.

As delayed or ineffective therapy due to antimicrobial resistance could result in a fatal progression of the disease, the assessment of resistance phenotypes and underlying molecular mechanisms in *V. vulnificus* is of great importance. Three large scale studies on the occurrence of antimicrobial resistance among environmental *V. vulnificus* isolates in the USA have been published (Han *et al*., 2007; Baker-Austin *et al*., 2009; Shaw *et al*., 2014). However, studies on European isolates have been scarce and included only few *V. vulnificus* isolates ($n \leq 12$) (Dalsgaard *et al*., 1996; Zanetti *et al*., 2001; Ripabelli *et al*., 2003).

In this study (Publication 4), a very low occurrence of antimicrobial resistance was observed among *V. vulnificus* isolates from German coastal waters as well as among clinical isolates from the Baltic Sea region. High efficiencies in *in vitro* killing of *V. vulnificus* were observed for quinolones, fluoroquinolones, phenicols, tetracyclines, folate pathway inhibitors, aminopenicillins with or without β-lactamase inhibitors, carbapenems, and third- and fourth-generation cephalosporins. The clinically relevant cefotaxime, ceftazidime, and tetracycline were under the most effective antimicrobial agents *in vitro*. Non-susceptibility was exclusively observed against aminoglycosides; predominantly to streptomycin and sporadically to kanamycin. All *V. vulnificus* isolates were susceptible to the aminoglycoside gentamicin, which

[7] http://emergency.cdc.gov/disasters/vibriovulnificus.asp
[8] http://emergency.cdc.gov/disasters/vibriovulnificus.asp

could represent an alternative for the treatment of children and pregnant women, as was also suggested by others (Shaw *et al.*, 2014).

A high occurrence of streptomycin non-susceptible isolates was also reported from the Chesapeake Bay (Shaw *et al.*, 2014) and South Carolina (Baker-Austin *et al.*, 2009). In contrast to this study and to the results of Shaw *et al.*, Baker-Austin reported a high occurrence of gentamicin non-susceptibility that may be partially explained by different breakpoints used for interpretation of the data. Both studies also reported a low to high frequency of bacterial resistance to tetracycline as well as to aminoglycosides and cepaholsporins that were not tested in the present work. These included the aminoglycosides amikacin and apramycin, as well as the cepaholsporins ceftriaxone, cefoxitin, cephalotin, and cephalexin.

The molecular mechanisms responsible for the observed non-susceptibility to streptomycin could not been elucidated in this study and would be an interesting subject for future studies. It is possible that *V. vulnificus* and *V. cholerae*, or maybe all species of the genus *Vibrio* possess an intrinsic resistance to streptomycin as opposed to an acquired and/or mutational resistance (Leclercq *et al.*, 2013). Compared to the international distributions of minimal inhibitory concentrations (MIC) against *Salmonella* spp. and *Escherichia coli* [9], MIC distributions of *V. vulnificus* and *V. cholerae* observed in this study are shifted towards higher MIC values (Figure 3).

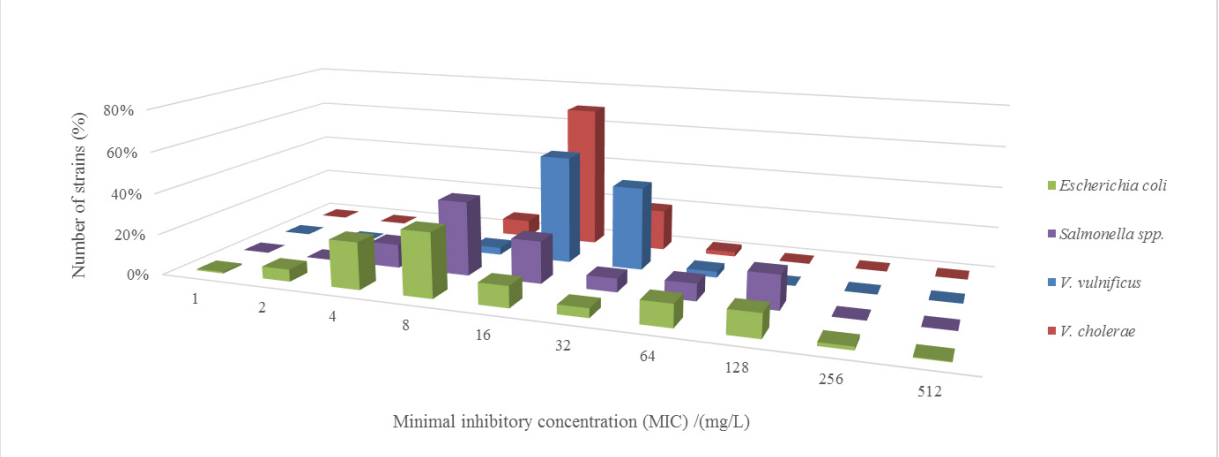

Figure 3: Comparison of MIC distributions of streptomycin against *V. vulnificus* and *V. cholerae* isolates from German coastal waters with international MIC distributions against *Escherichia coli* and *Salmonella* spp. published by the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[7]

[9] http://mic.eucast.org/Eucast2/

It is reassuring that the occurrence of antimicrobial resistance in *V. vulnificus* from German coastal waters is very low, given the severe consequences arising from antimicrobial resistance of this pathogen. With a constant fluctuation of the population and environment, it can be assumed that there is also a fluctuation of resistance mechanisms, which underlines the need for regular monitoring of antimicrobial resistance in *V. vulnificus*. Furthermore, the occurrence of putative carbapenemase producing *V. cholerae* in the North Sea and the Baltic Sea is of concern. The identity and genetic background of the responsible carbapenemase remains unclear, but location on mobile genetic elements would imply a higher risk for interspecies spread and could lead to the evolution of carbapenem resistant *V. vulnificus*.

3.6 Molecular Cloning in *Vibrio vulnificus* and Potential Applications

In another study from our laboratory, the cloning vector pVv3 was constructed based on a small plasmid (pVN-0126) originating from a clinical *V. vulnificus* strain. This vector together with an optimized electroporation protocol proved to be a suitable system for rapid and efficient transformation of *V. vulnificus*, *V. parahaemolyticus*, and *V. cholerae.* The functional expression of the green fluorescent protein (GFP) and the VvhA hemolysin in non-hemolytic *E. coli* and *V. vulnificus* strains demonstrated the applicability of pVv3 for molecular cloning and heterologous gene expression.

This transformation system represents a valuable tool for functional analyses of potential virulence genes and their contribution to *V. vulnificus* pathogenicity. Application of pVv3 allows overexpression of selected virulence genes or complementation of knockout-mutants. Wildtype strains, isogenic mutants and complemented mutants can be comparatively investigated for virulence-associated phenotypes, such as hemolysis, serum resistance, or cytotoxicity *in vitro*, or *in vivo* in an animal model.

Particularly interesting target genes for future studies are specific genes located on region XII (e.g., the chondroitinase gene or the arylsulfatase A gene cluster) or even the whole genomic region. The high prevalence of region XII in clinical isolates as well as within MLST cluster II strains, in this and other studies, implies a potential contribution to virulence (Cohen *et al*., 2007; Reynaud *et al*., 2013; Publication 1; Publication 3). However, its functional role in *V. vulnificus* pathogenesis, has not been demonstrated yet. In such investigations, the observed cluster specific sequence polymorphisms within the arylsulfatase A gene VVA1634 (Publication 3) should be considered. Other interesting target genes could be the mannitol fermentation operon or the different *nab* genes mediating synthesis of nonulosonic acids, such as sialic acids.

Since *V. vulnificus* pathogenesis is accomplished by the synergistic action of diverse virulence factors (Strom and Paranjpye, 2000; Anonymous, 2005), depletion of one particular factor may be partly complemented by the action of another. Therefore, apart from the functional analyses of single genes, also the successive introduction of several potential virulence genes into strains lacking these factors, or *vice versa* the successive knockout could be an interesting subject for future studies.

4 Summary

The Gram-negative bacterium *V. vulnificus* is ubiquitously distributed in coastal and estuarine waters with moderate salinity and sporadically causes severe foodborne or wound infections. Infections with this fast-growing pathogen are rare in Germany. However, forecasts assume a rising occurrence of *V. vulnificus* in German coastal waters, as well as increasing incidences of infection in the course of climate change. In view of these predictions, fundamental research and characterization of *V. vulnificus* present in German coastal waters regarding its pathogenic potential is of great importance for public health concerns. Furthermore, methods to identify potentially pathogenic strains of this bacterium are strongly needed to estimate the risk emanating from this pathogen.

In this work, the distribution of potential virulence genes, as well as of virulence-associated pheno- and genotypes was examined in clinical and environmental isolates from the Baltic Sea region to assess their suitability as potential pathogenicity markers. The most promising results were seen for genomic region XII, the *nanA* gene, and a mannitol fermentation operon, which were found in most clinical and relatively few environmental isolates. Genotypes that are described to correlate with the clinical origin in other studies, such as *vcg*-type C or 16S rRNA-type B were only rarely detected in clinical isolates from cases of the Baltic Sea region. However, these genotypes are strong indicators for phylogenetic lineages with a high pathogenicity potential. Phenotypic traits associated with virulence of *V. vulnificus,* such as resistance to human serum, strong hemolytic activity or cytotoxicity were exhibited by most isolates, underlining the destructive character of this pathogenic species.

The results led to the development and validation of a multiplex PCR that allows simultaneous amplification of genomic region XII, the *nanA* gene, the mannitol fermentation operon, as well as of the *vcg*-type C and 16S rRNA-type B alleles.

The prevalence of these potential pathogenicity markers was investigated in recent environmental isolates from the Baltic Sea as well as the North Sea. Together with phylogenetic analyses using multilocus sequence typing, this study showed that *V. vulnificus* populations in both areas vary considerably. The majority of North Sea isolates have to be considered as potential risk for human infection, although no *V. vulnificus* infections have been reported from this region so far. Therefore, increased awareness of this pathogen is needed in both, the Baltic Sea as well as the North Sea area, especially in summer when exposition to the pathogen is at its highest.

The characterization of *V. vulnificus* isolates was complemented by investigations on antimicrobial resistance patterns. All *V. vulnificus* isolates - irrespective of their origin - were susceptible to clinically relevant agents, such as tetracyclines, third-generation cephalosporins, and fluoroquinolones. No multidrug resistance was observed. Reduced susceptibility was exclusively found for streptomycin, which might be the result of an intrinsic resistance.

Finally, the applicability of a newly designed shuttle vector for molecular cloning in *Vibrio* spp. was demonstrated. The developed system represents an efficient tool for functional analyses of putative virulence genes.

5 Zusammenfassung

V. vulnificus ist ein Gram-negatives Bakterium, das ubiquitär in Küstengewässern und Ästuarien mit mäßigem Salzgehalt verbreitet ist und schwere Lebensmittel- und Wundinfektionen verursachen kann. Infektionen mit diesem schnell wachsenden Bakterium sind selten in Deutschland. Im Zuge des Klimawandels wird jedoch sowohl mit einem Anstieg des Vorkommens von *V. vulnificus* in deutschen Küstengewässern als auch mit einer steigenden Inzidenz an Infektionen gerechnet. In Anbetracht dieser Vorhersagen ist die grundlegende Untersuchung und Charakterisierung von *V. vulnificus* Isolaten in deutschen Küstengewässern in Bezug auf deren pathogenes Potential von großer Bedeutung für die öffentliche Gesundheit. Darüber hinaus werden dringend Methoden benötigt, die die Identifizierung von pathogenen Stämmen ermöglichen, um das von diesen Bakterien ausgehende Gesundheitsrisiko beurteilen zu können.

In dieser Arbeit wurde das Vorkommen mögliche Virulenzgene und virulenz-assoziierter Phäno- und Genotypen in klinischen Isolaten und Umweltisolaten der Ostseeregion untersucht, um ihre Eignung als potentielle Pathogenitätsmarker einzuschätzen. Die aussichtsreichsten Ergebnisse zeigten Untersuchungen der genomischen Region XII, des Gens *nanA* und des Mannitol Fermentations Operons. Diese Gene/Genregionen waren in den meisten klinischen Isolaten, aber nur in verhältnismäßig wenigen Umweltisolaten zu finden. Genotypen, die laut Literatur mit dem klinischen Ursprung von *V. vulnificus* Isolaten korrelieren sollten, wie z.B. der *vcg*-Typ C oder der 16S rRNA-Typ B, wurden nur selten in klinischen Isolaten aus dem Ostseeraum nachgewiesen. Jedoch deuten diese Genotypen stark auf eine Zugehörigkeit zu phylogenetischen Linien mit hohem Pathogenitätspotential hin. Die meisten Isolate wiesen phänotypische Merkmale auf, die mit der Virulenz von *V. vulnificus* assoziiert werden. Die weit verbreitete Resistenz gegenüber humanem Serum sowie die starke hämolytische Aktivität und Zytotoxizität heben den stark destruktiven Charakter dieser Spezies hervor.

Die Ergebnisse legten die Entwicklung und Validierung einer Multiplex PCR nahe, die die simultane Amplifikation der genomischen Region XII, des Gens *nanA*, des Mannitol Fermentations Operons und der *vcg*-Typ C und 16S rRNA-Typ B Allele erlaubt.

Schließlich wurde das Vorkommen dieser potentiellen Pathogenitätsmarker in kürzlich isolierten Umweltstämmen aus der Nordsee und Ostsee untersucht. Zusammen mit den phylogenetischen Analysen mittels Multilokus-Sequenz-Typisierung zeigten diese Untersuchungen, dass sich die *V. vulnificus* Populationen in beiden Regionen wesentlich unterscheiden. Obwohl bisher noch keine Infektionen aus dem Nordseeraum berichtet wurden, geht von den in dieser Studie untersuchten Nordseeisolaten ein potentielles Risiko für humane

Infektionen aus. Daher ist erhöhte Aufmerksamkeit für die Gesundheitsrisiken dieses pathogenen Erregers im Ostseeraum und Nordseeraum geboten. Dies gilt besonders in den Sommermonaten, wenn die Exposition mit diesem Bakterium am größten ist.

Die Charakterisierung der *V. vulnificus* Isolate wurde durch Antibiotikaresistenz-untersuchungen ergänzt. Alle *V. vulnificus* Isolate - unabhängig von deren Ursprung - waren empfindlich gegenüber klinisch relevanten antimikrobiellen Substanzen, wie z.B. Tetrazyklinen, Cephalosporinen der 3. Generation und Fluorochinolonen. Es traten keine Multiresistenzen auf und geringere Empfindlichkeit wurde nur gegenüber dem Aminoglycosid Streptomycin beobachtet, wobei es sich hierbei um eine intrinsische Resistenz handeln könnte.

Schließlich wurde die Anwendbarkeit eines neu konstruierten “Shuttle”-Vektors für die effiziente Klonierung von *Vibrio* Spezies nachgewiesen. Das entwickelte Klonierungssystem stellt damit ein effizientes Werkzeug für funktionelle Analysen potentieller Virulenzgene dar.

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7 Appendix

7.1 Supplementary Material

7.1.1 Publication 1

Table S1. *V. vulnificus* isolates included in this study

Strain	Country and year of isolation	Source (seawater Temp. [°C] / Sal. [‰]	Reference, Strain collection
VN-0010	Germany, 1994	Diseased human	RKI, BfR*
VN-0016	Germany, 1996	Seawater (SM/n.d.)	RKI, BfR*
VN-0092	Germany, 2011	Diseased human	Klinikum Greifswald
VN-0094	Denmark, 1994	Diseased human	(1)
VN-0095	Denmark, 1994	Diseased human	(1)
VN-0096	Denmark, 1994	Diseased human	RKI, BfR*
VN-0097	Denmark, 1994	Diseased human	(3)
VN-0098	Denmark, 1994	Diseased human	(3)
ATCC 33149	Japan	Diseased eel	ATCC
VN-0100	Germany, 2010	Seawater (SM/n.d.)	LAGuS
VN-0101	Germany, 2010	Seawater (SM/n.d.)	LAGuS
VN-0102	Germany, 2010	Seawater (SM/n.d.)	LAGuS
VN-0103	Germany, 2010	Seawater (SM/6.7)	LAGuS
VN-0104	Germany, 2010	Seawater (SM/n.d.)	LAGuS
VN-0105	Germany, 2010	Seawater (SM/n.d.)	LAGuS
VN-0108	Germany, 2010	Diseased human	LAGuS
VN-0112	Germany, 2010	Diseased human	LAGuS
VN-0119	Denmark, 1997	mussel	RKI, BfR*
VN-0120	Denmark, 1997	mussel	RKI, BfR*
ATCC 27562	USA	Diseased human	ATCC
VN-0125	Denmark, 1994	Diseased human	(1)
VN-0126	Denmark, 1994	Diseased human	(1)
VN-0127	Denmark, 1994	Diseased human	(1)
VN-0128	Denmark, 1994	Diseased human	(1)
VN-0129	Denmark, 1994	Diseased human	(1)
VN-0130	Denmark, 1994	Diseased human	(1)
VN-0131	Denmark, 1994	Diseased human	(1)

Table continued

Strain	Country and year of isolation	Source (seawater Temp. [°C] / Sal. [‰]	Reference, Strain collection
VN-0132	Denmark, 1994	Diseased human	(1)
VN-0133	Denmark, 1994	Diseased human	(1)
VN-0143	Sweden, 1991	Diseased eel	(3)
VN-0204	Denmark, 1994	Seawater	(3)
VN-0205	Germany, 1995	Seawater (SM/n.d.)	RKI, BfR*
VN-0206	Germany, 1995	Seawater (SM/n.d.)	RKI, BfR*
VN-0207	Germany, 1999	Seawater (22.0/n.d.)	RKI, BfR*
VN-0208	Germany, 1999	Seawater (13.0/n.d.)	RKI, BfR*
VN-0209	Germany, 1999	Seawater (17.0/n.d.)	RKI, BfR*
VN-0227	Germany, 2004	Seawater (21.7/7.4)	LAGuS
VN-0235	Germany, 2006	Seawater (24.2/6.8)	LAGuS
VN-0239	Germany, 2006	Seawater (24.0/7.2)	LAGuS
VN-0243	Germany, 2006	Seawater (24.2/6.8)	LAGuS
VN-0251	Germany, 2006	Seawater (20.0/7.1)	LAGuS
VN-0260	Germany, 2007	Seawater (20.4/7.4)	LAGuS
VN-0264	Germany, 2007	Seawater (19.2/7.1)	LAGuS
VN-0266	Germany, 2007	Seawater (n.d./8.1)	LAGuS
VN-0270	Germany, 2008	Seawater (16.2/6.3)	LAGuS
VN-0274	Germany, 2008	Seawater (20.9/7.6)	LAGuS
VN-0275	Germany, 2008	Seawater (18.6/7.8)	LAGuS
VN-0276	Germany, 2008	Seawater (16.7/7.4)	LAGuS
VN-0277	Germany, 2008	Seawater (17.9/7.0)	LAGuS
VN-0280	Germany, 2011	Seawater (19.5/5.4)	LAGuS
MO6-24	USA	Diseased human	(4,5)
CMCP6	South Korea	Diseased human	(2)
VN-0288	Germany, 2010	Diseased human	RKI, BfR*

*RKI, BfR, Strain collection of Robert Koch-Institute (RKI), now in collection of National Reference Laboratory for Monitoring Bacteriological Contamination of Bivalve Mollusks of BfR, LAGuS, Landesamt für Gesundheit und Soziales, Rostock, Germany.

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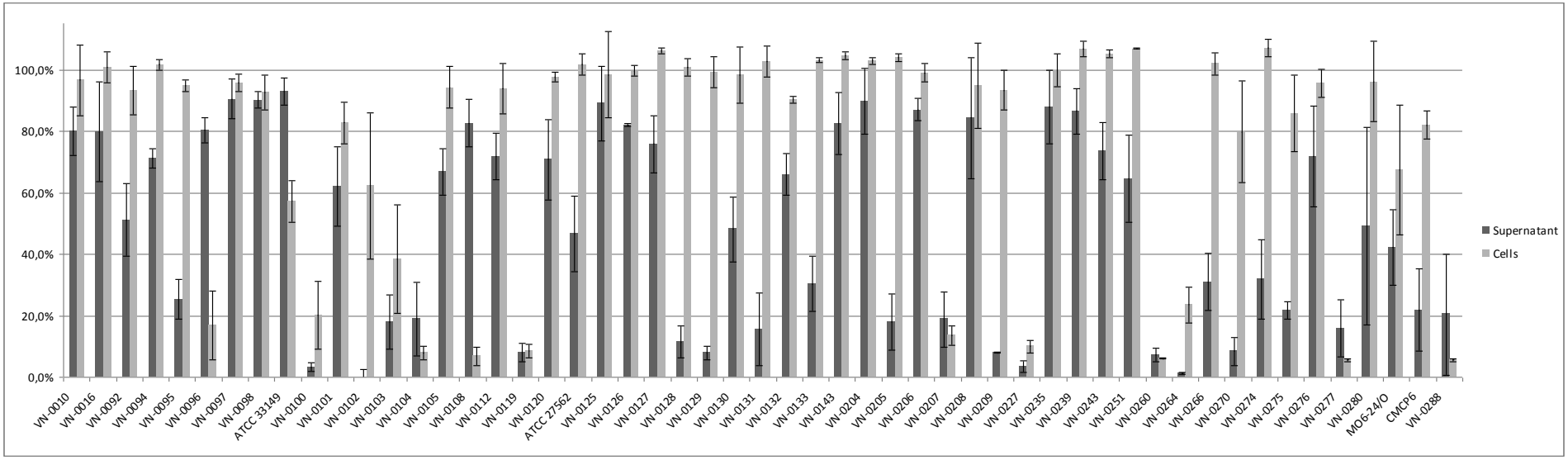
Table S2. Allelic profiles of the 53 isolates tested (Bold: new ST types/alleles)

Strain	Sequence type	Clonal complex (Burst)	*glp*	*gyrB*	*mdh*	*metG*	*purM*	*dtdS*	*lysA*	*pntA*	*pyrC*	*tnaA*
VN-0010	ST43	1	7	7	9	9	8	21	11	8	7	18
VN-0016	**ST103**	Singleton	**49**	**40**	**44**	2	8	22	**58**	5	11	**50**
VN-0092	**ST118**	Singleton	4	13	14	29	25	63	41	32	35	**46**
VN-0094	**ST110**	Singleton	38	1	12	31	27	23	36	1	48	**47**
VN-0095	**ST110**	Singleton	38	1	12	31	27	23	36	1	48	**47**
VN-0096	**ST111**	Singleton	**51**	**42**	41	**41**	8	22	4	**42**	14	**48**
VN-0097	**ST112**	Singleton	2	2	2	3	3	**65**	4	5	5	25
VN-0098	**ST112**	Singleton	2	2	2	3	3	**65**	4	5	5	25
ATCC 33149	**ST112**	Singleton	2	2	2	3	3	**65**	4	5	5	25
VN-0100	**ST128**	2	12	13	14	13	12	4	41	1	13	11
VN-0101	**ST113**	3	**50**	8	10	7	9	22	33	9	5	30
VN-0102	**ST114**	Singleton	47	**41**	11	23	8	**64**	**59**	5	14	**49**
VN-0103	**ST100**	4	13	14	15	7	9	34	6	15	19	39
VN-0104	**ST115**	2	12	**40**	14	13	12	4	41	1	13	11

Table continued												
Strain	**Sequence type**	**Clonal complex (Burst)**	***glp***	***gyrB***	***mdh***	***metG***	***purM***	***dtdS***	***lysA***	***pntA***	***pyrC***	***tnaA***
VN-0105	**ST116**	5	**49**	2	**44**	7	8	22	**57**	15	5	**50**
VN-0108	**ST117**	6	33	2	2	2	3	2	**62**	5	**53**	**40**
VN-0112	**ST118**	Singleton	4	13	14	29	25	**63**	41	32	35	**46**
VN-0119	**ST130**	Singleton	32	13	12	9	12	24	41	1	13	19
VN-0120	**ST119**	Singleton	1	3	32	5	2	**62**	3	4	**52**	7
ATCC 27562	ST3	Singleton	2	2	1	2	1	10	1	2	4	9
VN-0125	**ST112**	Singleton	2	2	2	3	3	**65**	4	5	5	25
VN-0126	**ST120**	1	7	7	12	9	8	21	11	**43**	7	18
VN-0127	**ST121**	Singleton	9	7	11	24	8	**61**	**56**	11	12	41
VN-0128	**ST110**	Singleton	38	1	12	31	27	23	36	1	48	**47**
VN-0129	**ST110**	Singleton	38	1	12	31	27	23	36	1	48	**47**
VN-0130	ST43	1	7	7	9	9	8	21	11	8	7	18
VN-0131	**ST122**	Singleton	48	11	**43**	24	8	22	**63**	13	12	42
VN-0132	ST43	1	7	7	9	9	8	21	11	8	7	18

Table continued												
Strain	**Sequence type**	**Clonal complex (Burst)**	***glp***	***gyrB***	***mdh***	***metG***	***purM***	***dtdS***	***lysA***	***pntA***	***pyrC***	***tnaA***
VN-0133	**ST131**	1	7	7	9	13	8	21	11	8	7	18
VN-0143	**ST112**	Singleton	2	2	2	3	3	**65**	4	5	5	25
VN-0204	**ST112**	Singleton	2	2	2	3	3	**65**	4	5	5	25
VN-0205	ST65	Singleton	13	14	15	7	9	40	6	25	5	4
VN-0206	**ST132**	5	**49**	**40**	**44**	7	8	22	57	15	5	**50**
VN-0207	**ST123**	Singleton	46	39	**42**	**40**	25	59	54	29	13	11
VN-0208	**ST105**	Singleton	33	28	11	2	3	2	17	13	51	25
VN-0209	**ST124**	Singleton	47	14	11	23	8	**64**	53	23	17	41
VN-0227	**ST125**	7	34	13	14	13	25	27	**61**	29	13	30
VN-0235	**ST133**	4	13	14	15	7	9	34	6	15	14	14
VN-0239	**ST133**	4	13	14	15	7	9	34	6	15	14	14
VN-0243	**ST107**	8	8	8	10	7	8	58	6	22	5	45
VN-0251	ST44	3	8	8	10	7	9	22	33	9	5	30
VN-0260	**ST126**	9	8	8	10	3	9	22	**55**	15	49	44

Table continued												
Strain	**Sequence type**	**Clonal complex (Burst)**	***glp***	***gyrB***	***mdh***	***metG***	***purM***	***dtdS***	***lysA***	***pntA***	***pyrC***	***tnaA***
VN-0264	**ST108**	Singleton	8	38	10	3	9	22	33	15	50	44
VN-0266	**ST113**	3	**50**	8	10	7	9	22	33	9	5	30
VN-0270	**ST133**	4	13	14	15	7	9	34	6	15	14	14
VN-0274	ST44	3	8	8	10	7	9	22	33	9	5	30
VN-0275	**ST133**	4	13	14	15	7	9	34	6	15	14	14
VN-0276	**ST134**	8	8	8	10	7	8	58	1	22	5	45
VN-0277	**ST127**	9	8	8	10	3	8	22	**55**	15	49	44
VN-0280	**ST109**	Singleton	45	2	20	2	9	**60**	26	20	17	43
MO6-24*	**ST32**	Singleton	5	3	18	14	14	7	2	17	22	13
CMCP6*			3	3	3	4	4			1	1	
VN-0288	**ST135**	7	34	13	14	13	25	27	41	29	13	11



Quantitative hemolysis assay. Hemolytic activity of intact cell suspension (light grey) and filtered culture supernatants (dark grey) of late exponential phase cultures towards human erythrocytes. After incubation for two hours at 37 °C, hemoglobin release was determined by measuring the absorbance at 570 nm. The means and standard deviations of three experiments are displayed.

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Table S1. Other *Vibrionaceae* and Non-*Vibrio* isolates used for exclusivity tests

Other *Vibrionaceae*	**No.**	**Strain IDs**
Vibrio alginolyticus	3	V144/11, V147/11, V50/12
Vibrio anguillarum	2	M74, LMG4410
Vibrio campbelli	2	CCUG 16330, LMG7910
Vibrio cholerae	5	V55/12, V178/12, NIH35A3, LMG4406, V179/12
Vibrio cincinnatiensis	3	V66/10, V73/10, V80/10,
Vibrio fischeri	1	LMG 4414 (DSM 507)
Vibrio fluvialis	4	V31/11, VN-0249, CH-1570, M45
Vibrio furnissii	2	VN-0341, VN-0342
Vibrio harveyi	3	V9/10, V88/11-2, V107/12
Vibrio hollisae	2	M106, M107
Vibrio metschnikovii	3	V148/12, V149/12, V168/12
Vibrio mimicus	4	V13/12, V14/12, V15/12, V16/12
Vibrio natriegens	1	LMG 10935
Vibrio navarrensis	3	V109/10, V110/10, V19/11
Vibrio orientalis	3	V113/10, V33/12, V106/12
Vibrio parahaemolyticus	4	V174/11, V76/12, V177/12, V180/12
Vibrio ponticus	1	V133/10
Vibrio proteolyticus	1	LMG 3772
Photobacterium damselae	3	M62, M63, M64

Table continued

Non-*Vibrio* isolates	No.	Strain IDs
Campylobacter coli	1	NCTC12668
Campylobacter jejuni	1	NCTC12662
Enterobacter cloacae	1	RKI 921/70
Escherichia coli	1	NCTC9001
Klebsiella sp.	1	RKI899b/73
Proteus mirabilis	1	DSM50903
Pseudomonas aeruginosa	1	ATCC27853
Salmonella enterica serovar Typhimurium	1	DSM11320
Staphylococcus aureus	1	ATCC25923
Yersinia enterocolitica	1	DSM13030
Yersinia intermedia	1	ATCC29909

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Supplementary Information

Virulence Profiles of *Vibrio vulnificus* in German Coastal Waters, a Comparison of North Sea and Baltic Sea Isolates

Table S1. Sampling sites, classification and bathing water quality.

Geographical region	Sampling Site No.	Sampling Site	No. of Strains	Classification (Coastal Waters) [a]	Bathing Water Quality [b]
North Sea	1	Borkum	1	euhaline open coastal waters	excellent
	2	Dyksterhusen	3	mesohaline inner coastal waters	good
	3	Jemgum	1	mesohaline inner coastal waters	no designated beach
	4	Burhave	8	mesohaline inner coastal waters	excellent
	5	Dedesdorf	9	mesohaline inner coastal waters	no designated beach
	6	Kleinensiel	2	mesohaline inner coastal waters	no designated beach
	7	Bremerhaven	16	mesohaline inner coastal waters	no designated beach
	8	Wremen	5	mesohaline inner coastal waters	excellent
	9	Altenbruch	3	mesohaline inner coastal waters	excellent
		k.A.	2		
Baltic Sea	10	Mönkeberg	1	mesohaline inner coastal waters	Excellent
	11	Kiel-Dietrichsdorf	1	mesohaline inner coastal waters	no designated beach
	12	Wohlenberger Wiek	2	mesohaline inner coastal waters	good
	13	Kühlungsborn	1	mesohaline open coastal waters	excellent
	14	Warnemünde	6	mesohaline open coastal waters	excellent
	15	Darss-Zingster Bodden chain, station 9	2	mesohaline inner coastal waters	no designated beach
	16	Darss-Zingster Bodden chain, station 8	2	mesohaline inner coastal waters	no designated beach
	17	Darss-Zingster Bodden chain, station 7	4	mesohaline inner coastal waters	no designated beach
	18	Greifswalder Bodden, station 5	2	mesohaline inner coastal waters	no designated beach
	19	Greifswalder Bodden, station 4	3	mesohaline inner coastal waters	no designated beach
	20	Greifswalder Bodden, station 3	5	mesohaline inner coastal waters	no designated beach

Table S1. *Cont.*

Geographical region	Sampling Site No.	Sampling Site	No. of Strains	Classification (Coastal Waters) [a]	Bathing Water Quality [b]
Baltic Sea	21	Greifswalder Bodden, station 2	4	mesohaline inner coastal waters	no designated beach
	22	Lubmin	4	mesohaline inner coastal waters	excellent
	23	Karlshagen	3	mesohaline open coastal waters	excellent
	24	Trassenheide	1	mesohaline open coastal waters	excellent
	25	Greifswalder Bodden, station 1	2	mesohaline inner coastal waters	no designated beach
	26	Binz	7	mesohaline open coastal waters	excellent
	27	Rügen	1	mesohaline inner coastal waters	no designated beach

[a] according to the European Water Framework Directive 2000/60/EC. [b] according to the requirements of the European Bathing Water Directive 2006/7/EC; results from 2011.

Table S2. Detailed sampling information of *V. vulnificus* isolates examined in this study.

Strain ID	Origin	Sampling Site No. [a]	Sampling Site Name	Seawater Temperature (°C)	Seawater Salinity (psu)	Sampling Date
VN-0279	B-sw	12	Wohlenberger Wiek	15,5	13	06.09.2011
VN-2813	N-sw	-	n.s.	n.s.	n.s.	16.05.2011
VN-2814	N-sw	-	n.s.	n.s.	n.s.	16.05.2011
VN-2961	B-sw	11	Kiel-Dietrichsdorf	18	n.s.	25.07.2011
VN-2969	B-sw	10	Mönkeberg	17,4	n.s.	25.07.2011
VN-3363	N-sd	4	Burhave	25,6	21,7	12.07.2010
VN-3364	N-sd	4	Burhave	25,6	21,7	12.07.2010
VN-3366	N-sd	4	Burhave	25,6	21,7	12.07.2010
VN-3367	N-sw	7	Bremerhaven	24,8	17,0	12.07.2010
VN-3368	N-sw	7	Bremerhaven	24,8	17,0	12.07.2010
VN-3369	N-sw	7	Bremerhaven	24,8	17,0	12.07.2010
VN-3373	N-sd	5	Dedesdorf	20,4	8,2	12.07.2010
VN-3374	N-sd	5	Dedesdorf	20,4	8,2	12.07.2010
VN-3378	N-sw	2	Dyksterhusen	20,4	20,5	20.07.2010

Table S2. *Cont.*

Strain ID	Origin	Sampling Site No. [a]	Sampling Site Name	Seawater Temperature (°C)	Seawater Salinity (psu)	Sampling Date
VN-3379	N-sw	2	Dyksterhusen	20,4	20,5	20.07.2010
VN-3394	N-sd	8	Wremen	21,7	22,5	10.08.2010
VN-3398	N-sw	8	Wremen	21,7	22,5	10.08.2010
VN-3403	N-sw	8	Wremen	21,7	22,5	10.08.2010
VN-3408	N-sw	2	Dyksterhusen	19	14,2	17.08.2010
VN-3410	N-sw	4	Burhave	18,7	25,1	18.08.2010
VN-3411	N-sd	4	Burhave	18,7	25,1	18.08.2010
VN-3412	N-sw	7	Bremerhaven	20,5	19,3	18.08.2010
VN-3415	N-sw	7	Bremerhaven	20,5	19,3	18.08.2010
VN-3418	N-sd	7	Bremerhaven	20,5	19,3	18.08.2010
VN-3419	N-sw	5	Dedesdorf	20,7	6,2	18.08.2010
VN-3426	N-sd	5	Dedesdorf	20,7	6,2	18.08.2010
VN-3442	N-sd	1	Borkum	16	29,8	24.08.2010
VN-3443	N-sd	4	Burhave	15	11,8	07.09.2010
VN-3444	N-sd	4	Burhave	15	11,8	07.09.2010
VN-3446	N-sd	7	Bremerhaven	15	11,8	07.09.2010
VN-3448	N-sw	7	Bremerhaven	17,5	14,6	07.09.2010
VN-3451	N-sw	7	Bremerhaven	17,5	14,6	07.09.2010
VN-3454	N-sd	7	Bremerhaven	17,5	14,6	07.09.2010
VN-3457	N-sd	7	Bremerhaven	17,5	14,6	07.09.2010
VN-3461	N-sw	5	Dedesdorf	17,7	1,9	07.09.2010
VN-3465	N-sd	5	Dedesdorf	17,7	1,9	07.09.2010
VN-3467	N-sd	5	Dedesdorf	17,7	1,9	07.09.2010
VN-3477	N-sw	7	Bremerhaven	14,1	14,9	05.10.2010
VN-3478	N-sd	7	Bremerhaven	14,1	14,9	05.10.2010
VN-3479	N-sd	5	Dedesdorf	15,6	5,3	05.10.2010

Table S2. *Cont.*

Strain ID	Origin	Sampling Site No. [a]	Sampling Site Name	Seawater Temperature (°C)	Seawater Salinity (psu)	Sampling Date
VN-3494	N-sw	7	Bremerhaven	17,5	14,6	07.09.2010
VN-3496	N-sw	7	Bremerhaven	17,5	14,6	07.09.2010
VN-3498	N-sd	4	Burhave	15	11,8	07.09.2010
VN-3500	N-sd	7	Bremerhaven	20,5	12,4	11.07.2011
VN-3506	N-sw	5	Dedesdorf	17,7	1,9	07.09.2010
VN-3518	N-sw	9	Altenbruch	n.s.	n.s.	12.06.2012
VN-3529	N-sw	6	Kleinensiel	n.s.	n.s.	02.08.2012
VN-3533	N-sw	8	Wremen	n.s.	n.s.	07.08.2012
VN-3536	N-sw	9	Altenbruch	n.s.	n.s.	07.08.2012
VN-3538	N-sw	6	Kleinensiel	n.s.	n.s.	13.08.2012
VN-3539	N-sw	3	Jemgum	n.s.	n.s.	14.08.2012
VN-3541	N-sw	8	Wremen	n.s.	n.s.	21.08.2012
VN-3542	N-sw	9	Altenbruch	n.s.	n.s.	06.09.2012
VN-3904	B-sd	19	Greifswalder Bodden, station 4	19,0	6,6	05.07.2011
VN-3905	B-sd	19	Greifswalder Bodden, station 4	19,0	6,6	05.07.2011
VN-3906	B-sd	18	Greifswalder Bodden, station 5	19,3	7,2	05.07.2011
VN-3909	B-sd	17	Darss-Zingster Bodden chain, station 7	19,2	7,9	05.07.2011
VN-3910	B-sd	17	Darss-Zingster Bodden chain, station 7	19,2	7,9	05.07.2011
VN-3912	B-sd	16	Darss-Zingster Bodden chain, station 8	19,0	7,1	05.07.2011
VN-3914	B-sd	15	Darss-Zingster Bodden chain, station 9	18,4	7,3	05.07.2011
VN-3915	B-sd	15	Darss-Zingster Bodden chain, station 9	18,4	7,3	05.07.2011
VN-3919	B-sd	25	Greifswalder Bodden, station 1	18,4	6,7	06.07.2011
VN-3921	B-sd	21	Greifswalder Bodden, station 2	18,9	6,6	06.07.2011
VN-3922	B-sw	20	Greifswalder Bodden, station 3	19,1	6,4	06.07.2011
VN-3924	B-sd	20	Greifswalder Bodden, station 3	19,1	6,4	06.07.2011
VN-3925	B-sd	12	Wohlenberger Wiek	18,9	10,7	01.08.2011

Table S2. *Cont.*

Strain ID	Origin	Sampling Site No. [a]	Sampling Site Name	Seawater Temperature (°C)	Seawater Salinity (psu)	Sampling Date
VN-3926	B-sd	25	Greifswalder Bodden, station 1	18,2	7,3	02.08.2011
VN-3927	B-sd	21	Greifswalder Bodden, station 2	19,5	6,2	02.08.2011
VN-3928	B-sw	20	Greifswalder Bodden, station 3	18,9	6,2	02.08.2011
VN-3929	B-sd	20	Greifswalder Bodden, station 3	18,9	5,9	02.08.2011
VN-3931	B-sd	20	Greifswalder Bodden, station 3	18,9	5,9	02.08.2011
VN-3932	B-sd	19	Greifswalder Bodden, station 4	19,7	6,3	02.08.2011
VN-3934	B-sd	17	Darss-Zingster Bodden chain, station 7	19,7	5,6	03.08.2011
VN-3935	B-sd	16	Darss-Zingster Bodden chain, station 7	19,7	5,6	03.08.2011
VN-3937	B-sd	16	Darss-Zingster Bodden chain, station 8	20,7	3	03.08.2011
VN-3946	B-sd	21	Greifswalder Bodden, station 2	13,4	6,7	11.10.2011
VN-3947	B-sd	21	Greifswalder Bodden, station 2	13,4	6,7	11.10.2011
VN-3948	B-sd	18	Greifswalder Bodden, station 5	12,6	7,6	11.10.2011
VN-3959	B-sw	22	Lubmin	26	6,5	05.07.2010
VN-3960	B-sw	23	Karlshagen	21,1	5,4	19.07.2010
VN-3961	B-sw	23	Karlshagen	21,1	5,4	19.07.2010
VN-3962	B-sw	22	Lubmin	20	6,4	19.07.2010
VN-3964	B-sw	26	Binz	n.s.	6,7	23.07.2010
VN-3965	B-sw	26	Binz	n.s.	6,7	23.07.2010
VN-3966	B-sw	26	Binz	n.s.	6,7	23.07.2010
VN-3968	B-sw	26	Binz	n.s.	6,7	23.07.2010
VN-3969	B-sw	26	Binz	n.s.	6,7	23.07.2010
VN-3970	B-sw	22	Lubmin	n.s.	6,3	02.08.2010
VN-3971	B-sw	24	Trassenheide	20,9	6,6	02.08.2010
VN-3972	B-sw	23	Karlshagen	21,5	6,5	02.08.2010
VN-3973	B-sw	13	Kühlungsborn	20,2	8,3	02.08.2010

Table S2. *Cont.*

Strain ID	Origin	Sampling Site No. [a]	Sampling Site Name	Seawater Temperature (°C)	Seawater Salinity (psu)	Sampling Date
VN-3974	B-sw	14	Warnemünde	22,8	8	04.08.2010
VN-3975	B-sw	14	Warnemünde	22,8	8	04.08.2010
VN-3976	B-sw	14	Warnemünde	22,8	8	04.08.2010
VN-3977	B-sw	14	Warnemünde	22,8	8	04.08.2010
VN-3978	B-sw	14	Warnemünde	22,8	8	04.08.2010
VN-3979	B-sd	14	Warnemünde	22,8	8	04.08.2010
VN-3980	B-sw	26	Binz	21	6,6	09.08.2010
VN-3981	B-sw	22	Lubmin	5,4	19,5	11.07.2011
VN-3982	B-sw	26	Binz	n.s.	6,7	23.07.2010
VN-5163	B-sw	27	Rügen	n.s.	n.s.	12.06.2011

N, North Sea; B, Baltic Sea; sw, seawater; sd, sediment; n.s., not specified. [a] Sampling site numbers shown in Figure 1.

Table S3. Allelic profiles of the 101 *V. vulnificus* isolates tested (new STs/alleles are displayed in red).

Strain ID	MLST ST	MLST-Cluster	Clonal Complex (SLV-Level)	Clonal Complex (TLV-Level)	*glp*	*gyrB*	*mdh*	*metG*	*purM*	*dtdS*	*lysA*	*pntA*	*pyrC*	*tnaA*
VN-0279	217	IIB	Singleton	Singleton	12	39	42	29	57	27	41	77	13	11
VN-2813	219	I	Singleton	Singleton	70	57	2	9	8	97	4	6	78	73
VN-2814	219	I	Singleton	Singleton	70	57	2	9	8	97	4	6	78	73
VN-2961	220	IIB	Singleton	Singleton	4	58	12	13	12	24	92	1	13	19
VN-2969	220	IIB	Singleton	Singleton	4	58	12	13	12	24	92	1	13	19
VN-3363	223	I	Singleton	Singleton	49	40	44	24	8	107	6	65	5	50
VN-3364	223	I	Singleton	Singleton	49	40	44	24	8	107	6	65	5	50
VN-3366	224	I	Singleton	Singleton	47	14	2	7	8	19	33	15	5	74
VN-3367	219	I	Singleton	Singleton	70	57	2	9	8	97	4	6	78	73
VN-3368	225	IIA	Singleton	Singleton	44	1	37	59	25	98	106	1	23	7
VN-3369	225	IIA	Singleton	Singleton	44	1	37	59	25	98	106	1	23	7

Table S3. *Cont.*

Strain ID	MLST ST	MLST-Cluster	Clonal Complex (SLV-Level)	Clonal Complex (TLV-Level)	*glp*	*gyrB*	*mdh*	*metG*	*purM*	*dtdS*	*lysA*	*pntA*	*pyrC*	*tnaA*
VN-3373	227	I	Singleton	Singleton	7	23	2	22	8	22	4	66	56	18
VN-3374	228	I	Singleton	Singleton	71	12	2	41	8	22	69	67	56	73
VN-3378	229	I	1	2	19	61	73	60	65	100	96	80	12	76
VN-3379	171	IIA	Singleton	Singleton	55	1	4	5	30	66	42	10	37	53
VN-3394	230	I	Singleton	Singleton	72	60	2	24	9	22	12	13	84	18
VN-3398	231	I	Singleton	Singleton	19	42	13	12	8	22	104	21	7	49
VN-3403	230	I	Singleton	Singleton	72	60	2	24	9	22	12	13	84	18
VN-3408	232	I	Singleton	Singleton	24	23	70	12	9	35	12	68	80	30
VN-3410	233	I	1	2	19	61	2	60	65	100	96	80	11	76
VN-3411	234	I	Singleton	Singleton	19	42	13	62	40	82	12	13	64	15
VN-3412	235	I	Singleton	Singleton	7	11	11	22	59	94	65	69	12	51
VN-3415	236	I	Singleton	Singleton	8	62	2	2	13	19	75	9	5	18
VN-3418	237	I	Singleton	Singleton	73	15	16	7	9	35	91	13	33	9
VN-3419	238	I	Singleton	Singleton	9	23	11	24	8	96	56	11	12	9
VN-3426	239	I	Singleton	4	74	11	2	22	60	103	12	20	81	50
VN-3442	239	I	Singleton	4	74	11	2	22	60	103	12	20	81	50
VN-3443	240	I	1	2	19	61	73	60	65	100	96	80	11	76
VN-3444	241	IIA	Singleton	Singleton	35	63	3	25	25	105	114	36	43	77
VN-3446	242	I	Singleton	Singleton	75	42	2	12	9	22	12	70	82	78
VN-3448	250	I	Singleton	Singleton	26	2	2	3	62	110	12	20	84	83
VN-3451	243	I	Singleton	Singleton	76	64	2	60	8	106	98	13	83	79
VN-3454	244	I	Singleton	Singleton	10	28	13	7	8	107	26	71	15	80
VN-3457	234	I	Singleton	Singleton	19	42	13	62	40	82	12	13	64	15
VN-3461	244	I	Singleton	Singleton	10	28	13	7	8	107	26	71	15	80
VN-3465	245	I	Singleton	Singleton	47	14	17	17	8	44	18	21	4	9
VN-3467	246	I	Singleton	Singleton	18	19	17	17	8	5	95	21	4	26

Table S3. *Cont.*

Strain ID	MLST ST	MLST-Cluster	Clonal Complex (SLV-Level)	Clonal Complex (TLV-Level)	*glp*	*gyrB*	*mdh*	*metG*	*purM*	*dtdS*	*lysA*	*pntA*	*pyrC*	*tnaA*
VN-3477	247	I	Singleton	Singleton	71	23	2	12	61	22	113	7	26	62
VN-3478	248	IIA	Singleton	Singleton	77	65	71	63	23	108	41	72	59	81
VN-3479	244	I	Singleton	Singleton	10	28	13	7	8	107	26	71	15	80
VN-3494	249	I	Singleton	Singleton	71	66	44	64	59	109	100	69	11	82
VN-3496	250	I	Singleton	Singleton	26	2	2	3	62	110	12	20	84	83
VN-3498	240	I	1	2	19	61	73	60	65	100	96	80	11	76
VN-3500	252	I	Singleton	Singleton	78	67	2	22	9	22	12	73	26	10
VN-3506	253	I	Singleton	Singleton	52	28	11	23	8	34	60	21	5	52
VN-3518	254	I	Singleton	Singleton	79	16	75	2	9	111	17	18	85	45
VN-3529	255	I	Singleton	Singleton	80	23	9	12	63	111	64	74	9	84
VN-3533	255	I	Singleton	Singleton	80	23	9	12	63	111	64	74	9	84
VN-3536	256	I	Singleton	Singleton	81	43	9	12	63	22	93	74	86	85
VN-3538	110	IIA	Singleton	Singleton	38	1	12	31	27	23	36	1	48	47
VN-3539	257	I	7	9	13	14	15	40	9	40	6	25	5	4
VN-3541	258	I	Singleton	4	74	26	2	22	60	103	12	75	81	18
VN-3542	259	I	Singleton	Singleton	17	68	73	22	65	112	112	25	11	18
VN-3904	133	I	6	3	13	14	15	7	9	34	6	15	14	14
VN-3905	287	I	2	5	47	14	11	61	8	64	53	23	17	41
VN-3906	260	I	Singleton	Singleton	82	2	20	2	8	107	110	71	87	86
VN-3909	261	I	Singleton	Singleton	47	12	74	22	8	2	6	76	50	87
VN-3910	262	IIB	Singleton	Singleton	12	39	14	42	25	24	92	41	13	11
VN-3912	263	IIB	3	6	4	13	14	7	25	27	61	77	13	11
VN-3914	113	I	4	7	50	8	10	7	9	22	33	9	5	30
VN-3915	264	I	Singleton	Singleton	69	14	11	7	9	113	105	78	88	39
VN-3919	265	I	Singleton	1	8	41	10	3	9	114	33	15	49	44
VN-3921	266	I	Singleton	Singleton	84	38	11	23	9	34	110	5	17	49

Table S3. *Cont.*

Strain ID	MLST ST	MLST-Cluster	Clonal Complex (SLV-Level)	Clonal Complex (TLV-Level)	*glp*	*gyrB*	*mdh*	*metG*	*purM*	*dtdS*	*lysA*	*pntA*	*pyrC*	*tnaA*
VN-3922	226	IIB	Singleton	Singleton	4	13	42	42	23	67	103	13	13	11
VN-3924	268	I	Singleton	3	13	14	15	7	9	5	6	15	14	45
VN-3925	217	IIB	Singleton	Singleton	12	39	42	29	57	27	41	77	13	11
VN-3926	251	I	2	5	47	14	11	61	8	99	53	23	17	41
VN-3927	269	I	Singleton	1	8	8	10	3	9	115	17	15	49	44
VN-3928	268	I	Singleton	3	13	14	15	7	9	5	6	15	14	45
VN-3929	268	I	Singleton	3	13	14	15	7	9	5	6	15	14	45
VN-3931	270	I	Singleton	1	8	40	10	3	9	22	33	15	49	44
VN-3932	271	I	Singleton	Singleton	47	41	11	23	8	64	59	5	5	49
VN-3934	226	IIB	Singleton	Singleton	4	13	42	42	23	67	103	13	13	11
VN-3935	272	IIB	3	6	4	13	14	40	25	27	61	77	13	11
VN-3937	144	I	Singleton	Singleton	47	14	2	7	41	19	17	46	57	30
VN-3946	273	I	5	1	8	8	10	3	9	115	41	15	15	44
VN-3947	274	I	Singleton	Singleton	85	40	2	65	13	81	1	46	5	44
VN-3948	226	IIB	Singleton	Singleton	4	13	42	42	23	67	103	13	13	11
VN-3959	275	I	Singleton	Singleton	18	14	11	2	3	116	62	5	53	25
VN-3960	126	I	Singleton	1	8	8	10	3	9	22	55	15	49	44
VN-3961	133	I	6	3	13	14	15	7	9	34	6	15	14	14
VN-3962	269	I	Singleton	1	8	8	10	3	9	115	17	15	49	44
VN-3964	128	IIB	Singleton	Singleton	12	13	14	13	12	4	41	1	13	11
VN-3965	113	I	4	7	50	8	10	7	9	22	33	9	5	30
VN-3966	276	I	4	7	86	8	10	7	9	22	33	9	5	30
VN-3968	128	IIB	Singleton	Singleton	12	13	14	13	12	4	41	1	13	11
VN-3969	132	I	Singleton	8	49	40	44	7	8	22	57	15	5	50
VN-3970	277	I	Singleton	8	49	40	44	2	8	22	58	5	5	50
VN-3971	278	I	Singleton	Singleton	87	42	11	7	8	82	57	65	5	45

Table S3. *Cont.*

Strain ID	MLST ST	MLST-Cluster	Clonal Complex (SLV-Level)	Clonal Complex (TLV-Level)	*glp*	*gyrB*	*mdh*	*metG*	*purM*	*dtdS*	*lysA*	*pntA*	*pyrC*	*tnaA*
VN-3972	277	I	Singleton	Singleton	49	40	44	2	8	22	58	5	5	50
VN-3973	268	I	Singleton	3	13	14	15	7	9	5	6	15	14	45
VN-3974	279	I	Singleton	Singleton	47	69	46	7	3	107	108	79	5	88
VN-3975	280	I	5	1	8	8	10	66	9	115	41	15	15	44
VN-3976	281	IIB	Singleton	Singleton	4	13	14	29	43	24	109	32	13	11
VN-3977	281	IIB	Singleton	Singleton	4	13	14	29	43	24	109	32	13	11
VN-3978	282	I	Singleton	Singleton	52	42	2	7	8	35	4	5	4	4
VN-3979	128	IIB	Singleton	Singleton	12	13	14	13	12	4	41	1	13	11
VN-3980	269	I	Singleton	1	8	8	10	3	9	115	17	15	49	44
VN-3981	283	I	Singleton	Singleton	45	2	20	2	8	60	26	20	17	43
VN-3982	284	I	6	3	13	14	15	7	9	34	6	15	14	39
VN-5163	65	I	7	9	13	14	15	7	9	40	6	25	5	4

MLST, multilocus sequence typing; ST, sequence type; SLV, single locus variant; TLV, triple locus variant.

Table S4. Primers and probes used for PCR amplification and sequencing.

Primer Name	Specificity/Gene Target/Designation	Sequence (5′ to 3′)	Amplicon (bp)	T_a (ºC)	Reference
SerE-F	specific for serovar E	TGTTGTTCTTGCCCACTCTC	665	64	[1]
SerE-R	specific for serovar E	CGCGCTTAGATTTGTCTCACC			[1]
Bt2-F	specific for biotype 2	AGAGATGGAAGAAACAGGCG	344		[1]
Bt2-R	specific for biotype 2	GGACAGATATAAGGGCAAATGG			[1]
vvhA-F	*V. vulnificus*-specific hemolysin	CGCCACCCACTTTCGGGCC	519		[1]
vvhA-R	*V. vulnificus*-specific hemolysin	CCGCGGTACAGGTTGGCGC			[1]
UtoxF	*toxR*	GASTTTGTTTGGCGYGARCAAGGTT	435	60	[2]
VvtoxR	*V. vulnificus*-specific *toxR*	AACGGAACTTAGACTCCGAC			[2]
vcg-typeC-F	virulence correlated gene clinical allele	AGCTGCCGATAGCGATCT	277	56	[3]
vcg-typeE-F	virulence correlated gene environmental allele	CTCAATTGACAATGATCT		47	[3]
vcg-typeC/E-R	virulence correlated gene	CGCTTAGGATGATCGGTG			[3]
VVA1612F	Region XII, 5′flanking region	ACCCTGATCGTTGGCTACTC	2.257	65	[4]
VVA1613R	Region XII, chondroitinase AC lyase	GGAGCGGTGTGATGGTGTTG			[4]
VVA1634F	Region XII, arylsulfatase A	TGACACCCAACCTAGACCAC	1.364	55	[4]
VVA1634R	Region XII, arylsulfatase A	ATTGATGCCAACCTGAG			[4]
VVA1612bF	Region XII, 5′flanking region	TGTGGAGAGCGGCAAGATCAAG	1.200	65	[4]
VVA1637R	Region XII, 3′flanking region	AACATCAACCAGCGAGTCGAAC			[4]
VVA1633a_F [a]	Region XII	CGTCATCACTCATGTCAAAGC	2483	60	this study
VVA1635c_R [a]	Region XII	GGTTTCATCGTCCCAAATGG			this study
VVA1633b_F [a]	Region XII	TCGAGATTGCAAACCGGACC			this study
VVA1635a_R [a]	Region XII	CGGCGTAGAGAATGATAACG			this study
VVA1635b_R [a]	Region XII	CGTACATCACATCCAACAGTTC			this study
VVA1634a_F [a]	Region XII, arylsulfatase A	GGCACGTTCGATCAACATTG			this study
VVA1634a_R [a]	Region XII, arylsulfatase A	TGATCGAACGTGCCATAGCC			this study
VVA1634b_F [a]	Region XII, arylsulfatase A	TCTATTTCGCCAACGTGAC			this study
VVA1634b_R [a]	Region XII, arylsulfatase A	GCAAAGTAATCGCGGATCTTG			this study

Table S4. *Cont.*

Primer Name	Specificity/Gene Target/Designation	Sequence (5′ to 3′)	Amplicon (bp)	T_a (ºC)	Reference
VVA1634c_F [a]	Region XII, arylsulfatase A	CCCCTATCAAACCAACAACC			this study
VVA1634d_F [a]	Region XII, arylsulfatase A	GCTGCTTTACCGATGTGCTC			this study
Vvu16S51-F [b]	16S rRNA gene	CAAGTCGAGCGGCAGCA	171	62	[5]
Vvu16S221-R [b]	16S rRNA gene	TCCTGACGCGAGAGGCC			[5]
Vvu16SA-P [b] (2091859)	16S rRNA gene type A allele	6-FAM-TGATAGCTTCGGCTCAA-MGBNFQ	probe		[5]
Vvu16SB-P [b] (2091860)	16S rRNA gene type B allele	VIC-CCCGTAGGCATCATGC-MGBNFQ	probe		[5]
nanA-F	sialic acid catabolism (SAC) cluster, aldolase,	TKATCGCCGCTCCYCATACA	745	58	[6]
nanA -R	sialic acid catabolism (SAC) cluster, aldolase,	GCAACGCCACCGTATTCAAC			[7]
Man IIA F	mannitol fermentation operon, enzyme IIA	GATGTTGGTGAACAACTTCTCTGC	243	61	[8]
Man IIA R	mannitol fermentation operon, enzyme IIA	TCTGAAGCCTGTTGGATGCC			[8]

T_a, annealing temperature. [a] used for gene sequencing; [b] used for Real-Time PCR.

Table S5. Genotypic and phenotypic characteristics of *V. vulnificus* strains from the Baltic Sea and North Sea.

Strain ID	Source	Sampling Site No. [a]	BT [b]	Serum resistance	Mannitol fermen-tation[c]	16S rRNA type	*vcg* type	Region XII	*nanA*	MLST -ST	MLST Cluste r	Risk Group [d]
VN-0279	B-sw	12	1	R	-	AB	E	+	+	217	IIB	2
VN-2813	N-sw	-	1	R	+	A	E	−	+	219	I	2
VN-2814	N-sw	-	1	R	+	A	E	−	+	219	I	2
VN-2961	B-sw	11	1	R	+	B	E	+	-	220	IIB	2
VN-2969	B-sw	10	1	R	+	AB	E	+	-	220	IIB	2
VN-3363	N-sd	4	1	R	+	A	E	+	-	223	I	2
VN-3364	N-sd	4	1	R	+	A	E	+	-	223	I	2
VN-3366	N-sd	4	1	R	-	A	E	-	-	224	I	1
VN-3367	N-sw	7	1	R	+	A	E	-	+	219	I	2
VN-3368	N-sw	7	1	I	+	B	C	-	+	225	IIA	2
VN-3369	N-sw	7	1	R	+	B	C	-	+	225	IIA	2
VN-3373	N-sd	5	1	R	-	A	E	-	-	227	I	1
VN-3374	N-sd	5	1	R	+	A	E	-	+	228	I	2
VN-3378	N-sw	2	1	R	-	A	E	-	+	229	I	2
VN-3379	N-sw	2	1	R	+	B	C	+	+	171	IIA	2
VN-3394	N-sd	8	1	R	+	A	E	+	+	230	I	2
VN-3398	N-sw	8	1	R	+	A	E	+	+	231	I	2
VN-3403	N-sw	8	1	R	+	A	E	+	+	230	I	2
VN-3408	N-sw	2	1	R	+	A	E	+	+	232	I	2
VN-3410	N-sw	4	1	R	-	A	E	-	+	233	I	2
VN-3411	N-sd	4	1	I	+	A	E	-	+	234	I	2
VN-3412	N-sw	7	1	R	+	A	E	-	+	235	I	2
VN-3415	N-sw	7	1	R	-	A	E	-	+	236	I	2

Table S5. *Cont.*

Strain ID	Source	Sampling Site No. [a]	BT [b]	Serum resistance	Mannitol fermen-tation[c]	16S rRNA type	*vcg* type	Region XII	*nanA*	MLST -ST	MLST Cluste r	Risk Group [d]
VN-3418	N-sd	7	1	R	+	A	E	+	+	237	I	2
VN-3419	N-sw	5	1	R	+	A	E	-	+	238	I	2
VN-3426	N-sd	5	1	R	+	A	E	+	+	239	I	2
VN-3442	N-sd	1	1	R	+	A	E	+	+	239	I	2
VN-3443	N-sd	4	1	R	-	A	E	-	+	240	I	2
VN-3444	N-sd	4	1	R	+	B	C	+	-	241	IIA	2
VN-3446	N-sd	7	1	I	+	A	E	-	+	242	I	2
VN-3448	N-sw	7	1	R	+	A	E	+	+	250	I	2
VN-3451	N-sw	7	1	I	-	A	E	-	-	243	I	1
VN-3454	N-sd	7	1	S	-	A	E	-	-	244	I	1
VN-3457	N-sd	7	1	R	+	A	E	-	+	234	I	2
VN-3461	N-sw	5	1	S	-	A	E	-	-	244	I	1
VN-3465	N-sd	5	1	R	-	A	E	-	-	245	I	1
VN-3467	N-sd	5	1	R	-	A	E	-	-	246	I	1
VN-3477	N-sw	7	1	I	+	A	C	+	+	247	I	2
VN-3478	N-sd	7	1	R	+	B	C	-	+	248	IIA	2
VN-3479	N-sd	5	1	S	-	A	E	-	-	244	I	1
VN-3494	N-sw	7	1	R	+	A	E	-	-	249	I	2
VN-3496	N-sw	7	1	R	+	A	E	+	+	250	I	2
VN-3498	N-sd	4	1	R	-	A	E	-	+	240	I	2
VN-3500	N-sd	7	1	R	+	A	E	+	+	252	I	2
VN-3506	N-sw	5	1	R	-	A	E	-	+	253	I	2
VN-3518	N-sw	9	1	R	-	A	E	-	-	254	I	1

Table S5. *Cont.*

Strain ID	Source	Sampling Site No. [a]	BT [b]	Serum resistance	Mannitol fermen-tation[c]	16S rRNA type	*vcg* type	Region XII	*nanA*	MLST -ST	MLST Cluste r	Risk Group [d]
VN-3529	N-sw	6	1	R	+	A	E	+	+	255	I	2
VN-3533	N-sw	8	1	R	+	A	E	-	+	255	I	2
VN-3536	N-sw	9	1	R	+	A	E	+	+	256	I	2
VN-3538	N-sw	6	1	R	+	B	E	+	+	110	IIA	2
VN-3539	N-sw	3	1	S	-	A	E	-	-	257	I	1
VN-3541	N-sw	8	1	R	+	A	E	+	+	258	I	2
VN-3542	N-sw	9	1	R	-	A	E	-	-	259	I	1
VN-3904	B-sd	20	1	R	-	A	E	-	-	133	I	1
VN-3905	B-sd	20	1	I	-	A	E	-	-	287	I	1
VN-3906	B-sd	19	1	R	-	A	E	-	-	260	I	1
VN-3909	B-sd	18	1	R	-	A	E	-	-	261	I	1
VN-3910	B-sd	18	1	R	-	AB	E	+	+	262	IIB	2
VN-3912	B-sd	17	1	R	-	AB	E	+	-	263	IIB	2
VN-3914	B-sd	16	1	I	-	A	E	-	-	113	I	1
VN-3915	B-sd	16	1	R	-	A	E	-	-	264	I	1
VN-3919	B-sd	26	1	I	-	A	E	-	-	265	I	1
VN-3921	B-sd	22	1	I	-	A	E	-	-	266	I	1
VN-3922	B-sw	21	1	R	-	AB	E	+	+	226	IIB	2
VN-3924	B-sd	21	1	R	-	A	E	-	-	268	I	1
VN-3925	B-sd	13	1	R	-	AB	E	+	+	217	IIB	2
VN-3926	B-sd	26	1	R	-	A	E	-	-	251	I	1
VN-3927	B-sd	22	1	I	-	A	E	-	-	269	I	1
VN-3928	B-sw	21	1	R	-	A	E	-	-	268	I	1

Table S5. *Cont.*

Strain ID	Source	Sampling Site No. [a]	BT [b]	Serum resistance	Mannitol fermen-tation[c]	16S rRNA type	*vcg* type	Region XII	*nanA*	MLST -ST	MLST Cluste r	Risk Group [d]
VN-3929	B-sd	21	1	R	-	A	E	-	-	268	I	1
VN-3931	B-sd	21	1	R	-	A	E	-	-	270	I	1
VN-3932	B-sd	20	1	R	-	A	E	-	-	271	I	1
VN-3934	B-sd	18	1	R	-	AB	E	+	+	226	IIB	2
VN-3935	B-sd	17	1	R	-	AB	E	+	-	272	IIB	2
VN-3937	B-sd	17	1	R	-	A	E	-	-	144	I	1
VN-3946	B-sd	22	1	I	-	A	E	-	-	273	I	1
VN-3947	B-sd	22	1	R	-	A	E	-	-	274	I	1
VN-3948	B-sd	19	1	R	-	AB	E	+	+	226	IIB	2
VN-3959	B-sw	23	1	R	-	A	E	-	-	275	I	1
VN-3960	B-sw	24	1	S	-	A	E	-	-	126	I	1
VN-3961	B-sw	24	1	R	-	A	E	-	-	133	I	1
VN-3962	B-sw	23	1	S	-	A	E	-	-	269	I	1
VN-3964	B-sw	27	1	R	-	AB	E	+	+	128	IIB	2
VN-3965	B-sw	27	1	I	-	A	E	-	-	113	I	1
VN-3966	B-sw	27	1	I	-	A	E	-	-	276	I	1
VN-3968	B-sw	27	1	R	-	AB	E	+	+	128	IIB	2
VN-3969	B-sw	27	1	R	+	A	E	+	+	132	I	2
VN-3970	B-sw	23	1	R	+	A	E	+	-	277	I	2
VN-3971	B-sw	25	1	R	+	A	E	-	-	278	I	2
VN-3972	B-sw	24	1	R	+	A	E	+	-	277	I	2
VN-3973	B-sw	14	1	R	-	A	E	-	-	268	I	1
VN-3974	B-sw	15	1	I	-	A	E	-	-	279	I	1

Table S5. *Cont.*

Strain ID	Source	Sampling Site No. [a]	BT [b]	Serum resistance	Mannitol fermen-tation[c]	16S rRNA type	*vcg* type	Region XII	*nanA*	MLST -ST	MLST Cluste r	Risk Group [d]
VN-3975	B-sw	15	1	R	-	A	E	-	-	280	I	1
VN-3976	B-sw	15	1	R	-	AB	E	+	+	281	IIB	2
VN-3977	B-sw	15	1	R	-	AB	E	+	+	281	IIB	2
VN-3978	B-sw	15	1	R	+	A	E	-	-	282	I	2
VN-3979	B-sd	15	1	R	-	AB	E	+	+	128	IIB	2
VN-3980	B-sw	27	1	R	-	A	E	-	-	269	I	1
VN-3981	B-sw	23	1	R	-	A	E	-	-	283	I	1
VN-3982	B-sw	27	1	R	-	A	E	-	-	284	I	1
VN-5163	B-sw	28	1	S	-	A	E	-	-	65	I	1

N, North Sea; B, Baltic Sea; sw, seawater; sd, sediment; R, resistant; I, intermediate resistant; S, susceptible; ST, sequence type. [a] Sampling site numbers shown in Figure 1. [b] Biotype assessed biochemically and by multiplex PCR. [c] Mannitol fermentation tested biochemically and by presence of mannitol fermentation operon (PCR). [d] Risk group 2 comprising strains with two or more pathogenicity markers, risk group 1 comprising strains without or with one pathogenicity marker.

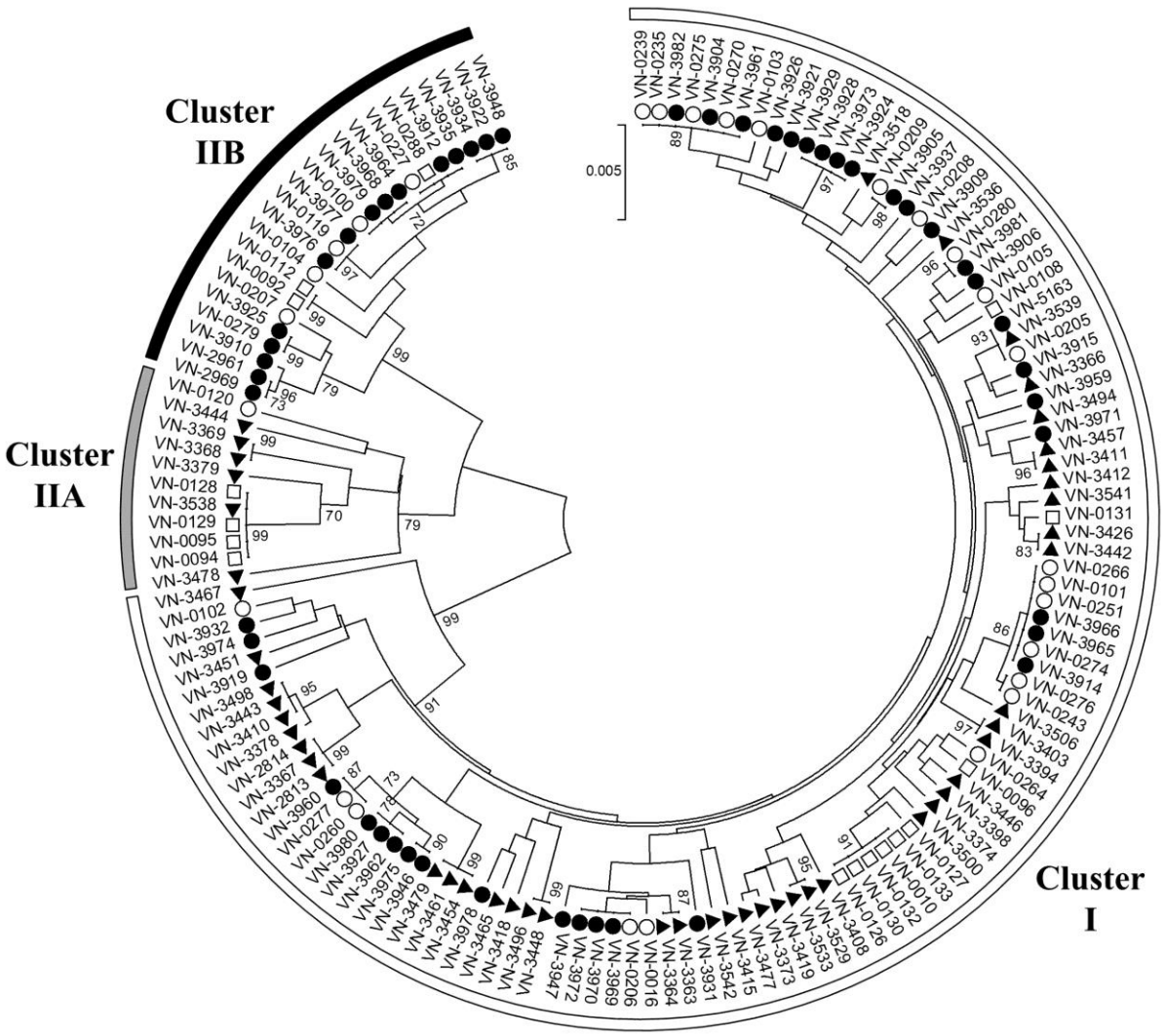

Figure S1. Population structure of *V. vulnificus* biotype 1 isolates from the North Sea (▲) and Baltic Sea (●) based on concatenated MLST sequences of three housekeeping genes (*gyrB, dtdS, and pyrC*). Bootstrap values above 70% are shown next to the branches. Semicircles around the tree highlight the association of strains to MLST cluster I (white), IIA (grey), and IIB (black). Sequences from clinical (□) and environmental (○) Baltic Sea isolates from a previous study [6] were included for comparison.

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A

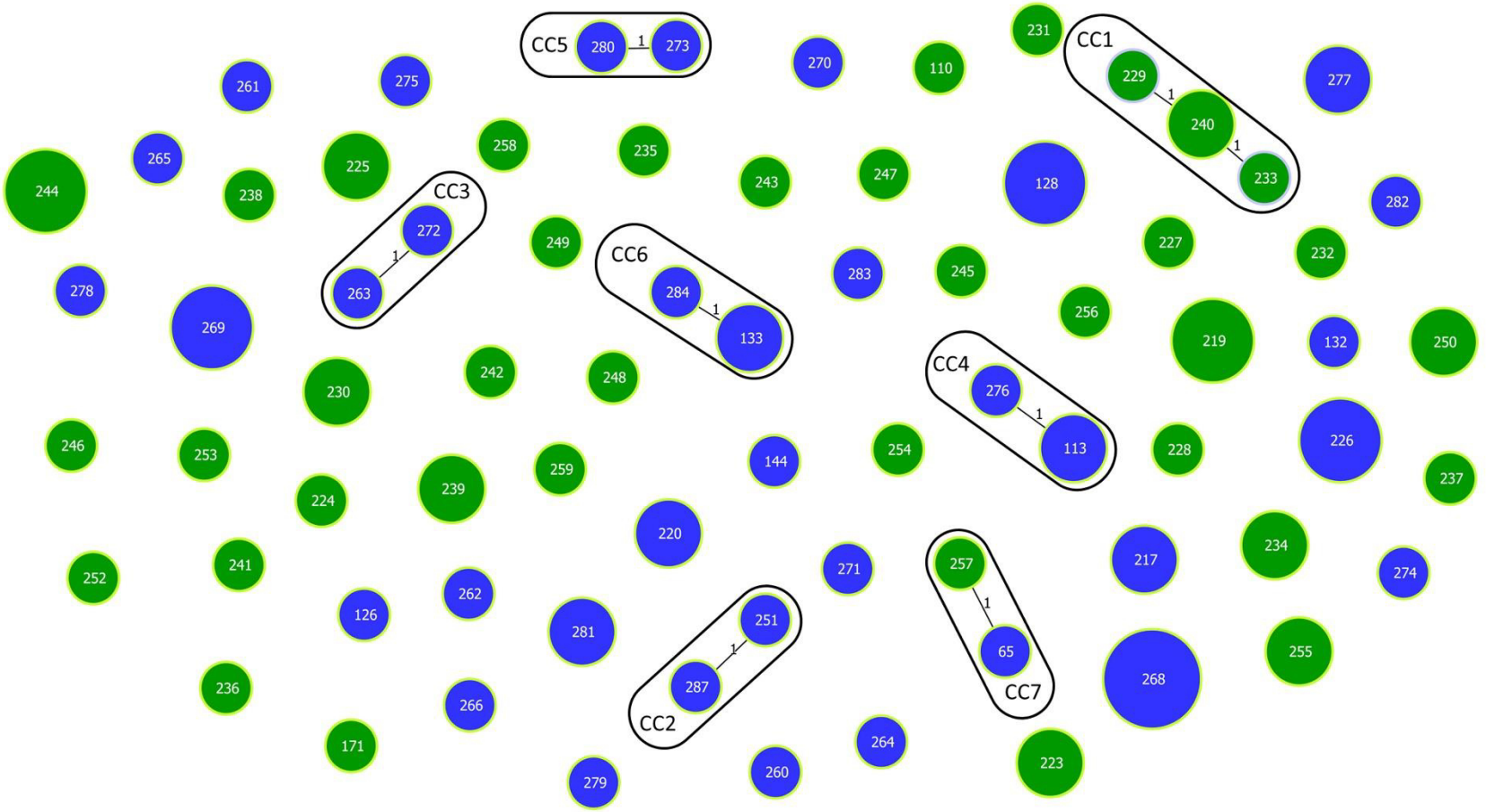

Figure 2. (*Cont.*)

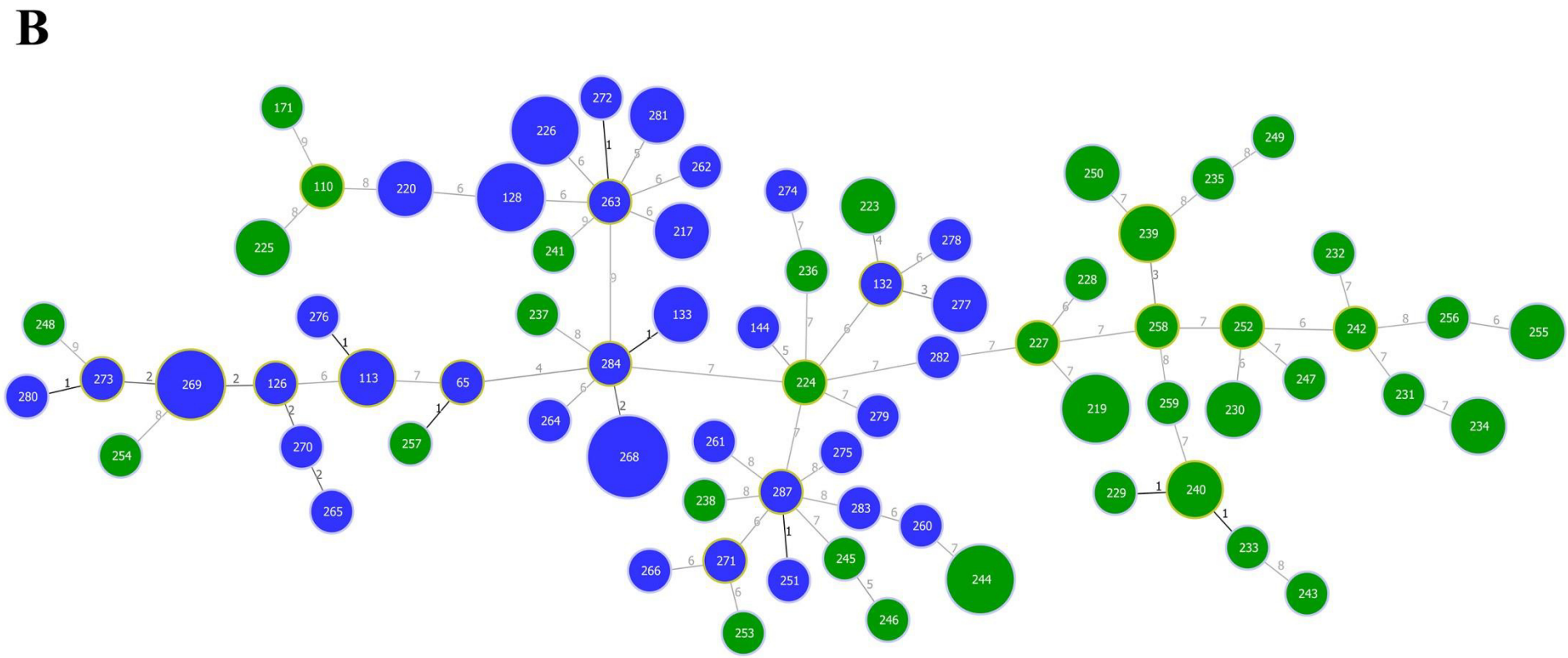

Figure 2. Population structure of *V. vulnificus* biotype 1 isolates from the North Sea and Baltic Sea obtained by goeBURST analysis based on MLST allelic profiles. Each sequence type (ST) is displayed as a circle with a size proportional to the number of isolates by which it is represented. The different colors indicate the geographical origin: North Sea (green) and Baltic Sea (blue). Single locus variants (SLVs) are connected via black lines. Light green halos around the circles indicate the respective founder of the group. (**A**) Population snapshot based on MLST allelic profiles. Clonal complexes (CC1–CC7) formed at the SLV level are highlighted by black edging. (**B**) Full Minimum Spanning Tree based on MLST allelic profiles. The number of different alleles between two STs is shown next to the connection lines.

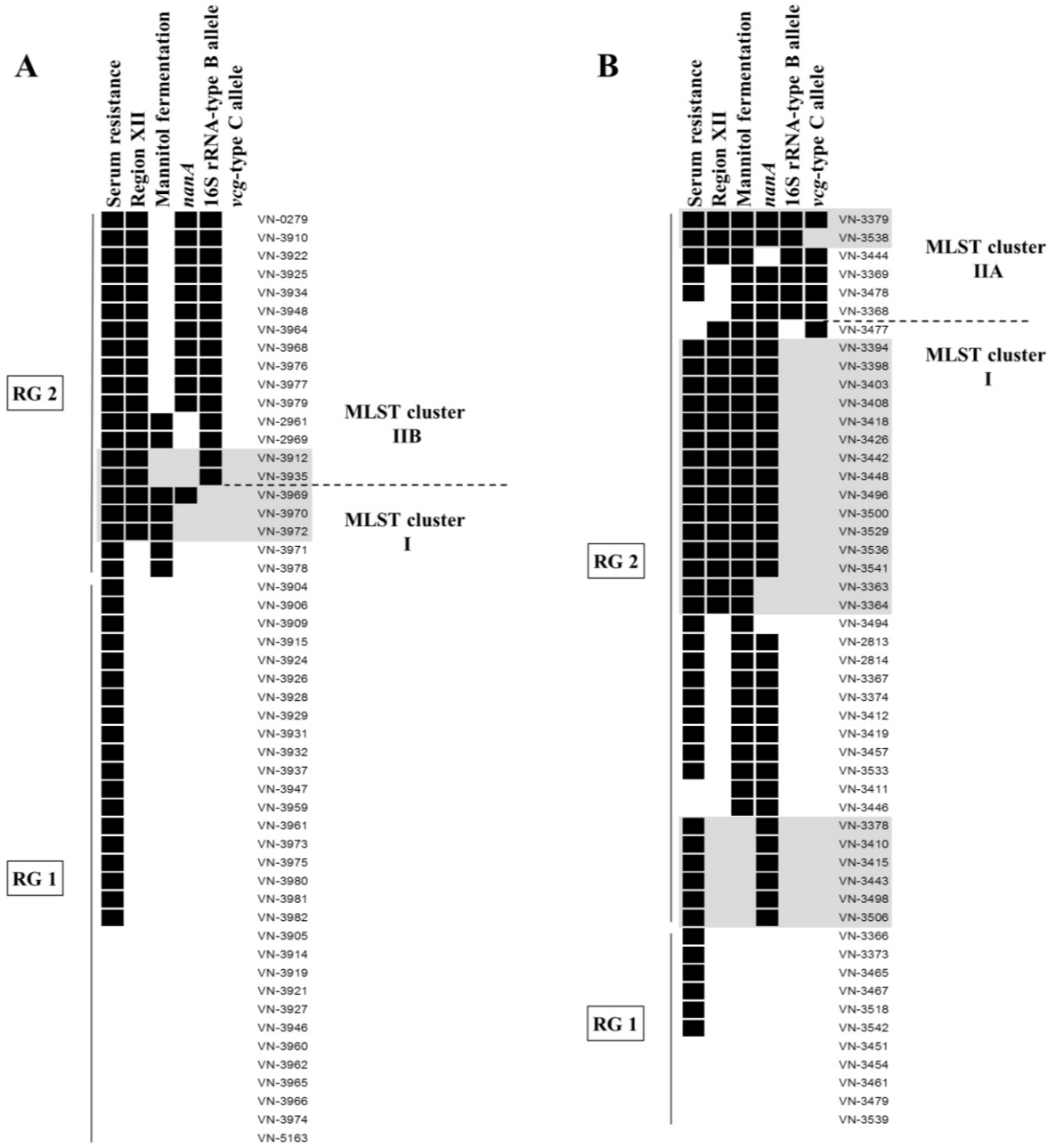

Figure 4. Combined results of MLST analysis and the investigation of virulence-associated traits and genotypes among *V. vulnificus* isolates from the Baltic Sea (**A**) and the North Sea (**B**). Presence of a pathogenicity marker is indicated by a black box. Strains rated as resistant (growth in 60%–80% human serum) are displayed as positive for serum resistance. Virulence profiles that have already been found in clinical isolates from the Baltic Sea region [11] are highlighted in grey. Risk Group 2 (RG2) comprises strains with two or more pathogenicity markers, while strains without or with one pathogenicity marker were assigned to Risk Group 1 (RG1) (see text). The figure was created using BioNumerics v7.5 (Applied Maths, Sint-Martens-Latem, Belgium).

7.1.4 Publication 4

Supplementary Table S1. Results of broth microdilution and disk diffusion assays of all *V. vulnificus* isolates[a]

	Strain source code[b]	Year	Minimal inhibitory concentration (MIC) [mg/L]													Inhibition zone diameter [mm]						Resistance Profile[c]
			AMP R ≥32	CHL R ≥32	CIP R ≥4	CST R >2	FFN R ≥16	CTX R ≥4	GEN R ≥16	KAN R ≥64	NAL R ≥32	STR R ≥64	CAZ R ≥16	TET R ≥16	TMP R ≥16	AMC R ≤13	SXT R ≤10	FEP R ≤18	LVX R ≤13	MEM R ≤19	IPM R ≤19	
Clinical																						
VN-0094	C-D-ext	1994	1	≤2	0.03	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	2	27	30	28	34	32	32	susceptible
VN-0095	C-D-ext	1994	1	≤2	0.015	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	1	28	30	32	36	36	36	susceptible
VN-0096	C-D-ext	1994	1	≤2	0.015	>4	≤2	≤0.06	0.5	≤4	≤4	4	≤0.25	≤1	≤0.5	28	35	30	32	40	34	susceptible
VN-0097	C-D-ext	1994	2	≤2	0.015	>4	≤2	0.12	4	16	≤4	**32**	0.5	≤1	≤0.5	30	30	22	32	24	28	(STR)
VN-0098	C-D-ext	1994	2	≤2	0.015	>4	≤2	0.12	4	16	≤4	64	0.5	≤1	1	22	34	26	32	32	31	STR
VN-0125	C-D-ext	1994	2	≤2	0.03	>4	≤2	0.12	4	**32**	≤4	64	0.5	≤1	1	24	30	26	32	32	34	STR, (KAN)
VN-0126	C-D-ext	1994	1	≤2	0.015	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	27	34	32	36	34	34	susceptible
VN-0127	C-D-ext	1994	1	≤2	0.015	>4	≤2	≤0.06	1	8	≤4	**32**	0.5	≤1	1	27	32	30	38	38	32	(STR)
VN-0128	C-D-ext	1994	1	≤2	0.03	>4	≤2	≤0.06	4	16	≤4	**32**	≤0.25	≤1	2	26	32	30	40	34	32	(STR)
VN-0129	C-D-ext	1994	2	≤2	0.03	>4	≤2	≤0.06	4	16	≤4	64	≤0.25	≤1	4	34	30	34	40	34	32	STR
VN-0130	C-D-ext	1994	1	≤2	0.015	>4	≤2	≤0.06	2	16	≤4	**32**	≤0.25	≤1	≤0.5	34	34	30	36	40	36	(STR)
VN-0131	C-D-ext	1994	1	≤2	0.03	>4	≤2	≤0.06	4	8	≤4	16	≤0.25	≤1	1	27	31	28	34	36	32	susceptible
VN-0132	C-D-ext	1994	2	≤2	0.015	>4	≤2	≤0.06	1	8	≤4	**32**	≤0.25	≤1	1	28	33	32	40	36	34	(STR)
VN-0133	C-D-ext	1994	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	**32**	≤0.25	≤1	1	27	31	30	38	36	32	(STR)
VN-0010	C-G-ext	1994	2	≤2	0.015	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	1	24	28	26	26	32	28	susceptible
VN-0108	C-G-ext	2010	2	≤2	≤0.008	>4	≤2	≤0.06	2	8	≤4	16	≤0.25	≤1	≤0.5	29	32	30	38	38	34	susceptible
VN-0112	C-G-ext	2010	1	≤2	0.06	>4	≤2	≤0.06	4	8	≤4	**32**	≤0.25	≤1	1	26	31	30	38	36	32	(STR)
VN-0288	C-G-ext	2010	1	≤2	0.015	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	1	38	38	38	40	44	40	susceptible
VN-0092	C-G-ext	2011	1	≤2	0.015	>4	≤2	≤0.06	1	16	≤4	**32**	≤0.25	≤1	≤0.5	26	31	26	36	37	34	(STR)
Environmental Baltic Sea																						
VN-3979	E-BS-sd	2010	1	≤2	0.015	>4	≤2	≤0.06	4	8	≤4	**32**	≤0.25	≤1	1	30	30	30	34	36	36	(STR)
VN-3904	E-BS-sd	2011	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	**32**	≤0.25	≤1	≤0.5	26	30	26	30	32	32	(STR)
VN-3905	E-BS-sd	2011	1	≤2	0.015	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	1	26	29	28	32	36	30	susceptible
VN-3906	E-BS-sd	2011	1	≤2	0.03	>4	≤2	≤0.06	2	8	≤4	16	0.5	≤1	1	26	30	28	36	36	34	susceptible
VN-3909	E-BS-sd	2011	2	≤2	0.015	>4	≤2	≤0.06	2	≤4	≤4	16	≤0.25	≤1	≤0.5	30	32	32	34	36	36	susceptible
VN-3910	E-BS-sd	2011	1	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	28	32	34	34	34	34	susceptible
VN-3912	E-BS-sd	2011	1	≤2	0.015	>4	≤2	≤0.06	0.5	≤4	≤4	8	≤0.25	≤1	1	34	34	34	50	50	44	susceptible
VN-3914	E-BS-sd	2011	1	≤2	0.015	>4	≤2	≤0.06	1	≤4	≤4	**32**	≤0.25	≤1	≤0.5	34	34	34	36	40	42	(STR)
VN-3915	E-BS-sd	2011	1	≤2	≤0.008	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	1	30	32	28	34	34	34	susceptible
VN-3919	E-BS-sd	2011	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	16	≤0.25	≤1	≤0.5	27	32	26	32	34	34	susceptible
VN-3921	E-BS-sd	2011	1	≤2	0.015	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	29	30	30	30	40	36	susceptible
VN-3924	E-BS-sd	2011	2	≤2	0.03	>4	≤2	0.12	1	≤4	≤4	16	0.5	≤1	1	25	30	29	32	30	30	susceptible
VN-3925	E-BS-sd	2011	2	≤2	0.03	>4	≤2	≤0.06	2	8	≤4	16	≤0.25	≤1	≤0.5	25	29	27	34	32	32	susceptible
VN-3926	E-BS-sd	2011	1	≤2	0.015	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	1	26	29	30	32	36	32	susceptible
VN-3927	E-BS-sd	2011	1	≤2	0.015	>4	≤2	≤0.06	2	16	≤4	**32**	≤0.25	≤1	≤0.5	30	32	30	36	36	34	(STR)
VN-3929	E-BS-sd	2011	2	≤2	0.015	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	1	26	30	30	34	26	30	susceptible
VN-3931	E-BS-sd	2011	1	≤2	0.015	>4	≤2	≤0.06	1	≤4	≤4	**32**	≤0.25	≤1	1	26	30	28	32	34	34	(STR)

2

Table continued

	Strain source code[b]	Year	Minimal inhibitory concentration (MIC) [mg/L]													Inhibition zone diameter [mm]						Resistance Profile[c]
			AMP R ≥32	CHL R ≥32	CIP R ≥4	CST R >2	FFN R ≥16	CTX R ≥4	GEN R ≥16	KAN R ≥64	NAL R ≥32	STR R ≥64	CAZ R ≥16	TET R ≥16	TMP R ≥16	AMC R ≤13	SXT R ≤10	FEP R ≤18	LVX R ≤13	MEM R ≤19	IPM R ≤19	
VN-3932	E-BS-sd	2011	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	**32**	≤0.25	≤1	2	26	28	26	32	32	32	(STR)
VN-3934	E-BS-sd	2011	1	≤2	0.03	>4	≤2	≤0.06	2	8	≤4	**32**	≤0.25	≤1	≤0.5	26	29	30	36	36	34	(STR)
VN-3935	E-BS-sd	2011	1	≤2	0.015	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	27	32	30	38	34	34	susceptible
VN-3937	E-BS-sd	2011	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	**32**	0.5	≤1	1	25	30	32	28	32	30	(STR)
VN-3946	E-BS-sd	2011	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	**32**	≤0.25	≤1	≤0.5	28	32	30	34	40	34	(STR)
VN-3947	E-BS-sd	2011	1	≤2	0.015	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	28	30	29	32	38	34	susceptible
VN-3948	E-BS-sd	2011	1	≤2	0.03	>4	≤2	≤0.06	2	8	≤4	**32**	≤0.25	≤1	≤0.5	25	28	28	30	34	32	(STR)
VN-0227	E-BS-sw	2004	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	**32**	≤0.25	≤1	1	34	40	50	50	50	50	(STR)
VN-0235	E-BS-sw	2006	1	≤2	0.015	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	26	32	26	32	36	32	susceptible
VN-0239	E-BS-sw	2006	2	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	**32**	0.5	≤1	≤0.5	25	32	30	34	34	30	(STR)
VN-0243	E-BS-sw	2006	1	≤2	≤0.008	>4	≤2	≤0.06	0.5	≤4	≤4	8	≤0.25	≤1	1	28	30	30	36	36	30	susceptible
VN-0251	E-BS-sw	2006	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	**32**	≤0.25	≤1	1	25	31	26	30	32	34	(STR)
VN-0260	E-BS-sw	2007	≤0.5	≤2	0.015	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	1	36	34	34	40	44	44	susceptible
VN-0264	E-BS-sw	2007	1	≤2	0.015	>4	≤2	≤0.06	1	≤4	≤4	8	≤0.25	≤1	≤0.5	33	33	30	40	40	40	susceptible
VN-0266	E-BS-sw	2007	1	≤2	≤0.008	>4	≤2	≤0.06	2	8	≤4	**32**	≤0.25	≤1	1	25	28	25	32	33	34	(STR)
VN-0270	E-BS-sw	2008	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	16	≤0.25	≤1	≤0.5	30	32	28	32	36	34	susceptible
VN-0274	E-BS-sw	2008	1	≤2	≤0.008	>4	≤2	≤0.06	2	8	≤4	16	≤0.25	≤1	1	28	32	28	32	36	34	susceptible
VN-0275	E-BS-sw	2008	1	≤2	0.015	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	≤0.5	28	34	28	36	36	34	susceptible
VN-0276	E-BS-sw	2008	2	≤2	0.03	>4	≤2	≤0.06	2	8	≤4	16	≤0.25	≤1	2	28	31	28	34	36	36	susceptible
VN-0277	E-BS-sw	2008	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	16	≤0.25	≤1	1	28	31	30	31	38	34	susceptible
VN-0100	E-BS-sw	2010	1	≤2	0.015	>4	≤2	0.12	2	8	≤4	**64**	≤0.25	≤1	1	28	30	28	34	38	34	STR
VN-0101	E-BS-sw	2010	2	≤2	≤0.008	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	1	26	28	28	34	34	33	susceptible
VN-0102	E-BS-sw	2010	1	≤2	0.015	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	≤0.5	26	29	22	30	32	32	susceptible
VN-0103	E-BS-sw	2010	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	16	≤0.25	≤1	≤0.5	26	30	28	32	34	30	susceptible
VN-0104	E-BS-sw	2010	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	16	≤0.25	≤1	1	29	29	28	32	34	34	susceptible
VN-0105	E-BS-sw	2010	2	≤2	0.015	>4	≤2	≤0.06	2	16	≤4	16	0.5	2	1	22	26	26	34	32	30	susceptible
VN-3959	E-BS-sw	2010	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	**32**	≤0.25	≤1	1	28	30	30	30	34	34	(STR)
VN-3960	E-BS-sw	2010	1	≤2	0.03	>4	≤2	≤0.06	1	8	≤4	**32**	0.5	≤1	1	30	31	28	32	38	34	(STR)
VN-3961	E-BS-sw	2010	1	≤2	0.015	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	≤0.5	25	31	26	30	36	32	susceptible
VN-3962	E-BS-sw	2010	1	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	8	≤0.25	≤1	≤0.5	30	34	50	50	40	30	susceptible
VN-3964	E-BS-sw	2010	1	≤2	0.015	>4	≤2	≤0.06	2	16	≤4	**32**	≤0.25	≤1	1	30	30	30	34	36	36	(STR)
VN-3965	E-BS-sw	2010	1	≤2	≤0.008	>4	≤2	≤0.06	4	8	≤4	**32**	≤0.25	≤1	≤0.5	28	34	36	34	30	30	(STR)
VN-3966	E-BS-sw	2010	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	**32**	≤0.25	≤1	1	32	34	34	38	40	38	(STR)
VN-3968	E-BS-sw	2010	1	≤2	0.015	>4	≤2	≤0.06	1	≤4	≤4	**32**	≤0.25	≤1	1	30	31	32	32	36	34	(STR)
VN-3969	E-BS-sw	2010	2	≤2	0.015	>4	≤2	≤0.06	2	16	≤4	16	≤0.25	≤1	1	26	30	28	36	36	36	susceptible
VN-3970	E-BS-sw	2010	2	≤2	≤0.008	>4	≤2	≤0.06	2	8	≤4	**32**	≤0.25	≤1	1	24	31	28	34	34	30	(STR)
VN-3971	E-BS-sw	2010	2	≤2	0.03	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	1	30	33	30	32	44	38	susceptible
VN-3972	E-BS-sw	2010	1	≤2	≤0.008	>4	≤2	≤0.06	2	8	≤4	16	≤0.25	≤1	1	28	32	26	38	36	34	susceptible
VN-3973	E-BS-sw	2010	1	≤2	0.015	>4	≤2	≤0.06	2	≤4	≤4	16	≤0.25	≤1	1	28	32	32	34	38	36	susceptible
VN-3974	E-BS-sw	2010	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	**32**	≤0.25	≤1	1	28	28	26	30	34	40	(STR)

Table continued

	Strain source code[b]	Year	Minimal inhibitory concentration (MIC) [mg/L]													Inhibition zone diameter [mm]						Resistance Profile[c]
			AMP R ≥32	CHL R ≥32	CIP R ≥4	CST R >2	FFN R ≥16	CTX R ≥4	GEN R ≥16	KAN R ≥64	NAL R ≥32	STR R ≥64	CAZ R ≥16	TET R ≥16	TMP R ≥16	AMC R ≤13	SXT R ≤10	FEP R ≤18	LVX R ≤13	MEM R ≤19	IPM R ≤19	
VN-3975	E-BS-sw	2010	1	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	28	30	26	32	34	35	susceptible
VN-3976	E-BS-sw	2010	1	≤2	≤0.008	4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	1	24	29	26	32	34	32	susceptible
VN-3977	E-BS-sw	2010	1	≤2	≤0.008	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	1	26	29	27	32	34	32	susceptible
VN-3978	E-BS-sw	2010	1	≤2	0.03	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	1	26	30	28	32	36	32	susceptible
VN-3980	E-BS-sw	2010	1	≤2	≤0.008	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	≤0.5	28	31	30	32	32	34	susceptible
VN-3982	E-BS-sw	2010	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	16	≤0.25	≤1	≤0.5	28	33	30	34	36	34	susceptible
VN-0279	E-BS-sw	2011	2	≤2	0.03	>4	≤2	≤0.06	2	8	≤4	**32**	≤0.25	≤1	1	27	30	30	30	30	30	(STR)
VN-0280	E-BS-sw	2011	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	16	≤0.25	≤1	1	28	30	28	32	34	34	susceptible
VN-2961	E-BS-sw	2011	≤0.5	≤2	0.03	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	30	32	30	32	32	30	susceptible
VN-2969	E-BS-sw	2011	≤0.5	≤2	0.03	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	≤0.5	30	32	30	34	40	36	susceptible
VN-3922	E-BS-sw	2011	1	≤2	0.03	>4	≤2	≤0.06	4	16	≤4	**32**	≤0.25	≤1	1	27	29	30	30	33	30	(STR)
VN-3928	E-BS-sw	2011	1	≤2	0.015	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	1	24	27	28	30	30	30	susceptible
VN-3981	E-BS-sw	2011	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	**32**	≤0.25	≤1	1	26	30	28	30	32	31	(STR)
Environmental North Sea																						
VN-10119	E-NS-bm	2012	1	≤2	≤0.008	>4	≤2	≤0.06	2	8	≤4	16	≤0.25	≤1	1	28	31	28	32	36	34	susceptible
VN-10121	E-NS-bm	2012	1	≤2	0.015	>4	≤2	≤0.06	2	16	≤4	**32**	≤0.25	≤1	1	27	30	30	36	38	34	(STR)
VN-3363	E-NS-sd	2010	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	16	≤0.25	≤1	1	27	30	30	38	40	34	susceptible
VN-3364	E-NS-sd	2010	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	16	≤0.25	≤1	1	26	30	28	31	34	32	susceptible
VN-3366	E-NS-sd	2010	≤0.5	≤2	0.015	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	1	40	40	42	40	48	45	susceptible
VN-3373	E-NS-sd	2010	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	**32**	≤0.25	≤1	1	24	30	30	30	32	30	(STR)
VN-3374	E-NS-sd	2010	1	≤2	0.015	>4	≤2	≤0.06	4	8	≤4	**32**	≤0.25	≤1	1	25	30	26	30	34	30	(STR)
VN-3394	E-NS-sd	2010	1	≤2	0.03	>4	≤2	≤0.06	2	8	≤4	**32**	≤0.25	≤1	1	26	31	31	34	34	34	(STR)
VN-3411	E-NS-sd	2010	1	≤2	0.03	>4	≤2	≤0.06	4	16	≤4	**32**	≤0.25	≤1	≤0.5	29	34	28	34	40	36	(STR)
VN-3418	E-NS-sd	2010	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	**32**	≤0.25	≤1	1	29	33	30	36	34	34	(STR)
VN-3426	E-NS-sd	2010	1	≤2	0.03	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	1	22	32	27	32	34	30	susceptible
VN-3442	E-NS-sd	2010	2	≤2	0.03	>4	≤2	≤0.06	2	8	≤4	**32**	≤0.25	≤1	≤0.5	26	32	28	32	34	32	(STR)
VN-3443	E-NS-sd	2010	1	≤2	0.06	>4	≤2	≤0.06	2	16	≤4	16	≤0.25	≤1	≤0.5	28	33	29	32	36	32	susceptible
VN-3444	E-NS-sd	2010	1	≤2	0.03	>4	≤2	≤0.06	2	8	≤4	16	≤0.25	≤1	≤0.5	26	30	29	32	36	32	susceptible
VN-3446	E-NS-sd	2010	1	≤2	0.03	>4	≤2	≤0.06	2	8	≤4	16	≤0.25	≤1	1	28	30	28	30	36	32	susceptible
VN-3454	E-NS-sd	2010	1	≤2	≤0.008	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	1	30	30	32	40	38	33	susceptible
VN-3457	E-NS-sd	2010	≤0.5	≤2	0.015	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	32	30	30	32	38	36	susceptible
VN-3465	E-NS-sd	2010	1	≤2	0.015	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	1	30	34	32	36	40	36	susceptible
VN-3467	E-NS-sd	2010	1	≤2	0.015	>4	≤2	≤0.06	2	8	8	16	≤0.25	≤1	1	28	32	32	38	40	36	susceptible
VN-3478	E-NS-sd	2010	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	16	≤0.25	≤1	1	29	32	32	34	36	34	susceptible
VN-3479	E-NS-sd	2010	1	≤2	≤0.008	>4	≤2	≤0.06	2	≤4	≤4	16	≤0.25	≤1	1	32	32	36	36	40	34	susceptible
VN-3498	E-NS-sd	2010	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	**32**	≤0.25	≤1	≤0.5	29	32	30	34	36	36	(STR)
VN-3500	E-NS-sd	2011	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	16	≤0.25	≤1	≤0.5	28	32	30	38	34	34	susceptible
VN-3367	E-NS-sw	2010	2	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	**32**	≤0.25	≤1	1	28	30	30	34	32	30	(STR)
VN-3368	E-NS-sw	2010	≤0.5	≤2	0.015	>4	≤2	≤0.06	0.5	≤4	≤4	8	≤0.25	≤1	1	50	50	50	50	50	50	susceptible
VN-3369	E-NS-sw	2010	1	≤2	0.03	>4	≤2	0.12	2	16	≤4	**32**	≤0.25	≤1	1	25	30	30	30	34	30	(STR)

Table continued

	Strain source code[b]	Year	Minimal inhibitory concentration (MIC) [mg/L]													Inhibition zone diameter [mm]						Resistance Profile[c]
			AMP R ≥32	CHL R ≥32	CIP R ≥4	CST R >2	FFN R ≥16	CTX R ≥4	GEN R ≥16	KAN R ≥64	NAL R ≥32	STR R ≥64	CAZ R ≥16	TET R ≥16	TMP R ≥16	AMC R ≤13	SXT R ≤10	FEP R ≤18	LVX R ≤13	MEM R ≤19	IPM R ≤19	
VN-3378	E-NS-sw	2010	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	**32**	≤0.25	≤1	1	26	30	30	30	38	32	(STR)
VN-3379	E-NS-sw	2010	1	≤2	0.015	>4	≤2	≤0.06	2	≤4	≤4	16	≤0.25	≤1	1	27	29	30	32	34	30	susceptible
VN-3398	E-NS-sw	2010	1	≤2	0.03	>4	≤2	≤0.06	2	16	≤4	**32**	≤0.25	≤1	2	28	31	28	34	34	32	(STR)
VN-3403	E-NS-sw	2010	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	16	≤0.25	≤1	1	26	32	32	36	36	32	susceptible
VN-3408	E-NS-sw	2010	1	≤2	0.015	>4	≤2	≤0.06	2	16	≤4	**32**	≤0.25	≤1	1	30	35	30	34	40	35	(STR)
VN-3410	E-NS-sw	2010	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	**32**	≤0.25	≤1	1	28	31	28	34	36	36	(STR)
VN-3412	E-NS-sw	2010	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	16	≤0.25	≤1	1	27	30	28	30	34	32	susceptible
VN-3415	E-NS-sw	2010	1	≤2	0.03	>4	≤2	≤0.06	1	8	≤4	**32**	≤0.25	≤1	2	30	34	32	34	40	36	(STR)
VN-3419	E-NS-sw	2010	1	≤2	0.03	>4	≤2	≤0.06	2	8	≤4	**32**	0.5	≤1	1	27	30	28	34	36	34	(STR)
VN-3448	E-NS-sw	2010	1	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	29	32	36	36	36	35	susceptible
VN-3451	E-NS-sw	2010	1	≤2	0.015	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	≤0.5	28	32	28	31	34	34	susceptible
VN-3461	E-NS-sw	2010	1	≤2	≤0.008	>4	≤2	≤0.06	2	8	≤4	**32**	≤0.25	≤1	≤0.5	30	34	34	40	38	34	(STR)
VN-3477	E-NS-sw	2010	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	16	≤0.25	≤1	1	26	30	28	34	36	34	susceptible
VN-3494	E-NS-sw	2010	1	≤2	0.015	>4	≤2	≤0.06	2	≤4	≤4	16	≤0.25	≤1	1	30	32	32	36	36	34	susceptible
VN-3496	E-NS-sw	2010	1	≤2	0.015	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	≤0.5	29	35	32	38	34	34	susceptible
VN-3506	E-NS-sw	2010	1	≤2	0.03	>4	≤2	≤0.06	2	16	≤4	**32**	≤0.25	≤1	1	30	30	30	36	36	36	(STR)
VN-2813	E-NS-sw	2011	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	**32**	≤0.25	≤1	1	28	34	34	38	36	34	(STR)
VN-2814	E-NS-sw	2011	1	≤2	0.015	>4	≤2	≤0.06	2	16	≤4	16	≤0.25	≤1	1	26	28	28	34	34	34	susceptible
VN-3518	E-NS-sw	2012	1	≤2	0.03	>4	≤2	≤0.06	2	8	≤4	16	≤0.25	≤1	≤0.5	28	30	28	34	34	32	susceptible
VN-3529	E-NS-sw	2012	1	≤2	0.03	>4	≤2	≤0.06	2	8	≤4	16	≤0.25	≤1	1	28	32	30	36	34	32	susceptible
VN-3533	E-NS-sw	2012	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	16	≤0.25	≤1	1	26	29	30	32	34	32	susceptible
VN-3536	E-NS-sw	2012	1	≤2	0.015	>4	≤2	≤0.06	2	8	≤4	**32**	≤0.25	≤1	1	28	34	32	36	40	36	(STR)
VN-3538	E-NS-sw	2012	1	≤2	0.03	>4	≤2	≤0.06	2	16	≤4	**32**	≤0.25	≤1	1	28	30	30	32	36	34	(STR)
VN-3539	E-NS-sw	2012	2	≤2	0.015	>4	≤2	≤0.06	4	8	≤4	**32**	≤0.25	≤1	≤0.5	26	30	30	34	34	30	(STR)
VN-3541	E-NS-sw	2012	1	≤2	0.015	>4	≤2	≤0.06	1	≤4	≤4	**32**	≤0.25	≤1	1	28	33	30	38	38	34	(STR)
VN-3542	E-NS-sw	2012	2	≤2	0.03	>4	≤2	0.12	2	8	≤4	**32**	≤0.25	≤1	1	27	30	26	34	34	34	(STR)

AMC, amoxicillin/clavulanic acid; AMP, ampicillin; CAZ, ceftazidime; CHL, chloramphenicol; CIP, ciprofloxacin; CST, colistin; CTX, cefotaxime; FEP, cefepime; FFN, florfenicol; GEN, gentamicin; IPM, imipenem; KAN, kanamycin; LVX, levofloxacin; MEM, meropenem; NAL, nalidixic acid; STR, streptomycin; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; TMP, trimethoprim.

[a] MIC values or inhibition zone diameters rated as resistant are shown in bolt and red, while those rated as intermediate resistant are shown in bolt.

[b] Strain source code is explained in Table 1.

[c] Resistance profile includes intermediate resistance shown in brackets.

Supplementary Table S2. Results of broth microdilution and disk diffusion assays of all *V. cholerae* isolates[a]

	Strain source code[b]	Year	Minimal inhibitory concentration (MIC) [mg/L]													Inhibition zone diameter [mm]						Resistance Profile[c]
			AMP R ≥32	CHL R ≥32	CIP R ≥4	CST R >2	FFN R ≥16	CTX R ≥4	GEN R ≥16	KAN R ≥64	NAL R ≥32	STR R ≥64	CAZ R ≥16	TET R ≥16	TMP R ≥16	AMC R ≤13	SXT R ≤10	FEP R ≤18	LVX R ≤13	MEM R ≤19	IPM R ≤19	
Clinical																						
VN-00297	C-G/A-ext	1995	2	≤2	≤0.008	>4	≤2	≤0.06	2	8	≤4	**32**	≤0.25	≤1	≤0.5	23	30	36	19	34	28	(STR)
VN-00298	C-G/A-ext	1995	2	≤2	≤0.008	>4	≤2	≤0.06	0.5	≤4	≤4	8	≤0.25	≤1	≤0.5	22	26	34	38	32	26	susceptible
VN-00301	C-G/A-ext	2000	2	≤2	≤0.008	≤2	≤2	≤0.06	1	≤4	≤4	**32**	≤0.25	≤1	1	28	34	40	36	36	36	(STR)
VN-00313	C-G/A-ext	2000	>32	≤2	≤0.008	>4	≤2	≤0.06	0.5	8	≤4	16	≤0.25	≤1	≤0.5	22.5	28	30	30	26	28	AMP
VN-00314	C-G/A-ext	2005	>32	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	20	27	30	36	24	25	AMP
VN-00168	C-G/A-ext	2010	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	1	19	25	32	36	24	26	susceptible
VN-00169	C-G/A-ext	2010	4	≤2	≤0.008	>4	≤2	≤0.06	0.5	≤4	≤4	16	≤0.25	≤1	1	18	22	30	36	26	26	susceptible
VN-00305	C-G/A-ext	2012	>32	≤2	≤0.008	>4	≤2	≤0.06	0.5	≤4	≤4	16	≤0.25	≤1	≤0.5	20	24	30	28	26	24	AMP
VN-00307	C-G/A-ext	2012	2	≤2	≤0.008	>4	≤2	≤0.06	0.5	≤4	≤4	8	≤0.25	≤1	≤0.5	20	26	26	34	24	26	susceptible
VN-00300	C-G/A-int	1999	4	≤2	≤0.008	>4	≤2	≤0.06	0.5	≤4	≤4	16	≤0.25	≤1	≤0.5	18	27	30	34	32	24	susceptible
VN-00302	C-G/A-int	2012	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	21	25	32	29	28	28	susceptible
VN-00303	C-ta-ext	2012	≤0.5	≤2	≤0.008	>4	≤2	≤0.06	1	8	≤4	**32**	≤0.25	≤1	≤0.5	27	25	34	34	30	28	(STR)
VN-00299	C-ta-int	1996	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	22	26	32	38	30	28	susceptible
VN-00210	C-ta-int	2011	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	18	25	28	34	24	26	susceptible
VN-00211	C-ta-int	2011	2	≤2	0.5	>4	≤2	≤0.06	1	≤4	>64	16	≤0.25	≤1	≤0.5	18	27	30	25	25	25	NAL
VN-00315	C-ta-int	2011	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	19	27	30	34	26	25	susceptible
VN-00304	C-ta-int	2012	2	≤2	0.5	>4	≤2	≤0.06	0.5	≤4	>64	16	≤0.25	≤1	≤0.5	22	25	30	24	30	26	NAL
VN-00308	C-ta-int	2012	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	1	18	25	28	36	24	26	susceptible
Environmental Baltic Sea																						
VN-03916	E-BS-sd	2011	4	≤2	≤0.008	>4	≤2	≤0.06	2	≤4	≤4	16	≤0.25	≤1	≤0.5	21	29	34	36	28	25	susceptible
VN-03939	E-BS-sd	2011	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	2	22	29	34	34	30	26	susceptible
VN-03940	E-BS-sd	2011	4	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	1	21	31	30	38	30	26	susceptible
VN-03955	E-BS-sd	2011	>32	≤2	≤0.008	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	1	18	26	28	30	26	24	AMP
VN-00167	E-BS-sw	2010	2	≤2	≤0.008	>4	≤2	≤0.06	0.5	≤4	≤4	16	≤0.25	≤1	≤0.5	18	25	30	34	26	24	susceptible
VN-00478	E-BS-sw	2010	2	≤2	≤0.008	>4	≤2	≤0.06	0.5	≤4	≤4	8	≤0.25	≤1	≤0.5	20	26	33	32	26	34	susceptible
VN-03963	E-BS-sw	2010	4	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	21	26	30	22	25	25	susceptible
VN-03967	E-BS-sw	2010	2	≤2	≤0.008	>4	≤2	≤0.06	0.5	≤4	≤4	16	≤0.25	≤1	≤0.5	20	26	30	30	26	25	susceptible
VN-00278	E-BS-sw	2011	2	≤2	≤0.008	>4	≤2	≤0.06	0.5	≤4	≤4	16	≤0.25	≤1	≤0.5	22	27	34	20	30	28	susceptible
VN-00455	E-BS-sw	2011	2	≤2	≤0.008	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	≤0.5	20	27	32	38	26	25	susceptible
VN-02995	E-BS-sw	2011	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	21	28	32	32	28	26	susceptible
VN-02997	E-BS-sw	2011	>32	≤2	≤0.008	>4	≤2	0.12	≤0.25	≤4	≤4	8	0.5	≤1	≤0.5	**14**	28	28	36	**20**	14	AMP, IPM (AMC, MEM)
VN-03903	E-BS-sw	2011	2	≤2	≤0.008	>4	≤2	≤0.06	2	≤4	≤4	16	≤0.25	≤1	1	21	27	32	34	29	25	susceptible
VN-03908	E-BS-sw	2011	4	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	1	20	29	30	34	30	24	susceptible
VN-03911	E-BS-sw	2011	2	≤2	≤0.008	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	1	22	27	34	40	30	27	susceptible
VN-03917	E-BS-sw	2011	4	≤2	≤0.008	>4	≤2	≤0.06	1	8	≤4	**32**	≤0.25	≤1	1	21	27	32	34	28	25	(STR)
VN-05169	E-BS-sw	2011	4	≤2	≤0.008	>4	≤2	≤0.06	2	≤4	≤4	**32**	≤0.25	≤1	1	19	26	26	30	26	26	(STR)

Table continued

	Strain source code[b]	Year	Minimal inhibitory concentration (MIC) [mg/L]													Inhibition zone diameter [mm]						Resistance Profile[c]
			AMP R ≥32	CHL R ≥32	CIP R ≥4	CST R >2	FFN R ≥16	CTX R ≥4	GEN R ≥16	KAN R ≥64	NAL R ≥32	STR R ≥64	CAZ R ≥16	TET R ≥16	TMP R ≥16	AMC R ≤13	SXT R ≤10	FEP R ≤18	LVX R ≤13	MEM R ≤19	IPM R ≤19	
VN-05170	E-BS-sw	2011	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	**32**	≤0.25	≤1	1	21	26	30	32	28	27	(STR)
VN-05171	E-BS-sw	2011	4	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	1	19	26	32	34	27	24	susceptible
VN-05172	E-BS-sw	2011	4	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	1	21	22	30	34	28	26	susceptible
VN-05173	E-BS-sw	2011	8	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	21	28	30	34	28	28	susceptible
VN-05174	E-BS-sw	2011	>32	≤2	≤0.008	>4	≤2	≤0.06	2	≤4	≤4	16	≤0.25	≤1	1	20	26	30	36	28	25	AMP
VN-05175	E-BS-sw	2011	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	21	27	30	36	30	28	susceptible
VN-05176	E-BS-sw	2011	4	≤2	≤0.008	>4	≤2	≤0.06	2	≤4	≤4	16	≤0.25	≤1	1	22	27	32	34	34	25	susceptible
VN-05177	E-BS-sw	2011	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	21	30	32	36	30	25	susceptible
VN-05178	E-BS-sw	2011	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	**32**	≤0.25	≤1	1	22	29	34	36	32	27	(STR)
VN-05183	E-BS-sw	2011	4	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	20	23	32	34	28	25	susceptible
VN-05184	E-BS-sw	2011	4	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	20	27	31	36	30	28	susceptible
VN-05185	E-BS-sw	2011	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	20	27	32	36	30	26	susceptible
VN-05187	E-BS-sw	2011	4	≤2	≤0.008	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	≤0.5	20	28	32	36	27	**22**	(IPM)
VN-00456	E-BS-sw	2012	2	≤2	≤0.008	>4	≤2	≤0.06	2	8	≤4	16	≤0.25	≤1	≤0.5	20	26	30	34	27	24	susceptible
VN-00457	E-BS-sw	2012	2	≤2	≤0.008	>4	≤2	≤0.06	0.5	≤4	≤4	16	≤0.25	≤1	≤0.5	24	27	32	30	30	25	susceptible
VN-00458	E-BS-sw	2012	4	≤2	≤0.008	>4	≤2	≤0.06	0.5	≤4	≤4	**32**	≤0.25	≤1	≤0.5	19	28	30	32	27	24	(STR)
VN-00459	E-BS-sw	2012	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	8	≤0.25	≤1	1	20	27	32	34	28	26	susceptible
VN-00460	E-BS-sw	2012	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	21	27	30	32	26	24	susceptible
VN-00461	E-BS-sw	2012	4	≤2	≤0.008	>4	≤2	≤0.06	0.5	≤4	≤4	16	≤0.25	≤1	≤0.5	20	26	30	30	26	23	susceptible
VN-00462	E-BS-sw	2012	4	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	21	28	30	32	28	24	susceptible
VN-05300	E-BS-sw	2012	2	≤2	≤0.008	>4	≤2	≤0.06	2	8	≤4	16	≤0.25	≤1	≤0.5	22	27	32	30	30	24	susceptible
VN-05301	E-BS-sw	2012	4	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	**32**	≤0.25	≤1	≤0.5	21	27	30	34	28	24	(STR)
VN-00463	E-BS-sw	2013	2	≤2	≤0.008	>4	≤2	≤0.06	2	≤4	≤4	16	≤0.25	≤1	1	20	27	32	30	28	28	susceptible
VN-00464	E-BS-sw	2013	4	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	21	27	32	34	28	24	susceptible
VN-00465	E-BS-sw	2013	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	**32**	≤0.25	≤1	≤0.5	21	26	30	32	28	24	(STR)
VN-00466	E-BS-sw	2013	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	21	27	32	32	30	26	susceptible
VN-00467	E-BS-sw	2013	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	1	20	25	30	30	26	24	susceptible
VN-00468	E-BS-sw	2013	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	1	20	27	30	30	28	24	susceptible
VN-00469	E-BS-sw	2013	4	≤2	≤0.008	>4	≤2	≤0.06	0.5	≤4	≤4	16	≤0.25	≤1	1	21	30	32	34	30	27	susceptible
VN-00470	E-BS-sw	2013	2	≤2	≤0.008	>4	≤2	≤0.06	0.5	≤4	≤4	16	≤0.25	≤1	1	20	27	28	30	27	23	susceptible
VN-00471	E-BS-sw	2013	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	8	≤0.25	≤1	≤0.5	20	28	30	34	28	28	susceptible
VN-00472	E-BS-sw	2013	2	≤2	≤0.008	>4	≤2	≤0.06	2	≤4	≤4	16	≤0.25	≤1	≤0.5	21	22	30	30	28	24	susceptible
VN-00473	E-BS-sw	2013	4	≤2	≤0.008	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	1	20	27	30	30	26	23	susceptible
VN-00474	E-BS-sw	2013	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	19	28	30	32	28	26	susceptible
VN-04241	E-BS-sw	2013	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	19	26	32	36	26	24	susceptible
VN-04247	E-BS-sw	2013	8	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	**32**	≤0.25	≤1	≤0.5	18	28	30	34	24	**20**	(STR, IPM)
VN-04250	E-BS-sw	2013	2	≤2	≤0.008	>4	≤2	≤0.06	2	8	≤4	16	≤0.25	≤1	1	20	25	32	30	28	26	susceptible
VN-04251	E-BS-sw	2013	4	≤2	≤0.008	>4	≤2	≤0.06	2	≤4	≤4	**32**	≤0.25	≤1	1	21	27	32	32	28	26	(STR)
VN-00475	E-BS-sw	2014	4	≤2	≤0.008	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	≤0.5	20	27	26	30	30	24	susceptible
VN-00476	E-BS-sw	2014	2	≤2	≤0.008	>4	≤2	≤0.06	0.5	8	≤4	16	≤0.25	≤1	1	38	26	30	30	26	23	susceptible

Table continued

	Strain source code[b]	Year	Minimal inhibitory concentration (MIC) [mg/L]													Inhibition zone diameter [mm]						Resistance Profile[c]
			AMP R ≥32	CHL R ≥32	CIP R ≥4	CST R >2	FFN R ≥16	CTX R ≥4	GEN R ≥16	KAN R ≥64	NAL R ≥32	STR R ≥64	CAZ R ≥16	TET R ≥16	TMP R ≥16	AMC R ≤13	SXT R ≤10	FEP R ≤18	LVX R ≤13	MEM R ≤19	IPM R ≤19	
VN-00477	E-BS-sw	2014	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	20	26	32	36	28	24	susceptible
VN-03901	E-BS-sw/sd	2011	2	≤2	≤0.008	>4	≤2	≤0.06	1	8	≤4	**32**	≤0.25	≤1	≤0.5	22	28	32	36	26	26	(STR)
VN-03902	E-BS-sw/sd	2011	2	≤2	≤0.008	>4	≤2	≤0.06	2	8	≤4	16	≤0.25	≤1	≤0.5	25	30	34	40	32	30	susceptible
VN-03907	E-BS-sw/sd	2011	2	≤2	≤0.008	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	≤0.5	21	28	34	36	30	26	susceptible
VN-03913	E-BS-sw/sd	2011	4	≤2	≤0.008	>4	≤2	≤0.06	2	≤4	≤4	**32**	≤0.25	≤1	1	21	29	34	36	28	26	(STR)
VN-03918	E-BS-sw/sd	2011	4	≤2	≤0.008	>4	≤2	≤0.06	2	≤4	≤4	**32**	≤0.25	≤1	1	20	26	34	38	28	26	(STR)
VN-03923	E-BS-sw/sd	2011	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	20	28	34	38	26	25	susceptible
VN-03936	E-BS-sw/sd	2011	4	≤2	≤0.008	>4	≤2	≤0.06	2	≤4	≤4	16	≤0.25	≤1	≤0.5	20	29	30	34	30	24	susceptible
VN-03938	E-BS-sw/sd	2011	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	1	21	28	30	34	32	26	susceptible
VN-03941	E-BS-sw/sd	2011	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	19	28	34	34	28	24	susceptible
VN-03942	E-BS-sw/sd	2011	**>32**	≤2	≤0.008	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	≤0.5	19	27	28	32	26	24	AMP
VN-03943	E-BS-sw/sd	2011	2	≤2	≤0.008	>4	≤2	≤0.06	1	8	≤4	**32**	≤0.25	≤1	1	21	27	30	32	26	25	(STR)
VN-03944	E-BS-sw/sd	2011	2	≤2	≤0.008	>4	≤2	≤0.06	1	8	≤4	**32**	≤0.25	≤1	≤0.5	22	28	28	30	26	25	(STR)
VN-03949	E-BS-sw/sd	2011	4	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	21	27	30	32	28	25	susceptible
VN-03950	E-BS-sw/sd	2011	4	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	21	25	30	32	26	24	susceptible
VN-03951	E-BS-sw/sd	2011	2	≤2	≤0.008	>4	≤2	≤0.06	0.5	≤4	≤4	8	≤0.25	≤1	≤0.5	25	27	34	38	30	29	susceptible
VN-03952	E-BS-sw/sd	2011	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	22	28	34	34	30	26	susceptible
VN-03953	E-BS-sw/sd	2011	2	≤2	≤0.008	>4	≤2	≤0.06	0.5	≤4	≤4	16	≤0.25	≤1	1	21	27	32	34	28	25	susceptible
VN-03954	E-BS-sw/sd	2011	**>32**	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	8	≤0.25	≤1	1	19	26	26	26	24	25	AMP
VN-03956	E-BS-sw/sd	2011	2	≤2	≤0.008	>4	≤2	≤0.06	2	≤4	≤4	**32**	≤0.25	≤1	1	22	25	32	32	28	26	(STR)
VN-03957	E-BS-sw/sd	2011	2	≤2	≤0.008	>4	≤2	≤0.06	0.5	≤4	≤4	8	≤0.25	≤1	1	28	29	34	36	28	26	susceptible
VN-03958	E-BS-sw/sd	2011	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	1	23	28	32	34	29	28	susceptible
Environmental North Sea																						
VN-10012	E-NS-bm	2011	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	18	27	30	34	24	26	susceptible
VN-10013	E-NS-bm	2011	2	≤2	≤0.008	>4	≤2	≤0.06	0.5	≤4	≤4	16	≤0.25	≤1	≤0.5	18	27	30	34	24	26	susceptible
VN-10127	E-NS-bm	2012	**>32**	≤2	≤0.008	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	≤0.5	21	28	32	30	26	25	AMP
VN-10130	E-NS-bm	2012	**>32**	≤2	≤0.008	>4	≤2	≤0.06	2	≤4	≤4	16	≤0.25	≤1	1	19	27	30	30	28	26	AMP
VN-10131	E-NS-bm	2012	**>32**	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	1	21	27	30	31	28	25	AMP
VN-10133	E-NS-bm	2012	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	21	28	32	36	30	28	susceptible
VN-10137	E-NS-bm	2012	4	≤2	≤0.008	>4	≤2	≤0.06	2	8	≤4	**32**	≤0.25	≤1	1	20	28	34	32	28	34	(STR)
VN-10143	E-NS-bm	2012	4	≤2	≤0.008	**4**	≤2	≤0.06	2	16	≤4	16	≤0.25	≤1	≤0.5	21	32	34	36	30	27	susceptible
VN-10144	E-NS-bm	2012	4	≤2	≤0.008	≤2	≤2	≤0.06	2	≤4	≤4	**32**	≤0.25	≤1	1	25	34	34	46	30	28	(STR)
VN-10145	E-NS-bm	2012	2	≤2	≤0.008	≤2	≤2	≤0.06	4	8	≤4	**32**	≤0.25	≤1	≤0.5	23	34	34	42	34	30	(STR)
VN-10146	E-NS-bm	2012	2	≤2	≤0.008	≤2	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	1	25	34	50	50	40	28	susceptible
VN-10150	E-NS-bm	2012	4	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	1	19	27	32	36	26	24	susceptible
VN-10156	E-NS-bm	2012	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	21	28	32	30	30	24	susceptible
VN-10159	E-NS-bm	2012	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	20	29	31	32	28	24	susceptible
VN-10162	E-NS-bm	2012	4	≤2	≤0.008	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	≤0.5	19	27	28	32	28	24	susceptible
VN-10191	E-NS-bm	2013	4	≤2	≤0.008	>4	≤2	≤0.06	4	8	≤4	**64**	≤0.25	≤1	≤0.5	20	28	31	36	28	25	STR
VN-10192	E-NS-bm	2013	4	≤2	≤0.008	>4	≤2	≤0.06	2	16	≤4	**64**	≤0.25	≤1	≤0.5	20	30	33	40	32	28	STR

Table continued

	Strain source code[b]	Year	Minimal inhibitory concentration (MIC) [mg/L]													Inhibition zone diameter [mm]						Resistance Profile[c]
			AMP R ≥32	CHL R ≥32	CIP R ≥4	CST R >2	FFN R ≥16	CTX R ≥4	GEN R ≥16	KAN R ≥64	NAL R ≥32	STR R ≥64	CAZ R ≥16	TET R ≥16	TMP R ≥16	AMC R ≤13	SXT R ≤10	FEP R ≤18	LVX R ≤13	MEM R ≤19	IPM R ≤19	
VN-10196	E-NS-bm	2013	2	≤2	0.06	>4	≤2	≤0.06	1	16	≤4	16	≤0.25	≤1	≤0.5	22	29	34	30	28	25	susceptible
VN-10197	E-NS-bm	2013	2	≤2	≤0.008	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	≤0.5	20	26	32	36	26	25	susceptible
VN-10198	E-NS-bm	2013	4	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	21	27	32	34	27	25	susceptible
VN-10204	E-NS-bm	2013	2	≤2	≤0.008	>4	≤2	≤0.06	0.5	≤4	≤4	16	≤0.25	≤1	≤0.5	24	28	34	36	30	28	susceptible
VN-10205	E-NS-bm	2013	2	≤2	≤0.008	>4	≤2	≤0.06	4	8	≤4	**32**	≤0.25	≤1	≤0.5	23	29	34	34	30	27	(STR)
VN-10206	E-NS-bm	2013	2	≤2	≤0.008	>4	≤2	≤0.06	1	8	≤4	8	≤0.25	≤1	≤0.5	23	29	34	36	30	28	susceptible
VN-10207	E-NS-bm	2013	2	≤2	≤0.008	>4	≤2	≤0.06	2	≤4	≤4	**32**	≤0.25	≤1	≤0.5	23	28	34	34	34	27	(STR)
VN-10208	E-NS-bm	2013	2	≤2	≤0.008	>4	≤2	≤0.06	2	≤4	≤4	16	≤0.25	≤1	1	23	29	32	36	30	29	susceptible
VN-10320	E-NS-bm	2014	2	≤2	0.06	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	≤0.5	24	29	36	30	32	28	susceptible
VN-02808	E-NS-sw	2011	>32	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	**32**	≤0.25	≤1	≤0.5	**15**	29	28	35	19	15	AMP, IPM, MEM (AMC, STR)
VN-02825	E-NS-sw	2011	>32	≤2	≤0.008	>4	≤2	≤0.06	0.5	≤4	≤4	8	0.5	≤1	≤0.5	**14**	30	26	34	**20**	15	AMP, IPM (AMC, MEM)
VN-02923	E-NS-sw	2011	>32	≤2	≤0.008	>4	≤2	≤0.06	0.5	≤4	≤4	16	≤0.25	≤1	≤0.5	**14**	30	28	26	**20**	16	AMP, IPM (AMC, MEM)
VN-04216	E-NS-sw	2013	2	≤2	≤0.008	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	≤0.5	21	27	31	32	28	26	susceptible
VN-04219	E-NS-sw	2013	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	22	28	28	34	32	26	susceptible
VN-04223	E-NS-sw	2013	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	21	28	32	32	30	28	susceptible
VN-04226	E-NS-sw	2013	>32	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	8	≤0.25	≤1	≤0.5	22	30	32	34	30	26	AMP
VN-04231	E-NS-sw	2013	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	23	30	32	36	32	30	susceptible
VN-04233	E-NS-sw	2013	8	≤2	≤0.008	>4	≤2	≤0.06	0.5	≤4	≤4	16	≤0.25	≤1	≤0.5	20	27	30	30	28	24	susceptible
VN-04261	E-NS-sw	2013	>32	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	**32**	≤0.25	≤1	≤0.5	20	28	30	34	28	25	AMP (STR)
VN-03213	E-NS-sw	2014	2	≤2	≤0.008	>4	≤2	≤0.06	2	16	≤4	**32**	≤0.25	≤1	1	18	28	30	34	28	25	(STR)
VN-03012	E-NS-sw	2009 - 2011	4	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	0.5	≤1	≤0.5	19	29	32	32	28	24	susceptible
VN-03503	E-NS-sw/sd	2009	2	≤2	≤0.008	>4	≤2	≤0.06	0.5	≤4	≤4	8	≤0.25	≤1	2	24	27	34	34	30	28	susceptible
VN-03301	E-NS-sw/sd	2010	4	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	19	32	32	34	26	25	susceptible
VN-03361	E-NS-sw/sd	2010	2	≤2	≤0.008	>4	≤2	≤0.06	1	8	≤4	**32**	≤0.25	≤1	≤0.5	20	27	30	34	26	26	(STR)
VN-03377	E-NS-sw/sd	2010	2	≤2	≤0.008	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	1	20	28	31	38	28	26	susceptible
VN-03405	E-NS-sw/sd	2010	4	≤2	≤0.008	>4	≤2	≤0.06	2	≤4	≤4	**32**	≤0.25	≤1	≤0.5	19	22	30	30	26	25	(STR)
VN-03407	E-NS-sw/sd	2010	4	≤2	≤0.008	>4	≤2	≤0.06	0.5	≤4	≤4	8	≤0.25	≤1	≤0.5	19	28	30	32	26	24	susceptible
VN-03428	E-NS-sw/sd	2010	1	≤2	≤0.008	>4	≤2	≤0.06	2	≤4	≤4	**32**	≤0.25	≤1	1	24	26	32	34	28	28	(STR)
VN-03460	E-NS-sw/sd	2010	4	≤2	≤0.008	>4	≤2	≤0.06	2	8	≤4	16	≤0.25	≤1	≤0.5	20	28	32	34	26	25	susceptible
VN-03469	E-NS-sw/sd	2010	4	≤2	≤0.008	>4	≤2	≤0.06	1	8	≤4	64	≤0.25	≤1	≤0.5	20	26	30	34	30	24	STR
VN-03470	E-NS-sw/sd	2010	4	≤2	≤0.008	>4	≤2	≤0.06	2	8	≤4	**32**	0.5	≤1	≤0.5	20	28	30	40	24	25	(STR)
VN-03471	E-NS-sw/sd	2010	2	≤2	≤0.008	>4	≤2	≤0.06	0.5	≤4	≤4	16	≤0.25	≤1	≤0.5	19	28	30	32	26	25	susceptible
VN-03472	E-NS-sw/sd	2010	4	≤2	≤0.008	>4	≤2	≤0.06	4	≤4	≤4	**32**	≤0.25	≤1	≤0.5	20	28	30	32	28	24	(STR)
VN-03475	E-NS-sw/sd	2010	4	≤2	≤0.008	>4	≤2	≤0.06	1	8	≤4	**32**	≤0.25	≤1	≤0.5	20	28	30	38	28	24	(STR)
VN-03492	E-NS-sw/sd	2011	4	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	**32**	≤0.25	≤1	≤0.5	20	28	32	36	26	25	(STR)

Table continued

	Strain source code[b]	Year	Minimal inhibitory concentration (MIC) [mg/L]													Inhibition zone diameter [mm]						Resistance Profile[c]
			AMP R ≥32	CHL R ≥32	CIP R ≥4	CST R >2	FFN R ≥16	CTX R ≥4	GEN R ≥16	KAN R ≥64	NAL R ≥32	STR R ≥64	CAZ R ≥16	TET R ≥16	TMP R ≥16	AMC R ≤13	SXT R ≤10	FEP R ≤18	LVX R ≤13	MEM R ≤19	IPM R ≤19	
Retail																						
VN-05008	R-G-bm	2009	4	≤2	≤0.008	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	1	21	28	32	32	30	26	susceptible
VN-05010	R-G-bm	2009	2	≤2	≤0.008	>4	≤2	≤0.06	1	16	≤4	16	≤0.25	≤1	1	22	26	32	33	28	24	susceptible
VN-00014	R-G-cr	2008	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	1	20	26	30	34	29	27	susceptible
VN-00015	R-G-cr	2008	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	19	25	30	38	24	26	susceptible
VN-00161	R-G-cr	2009	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	8	≤0.25	≤1	≤0.5	18	29	30	38	28	26	susceptible
VN-05066	R-G-cr	2011	2	≤2	≤0.008	>4	≤2	≤0.06	2	≤4	≤4	16	≤0.25	≤1	≤0.5	21	27	32	32	28	25	susceptible
VN-05095	R-G-cr	2011	2	≤2	≤0.008	>4	≤2	≤0.06	4	16	≤4	**64**	≤0.25	≤1	≤0.5	21	25	30	30	28	28	STR
VN-05096	R-G-cr	2011	2	≤2	≤0.008	4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	22	28	35	32	30	28	susceptible
VN-05102	R-G-cr	2011	2	≤2	0.06	>4	≤2	≤0.06	0.5	8	≤4	16	≤0.25	≤1	≤0.5	21	27	32	26	26	23	susceptible
VN-05109	R-G-cr	2011	**>32**	≤2	≤0.008	>4	≤2	≤0.06	2	≤4	≤4	**32**	≤0.25	≤1	≤0.5	19	27	28	34	28	24	AMP (STR)
VN-05221	R-G-cr	2011	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	20	27	32	36	27	25	susceptible
VN-05222	R-G-cr	2011	2	≤2	≤0.008	>4	≤2	≤0.06	1	8	≤4	**32**	≤0.25	≤1	≤0.5	19	26	30	32	27	23	(STR)
VN-05223	R-G-cr	2011	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	20	26	30	36	24	25	susceptible
VN-00434	R-G-cr	2012	2	≤2	≤0.008	>4	≤2	≤0.06	1	8	≤4	16	≤0.25	≤1	≤0.5	18	25	30	34	24	27	susceptible
VN-00435	R-G-cr	2012	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	1	21	25	30	34	30	24	susceptible
VN-00436	R-G-cr	2012	4	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	20	27	30	36	26	25	susceptible
VN-00437	R-G-cr	2012	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	22	28	32	36	30	25	susceptible
VN-00438	R-G-cr	2012	2	≤2	0.06	>4	≤2	≤0.06	1	≤4	16	16	≤0.25	≤1	1	23	27	32	30	27	25	susceptible
VN-00439	R-G-cr	2012	**>32**	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	1	21	27	32	30	26	26	AMP
VN-00440	R-G-cr	2012	4	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	22	29	28	34	28	26	susceptible
VN-00441	R-G-cr	2012	2	≤2	≤0.008	>4	≤2	≤0.06	2	8	≤4	16	≤0.25	≤1	≤0.5	21	28	32	34	28	25	susceptible
VN-00442	R-G-cr	2013	2	≤2	≤0.008	>4	≤2	≤0.06	2	≤4	≤4	16	≤0.25	≤1	≤0.5	21	29	30	36	28	25	susceptible
VN-05417	R-G-cr	2013	2	≤2	0.03	>4	≤2	≤0.06	0.5	≤4	≤4	16	≤0.25	≤1	≤0.5	22	28	32	28	28	28	susceptible
VN-00446	R-G-cr	2014	**>32**	≤2	≤0.008	>4	≤2	≤0.06	2	≤4	≤4	16	≤0.25	≤1	≤0.5	20	26	30	33	28	24	AMP
VN-00449	R-G-cr	2014	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	1	25	27	30	34	30	30	susceptible
VN-00451	R-G-cr	2014	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	21	29	32	34	26	25	susceptible
VN-00452	R-G-cr	2014	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	20	26	34	34	26	24	susceptible
VN-00454	R-G-cr	2014	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	22	28	34	34	28	25	susceptible
VN-00001	R-G-fi	2008	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	1	18	27	30	32	23	26	susceptible
VN-00432	R-G-fi	2012	≤0.5	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	**32**	≤0.25	≤1	≤0.5	24	27	34	38	26	30	(STR)
VN-00433	R-G-fi	2012	1	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	≤0.5	24	26	34	38	28	30	susceptible
VN-00443	R-G-fi	2013	**>32**	≤2	0.06	≤2	≤2	≤0.06	0.5	≤4	16	**32**	≤0.25	≤1	≤0.5	22	30	34	32	32	26	AMP (STR)
VN-00444	R-G-fi	2013	2	≤2	≤0.008	>4	≤2	≤0.06	2	≤4	≤4	16	≤0.25	≤1	≤0.5	21	28	34	36	28	25	susceptible
VN-00445	R-G-fi	2013	2	≤2	≤0.008	>4	≤2	≤0.06	1	≤4	≤4	16	≤0.25	≤1	1	20	26	30	35	28	24	susceptible
VN-00450	R-G-fi	2014	2	≤2	0.06	>4	≤2	≤0.06	0.5	≤4	≤4	16	≤0.25	≤1	**>32**	23	20	36	32	30	26	TMP

AMC, amoxicillin/clavulanic acid; AMP, ampicillin; CAZ, ceftazidime; CHL, chloramphenicol; CIP, ciprofloxacin; CST, colistin; CTX, cefotaxime; FEP, cefepime; FFN, florfenicol; GEN, gentamicin; IPM, imipenem; KAN, kanamycin; LVX, levofloxacin; MEM, meropenem; NAL, nalidixic acid; STR, streptomycin; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; TMP, trimethoprim.

[a] MIC values or inhibition zone diameters rated as resistant are shown in bolt and red, while those rated as intermediate resistant are shown in bolt.

[b] Strain source code is explained in Table 1.
[c] Resistance profile includes intermediate resistance shown in brackets.

Supplementary Table S3. Interpretive criteria used for broth microdilution and disk diffusion antimicrobial susceptibility testing of *Vibrio* spp.

Antimicrobial Subclass	Antimicrobial agent	Abbre-viation	Test range (mg/L)	MIC breakpoint (mg/L)			Disk content (µg)	Zone diameter breakpoint (mm)			Reference
				S	I	R		S	I	R	
β-Lactams											
Aminopenicillins	Ampicillin	AMP	0.5-32	≤8	16	≥32	10	≥17	14-16	≤13	M45-A2, (CLSI, 2010a)
Aminopenicillins/β-lactamase inhibitors	Amoxicillin/ Clavulanic Acid	AMC	-	≤8/4	16/8	≥32/16	20/10	≥18	14-17	≤13	M45-A2, (CLSI, 2010a)
Penicillinase-stable penicillins	Temocillin	TMC	0.5-128	≤ 8		>8	-	-	-	-	(Andrews, 2009)
3rd Gen. cephalosporins	Cefotaxime	CTX	0.06-4	≤1	2	≥4	30	≥26	23-25	≤22	M45-A2, (CLSI, 2010a)
	Ceftazidime	CAZ	0.25-16	≤4	8	≥16	30	≥21	18-20	≤17	M45-A2, (CLSI, 2010a)
4th Gen. cephalosporins	Cefepime	FEP	0.06-32	≤2	4-8[a]	≥16	30	≥25	19-24 [a]	≤18	M100-S25, (CLSI, 2015)
Cephamycins	Cefoxitin	FOX	0.5-64	≤8	16	≥32	30	≥18	15-17	≤14	M45-A2, (CLSI, 2010a)
Carbapenems	Ertapenem	ETP	0.015-2	≤0,5	1	≥2	10	≥22	19-21	≤18	M100-S25, (CLSI, 2015)
	Imipenem	IPM	0.12-16	≤1	2	≥4	10	≥23	20-22	≤19	M100-S25, (CLSI, 2015)
	Meropenem	MEM	-	≤1	2	≥4	10	≥23	20-22	≤19	M100-S25, (CLSI, 2015)
Monobactams	Aztreonam	ATM	-	≤4	8	≥16	30	≥21	18-20	≤17	M100-S25, (CLSI, 2015)
Non β-Lactams											
Phenicols	Florfenicol	FFN	2-64	≤4	8	≥16		-	-	-	Vet01-S2, *S. cholerasuis* (CLSI, 2013)
	Chloramphenicol	CHL	2-64	≤8	16	≥32	30	≥18	13-17	≤12	M45-A2, (CLSI, 2010a)
Quinolones	Nalidixic acid	NAL	4-64	≤16	-	≥32	30	≥19	14-18	≤13	M100-S25, (CLSI, 2015)
Fluoroquinolones	Ciprofloxacin	CIP	0.008-8	≤1	2	≥4	5	≥21	16-20	≤15	M45-A2, (CLSI, 2010a)
	Levofloxacin	LVX	-	≤2	4	≥8	5	≥17	14-16	≤13	M45-A2, (CLSI, 2010a)
Aminoglycosides	Gentamicin	GEN	0.25-32	≤4	8	≥16	10	≥15	13-14	≤12	M100-S25, (CLSI, 2015)
	Kanamycin	KAN	4-128	≤16	32	≥64	30	≥18	14-17	≤13	M100-S25, (CLSI, 2015)
	Streptomycin	STR	2-128	≤16	32	≥64	-	-	-	-	(National Food Institute, 2013;Shaw et al., 2014)
Tetracyclines	Tetracycline	TET	1-64	≤4	8	≥16	30	≥15	12-14	≤11	M45-A2, (CLSI, 2010a)
Folate pathway inhibitors	Trimethoprim	TMP	0.5-32	≤8		≥16	5	≥16	11-15	≤10	M100-S25, (CLSI, 2015)
	Sulfamethoxazole / Trimethoprim	SXT	-	≤2/38	-	≥4/76	1.25/23.75	≥16	11-15	≤10	M45-A2, (CLSI, 2010a)
Polymyxins	Colistin	CST	2-4	≤2		>2	-	-	-	-	(EUCAST, 2015)

[a] SDD (susceptible dose-dependent) Breakpoints shown are clinical breakpoints. Clinical breakpoints specific for *Vibrio* spp. described in CLSI document M45-A2 (CLSI, 2010a) derived from breakpoints specific for *Enterobacteriaceae* given in CLSI document M100-S20 (CLSI, 2010b). Subsequent to the approval of document M45-A2 in 2010 carbapenem-specific breakpoints described for *Enterobacteriaceae* were changed (CLSI, 2010c). Thus in case of carbapenems as well as in other cases where specific breakpoints for *Vibrio* spp. were obsolete or not available, latest

breakpoints for *Enterobacteriaceae* were used (CLSI, 2015). Other interpretive criteria were used for colistin (EUCAST clinical breakpoints for *Enterobacteriaceae* (http://www.eucast.org.) (EUCAST, 2015), temocillin (BSAC interpretive criteria for systemic infections) (Andrews, 2009) and streptomycin (based on different studies of *Vibrio* spp. (Shaw et al., 2014) and *E. coli* (National Food Institute, 2013), as no CLSI breakpoints were available.

Supplementary Table 4. Primers used for species identification, characterization and detection of resistance determinants

Gene/Target	Primer name	Sequence (5'to 3')	Amplicon (bp)	T_a (°C)	Reference
Species Identification and Characterization					
toxR	UtoxF	GASTTTGTTTGGCGYGARCAAGGT T		59/60	(Bauer and Roervik, 2007)
toxR of *V. cholerae*	VctoxR	GGTTAGCAACGATGCGTAAG	640	59/60	(Bauer and Roervik, 2007)
toxR of *V. vulnificus*	VvtoxR	AACGGAACTTAGACTCCGAC	435	60	(Bauer and Roervik, 2007)
O139 *rfb*	O139F	AGCCTCTTTATTACGGGTGG	449	59	(Hoshino et al., 1998)
O139 *rfb*	O139R	GTCAAACCCGATCGTAAAGG			(Hoshino et al., 1998)
O1 *rfb*	O1F	GTTTCACTGAACAGATGGG	192		(Hoshino et al., 1998)
O1 *rfb*	O1R	GGTCATCTGTAAGTACAAC			(Hoshino et al., 1998)
ctxA	ctxA1	CTCAGACGGGATTTGTTAGGCAC G	301		(Shirai et al., 1991)
ctxA	ctxA2	TCTATCTCTGTAGCCCCTATTACG			(Shirai et al., 1991)
Detection of Resistance Determinants					
Streptomycin Resistance Determinants					
strA	strA-F	TTGATGTGGTGTCCCGCAATGC	383	57	(Hochhut et al., 2001)
strA	strA-R	CCAATCGCAGATAGAAGGCAA			(Hochhut et al., 2001)
strB	strB-F	CCGCGATAGCTAGATCGCGTT	515	60.5	(Ramachandran et al., 2007)
strB	strB-R	CGACTACCAGGCGACCGAAAT			(Ramachandran et al., 2007)
aadA1-like	aadA1a-F	GTGGATGGCGGCCTGAAGCC	526	70	(Sandvang et al., 1997)
aadA1-like	aadA1a-B	ATTGCCCAGTCGGCAGCG			(Sandvang et al., 1997)
aadA2	aadA2-F	TGTTGGTTACTGTGGCCGTA	622	60	(Walker et al., 2001)
aadA2	aadA2-B	GATCTCGCCTTTCACAAAGC			(Walker et al., 2001)
rpsL of *V. cholerae*	Vc-rpsL-F[a]	GAATTTTGCGTCCCTATTTGTG	500	60	This study
rpsL of *V. cholerae*	Vc-rpsL-R[a]	GGCCTTACTTAACGCTTCTC			This study
rpsL of *V. vulnificus*	Vv-rpsL-F[a]	TTGCGTGGTTGGGGATTAG	480	60	This study
rpsL of *V. vulnificus*	Vv-rpsL-R[a]	AGTGTTTGGCCTTACTTAACG			This study

Table continued

Gene/Target	Primer name	Sequence (5'to 3')	Amplicon (bp)	T_a (ºC)	Reference
Class A Carbapenemases					
IMI1-3, NMC-A	IMI(NMC)-F1	TGCGGTCGATTGGAGATAAA	399	50	(Hong et al., 2012)
	IMI(NMC)-R1	CGATTCTTGAAGCTTCTGCG			(Hong et al., 2012)
SME1-3	SME-F1	ACTTTGATGGGAGGATTGGC	551		(Hong et al., 2012)
	SME-R1	ACGAATTCGAGCATCACCAG			(Hong et al., 2012)
NMC-A	NMC1	GCATTGATATACCTTTAGCAGAGA	2,158	50	(Radice et al., 2004)
	NMC4	CGGTGATAAAATCACACTGAGCATA			(Radice et al., 2004)
SME	IRS-5	AGATAGTAAATTTTATAG	1,138	50	(Queenan et al., 2000)
	IRS-6	CTCTAACGCTAATAG			(Queenan et al., 2000)
IMI	IMI-A	ATAGCCATCCTTGTTTAGCTC	818	50	(Aubron et al., 2005)
	IMI-B	TCTGCGATTACTTTATCCTC			(Aubron et al., 2005)
KPC 1-5	multi KPC-F	CATTCAAGGGCTTTCTTGCTGC	538	56	(Dallenne et al., 2010)
	multi KPC-R	ACGACGGCATAGTCATTTGC			(Dallenne et al., 2010)
Class B carbapenemases					
IMP variants except IMP-9, IMP-16, IMP-18, IMP-22 and IMP-25	multi IMP F	TTGACACTCCATTTACDG	139	56	(Dallenne et al., 2010)
	multi IMP R	GATYGAGAATTAAGCCACYCT			(Dallenne et al., 2010)
VIM variants including VIM-1 and VIM-2	multi VIM 1-2 F	GATGGTGTTTGGTCGCATA	390		(Dallenne et al., 2010)
	multi VIM 1-2 R	CGAATGCGCAGCACCAG			(Dallenne et al., 2010)
NDM-1	NDM-F	GGTTTGGCGATCTGGTTTTC	621	52	(Poirel et al., 2011)
	NDM-R	CGGAATGGCTCATCACGATC			(Poirel et al., 2011)
OXA-carbapenemases (class D)					
OXA-48-like	multi OXA-48 F	GCTTGATCGCCCTCGATT	281	56	(Dallenne et al., 2010)
	multi OXA-48 R	GATTTGCTCCGTGGCCGAAA			(Dallenne et al., 2010)

Table continued

Gene/Target	Primer name	Sequence (5'to 3')	Amplicon (bp)	T_a (ºC)	Reference
AmpC β-Lactamases					
ACC	ACC F	AACAGCCTCAGCAGCCGGTTA	346	64	(Perez-Perez and Hanson, 2002)
	ACC B	TTCGCCGCAATCATCCCTAGC			(Perez-Perez and Hanson, 2002)
LAT-1-4, CMY-2-7,	CIT F	TGGCCAGAACTGACAGGCAAA	462		(Perez-Perez and Hanson, 2002)
BIL-1,	CIT B	TTTCTCCTGAACGTGGCTGGC			(Perez-Perez and Hanson, 2002)
DHA-1, DHA-2	DHA F	AACTTTCACAGGTGTGCTGGGT	405		(Perez-Perez and Hanson, 2002)
	DHA B	CCGTACGCATACTGGCTTTGC			(Perez-Perez and Hanson, 2002)
MIR-1T, ACT-1	EBC F	TCGGTAAAGCCGATGTTGCGG	302		(Perez-Perez and Hanson, 2002)
	EBC B	CTTCCACTGCGGCTGCCAGTT			(Perez-Perez and Hanson, 2002)
FOX-1-5b	FOX F	AACATGGGGTATCAGGGAGATG	190		(Perez-Perez and Hanson, 2002)
	FOX B	CAAAGCGCGTAACCGGATTGG			(Perez-Perez and Hanson, 2002)
MOX-1, MOX-2, CMY-	MOX F	GCTGCTCAAGGAGCACAGGAT	520		(Perez-Perez and Hanson, 2002)
1,CMY-8-11	MOX B	CACATTGACATAGGTGTGGTG			(Perez-Perez and Hanson, 2002)
Other β-Lactamases					
bla$_{OXA\text{-}1like}$	OXA1-F	AGCAGCGCCAGTGCATCA	708	59	(Guerra et al., 2000)
bla$_{OXA\text{-}1like}$	OXA1-B	ATTCGACCCCAAGTTTCC			(Guerra et al., 2000)
bla$_{pse1}$	pse1-F	CGCTTCCCGTTAACAAGTAC	419	65	(Sandvang et al., 1997)
bla$_{pse1}$	pse1-B	CTGGTTCATTTCAGATAGCG			(Sandvang et al., 1997)
bla$_{tem1\ like}$	OT-1	TTGGGTGCACGAGTGGGT	503	55	(Arlet and Philippon, 1991)
bla$_{tem1\ like}$	OT-2	TAATTGTTGCCGGGAAGC			(Arlet and Philippon, 1991)
Integrons					
intI1, class 1 integron	Int1F	CCTGCACGGTTCGAATG	497	59	(Kitiyodom et al., 2010)
intI1, class 1 integron	Int1R	TCGTTTGTTCGCCCAGC			(Kitiyodom et al., 2010)

T_a annealing temperature;

[a] used for amplification and sequencing

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7.2 Publications and Congress Presentations

2015 **Bier N**., Jäckel C., Dieckmann R., Brennholt N., Böer S.I., and Strauch E. Virulence profiles of *Vibrio vulnificus* in German coastal waters, a comparison of North Sea and Baltic Sea isolates. International Journal of Environmental Research and Public Health. 12(12): 15943-15959. doi: 10.3390/ijerph121215031.

2015 **Bier N.,** Schwartz K., Guerra B., and Strauch E. Survey on antimicrobial resistance patterns in *Vibrio vulnificus* and *Vibrio cholerae* non-O1/non-O139 in Germany reveals carbapenemase-producing *Vibrio cholerae* in coastal waters. Frontiers in Microbiology. 6(1179). doi: 10.3389/fmicb.2015.01179.

2015 **Bier N**., Diescher S., and Strauch E. Multiplex PCR for detection of virulence markers of *Vibrio vulnificus*. Letters in Applied Microbiology, 60(5): 414-420. doi: 10.1111/lam.12394.

2014 **Speaker International conference *Vibrio 2014*** **Edinburgh, UK**

Bier N.: "Distribution of virulence-associated traits among *Vibrio vulnificus* isolates from the Baltic Sea region"

2014 Klevanskaa K., **Bier N**., Stingl K., Strauch E., and Hertwig S. PVv3, a new shuttle vector for gene expression in *Vibrio vulnificus*. Applied and Environmental Microbiology, 80(4): 1477-1481. doi:10.1128/AEM.03720-13

2014 Schirmeister F., Dieckmann R., Bechlars S., **Bier N**., Faruque S. M., and Strauch E. Genetic and phenotypic analysis of *Vibrio cholerae* non-O1, non-O139 isolated from German and Austrian patients. European Journal of Clinical Microbiology & Infectious Diseases, 33(5): 767-778. doi: 10.1007/s10096-013-2011-9.

2014 Huehn S., Eichhorn C., Urmersbach S., Breidenbach J., Bechlars S., **Bier N**., Alter T., Bartelt E., Frank C., Oberheitmann B., Gunzer F., Brennholt N., Böer S.I., Appel B., Dieckmann R., and Strauch E. Pathogenic vibrios in

environmental, seafood and clinical sources in Germany. International Journal of Medical Microbiology, 304: 843–850. doi: 10.1016/j.ijmm.2014.07.010.

2013 **Bier N**., Bechlars S., Diescher S., Klein F., Hauk G., Duty O., Strauch E., and Dieckmann R. Genotypic diversity and virulence characteristics of clinical and environmental *Vibrio vulnificus* isolates from the Baltic Sea region. Applied and Environmental Microbiology, 79(12): 3570–3581. doi: 10.1128/AEM.00477-13.

7.3 Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die hier vorgelegte Arbeit selbstständig verfasst habe und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet habe. Ich versichere, dass diese Arbeit in dieser oder anderer Form keiner anderen Prüfungsbehörde vorgelegt wurde.

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