Cloning and molecular characterization of a unique hemolysin gene of *Vibrio pommerensis* sp. nov.: development of a DNA probe for the detection of the hemolysin gene and its use in identification of related *Vibrio* spp. from the Baltic Sea

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Abstract

A group of hemolytic *Vibrio* strains was isolated from surface water of the Baltic Sea in 1995. A typical representative strain, CH-291, was found to lyse washed human and animal erythrocytes. Hemolysis was found to be calcium-dependent and occurred over a temperature range from 25 to 37°C. The hemolysin-encoding genes were identified by screening a genomic library of total DNA from strain CH-291. A cloned chromosomal DNA fragment of 15.6 kb conferred to *Escherichia coli* DH5α a hemolytic phenotype. Hybridization and sequence analysis showed the cloned sequence to be unique to these Baltic Sea *Vibrio* isolates and therefore provides a useful marker for their identification. Moreover, the cloned 15.6-kb DNA fragment possessed structural features typical for genetic islands, including a decreased GC content and a flanking cryptic insertion sequence element.

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1. Introduction

Bacteria of the genus *Vibrio* occurring in the Baltic Sea have been isolated and characterized for several years. Their significance is not only restricted to human infections, since pathogenic *Vibrio* species like *Vibrio anguillarum* or *Vibrio vulnificus* are able to cause wound or systemic infections with fatal outcomes in fish. During a survey to determine the presence of the human and fish pathogen *V. vulnificus* in the German Baltic Sea several atypical *Vibrio* strains, which seemed to be closely related, were isolated. These bacteria could not be classified by their biochemical properties or on the basis of their 16S rDNA sequences as belonging to any already described *Vibrio* species. The name *Vibrio pommerensis* sp. nov. was proposed for naming of the strains [1]. The strains showed a hemolytic phenotype on washed sheep blood agar plates [1]. A typical representative of *V. pommerensis* sp. nov. is strain CH-291 (DSM number 15800). Because hemolysins are important virulence factors and their corresponding genes are used for identification of certain *Vibrio* species [2,3], the present work describes the cloning and the characterization of a hemolysin-encoding region from the *V. pommerensis* sp. nov. strain CH-291 in an *Escherichia coli* K12 background. Moreover this region was used as a probe for differentiation of the *V. pommerensis* sp. nov. from several other *Vibrio* species in hybridization experiments.

2. Materials and methods

2.1. Bacterial strains

*Vibrio* and *Aeromonas* strains used in this study are listed in Table 1. *E. coli* strain DH5α® (BD Biosciences Clontech, Heidelberg, Germany) was used for cloning ex-
periments. Mutagenesis of the plasmid pH2 was carried out by using the transposon Tn1725 carried by the plasmid pRU669 of strain E1019 as described elsewhere [4]. Plasmid vectors pUC19 [5], and pBluescript II SK+ (Stratagene Europe, Amsterdam, The Netherlands) were used in cloning experiments.

2.2. DNA manipulations and sequence analysis

DNA manipulations (DNA extraction, DNA electrophoresis, restriction digestions, ligations) and transformations were performed using standard methods [5]. Restriction and DNA-modifying enzymes were obtained from New England Biolabs (NEB, Frankfurt am Main, Germany) or MBI Fermentas (MBI Fermentas, St. Leon-Rot, Germany). Hybridization experiments were carried out overnight at 65°C in 2×SSC buffer [5] containing 5% (w/v) dextran sulfate, 0.1% (w/v) sodium dodecyl sulfate and 0.5% (w/v) blocking reagent. 500 ng of target DNA was blotted onto Hybond N+ nylon membranes (Amersham Pharmacia Biotech Europe, Freiburg, Germany) for hybridization experiments. For the labeling of DNA probes and the detection of hybridization signals by chemoluminescence, the Renaissance kit (DuPont NEN, Boston, MA, USA) was used. Oligonucleotides used were purchased from Amersham Pharmacia Biotech Europe (Freiburg, Germany) and TibMolBiol (Berlin, Germany). DNA sequencing was conducted using the ABI Taq dye deoxy cycle sequencing kit and an ABI 373 DNA-sequencer (PE Applied Biosystems, Darmstadt, Germany).

Primer sequences for sequencing of insertion sites of Tn1725 were Tn1725/5P (TGGAAGAGCGGAAGTTTCGG) and Tn1725/3P (CCTTAGAAAGCCTAGTCAAAGAGC). Partial amplification of the vah1 gene from V. anguillarum was achieved with the primers VaHF (ACCGATGCCATCGCTCAAGA) and VaHR (GGATATTGACCGAGAGTCA). The positions of the primer binding sites are base pairs 945–964 and 1434–1417, respectively, in EMBL accession number S83534.

2.3. Genomic library of Vibrio sp. strain CH-291

Total DNA from CH-291 was partially digested with restriction endonuclease Sau3AI, size-fractionated (5–18 kb) in a sucrose gradient and dephosphorylated prior to ligation in plasmid pUC19 by standard protocols [5]. Constructs were transformed into E. coli K12 strain DH5α, plated onto ampicillin-containing washed sheep blood agar plates and incubated at 37°C for 36 h.

2.4. Determination of the GC content of strain CH-291

The GC content was estimated as already described [6].

2.5. Detection of hemolysis on washed blood agar plates

Washed blood agar plates were prepared as follows. Blood was washed with phosphate-buffered saline (PBS) three times and added to Columbia agar (BD Difco, Heidelberg, Germany) [7] to a final concentration of 5%. Fur-
thermore, CaCl₂ was added to a final concentration of 10 mM. Plates were incubated at 37°C. Hemolysis caused by the Vibrio strains was evaluated visually after an incubation time of 24 h, hemolysis caused by the E. coli strains after 36 h. Negative controls for the evaluation of hemolysis were Vibrio sp. CH-355 and E. coli K12 DH5α (pUC19).

2.6. Detection of hemolysis due to culture supernatant of Vibrio spp.

A volume of 970 μl sterile filtered supernatant of an overnight bacterial liquid culture was mixed with 10 μl of a 1 M CaCl₂ solution and 20 μl blood (normally from sheep, washed three times in PBS solution with a pH of 7.4) and incubated at 37°C for 20 min. Following centrifugation (800 × g for 10 min) the optical density at 540 nm of the supernatant was measured in a spectrophotometer. The negative control was the non-hemolytic Vibrio sp. strain CH-355. The value for complete lysis of erythrocytes (OD > 2) was obtained by using distilled water instead of culture supernatant. A value of more than 0.2 was interpreted as positive for hemolysis, since the negative controls showed values of 0–0.2.

2.7. Detection of cell wall-associated hemolysin of Vibrio spp.

Bacteria of 1 ml overnight culture were centrifuged (800 × g for 10 min), the supernatant discarded, the pellet was washed with 1 ml PBS solution (pH 7.4), and resuspended in 970 μl of a PBS solution (pH 7.4). The addition of CaCl₂ and blood etc. was carried out as stated above.

2.8. Detection of hemolysin in lysates of E. coli cells

The E. coli cell disruption of 5 ml overnight liquid culture (LB medium) was conducted in 12-ml tubes (Greiner) at 4°C using ultrasound with the sonicator Sonoplus HD 70 with the micro tip MS 73 (Bandelin Elektronik). The microtip was dipped 1.5 cm into the culture and the following parameters were applied: power 70%, cycle 40%, time 30 s. Following centrifugation (15000 × g for 10 min) 970 μl of the supernatant was used for detection of hemolysis as described above for the culture supernatants. The negative control was E. coli DH5α (pBluescript II SK⁺).

3. Results and discussion

3.1. Hemolysis caused by strain CH-291

Strain CH-291 produced zones of hemolysis on washed blood agar plates supplemented with either human, chicken, sheep, horse, cattle, codfish or plaice erythrocytes at a temperature range of 28–37°C with a zone of hemolysis of 1–5 mm after 24 h of incubation (see Fig. 1 for hemolysis of sheep erythrocytes). The hemolysis was CaCl₂-dependent since the absence of CaCl₂ prevented a clear hemolytic phenotype. Moreover, the strain exported hemolysins into the surrounding medium and showed membrane-associated hemolysis as well. There was no hemolytic activity of the culture supernatant after heating at 80°C for 10 min.

In order to characterize the hemolysin genes in strain CH-291 we investigated whether the strain harbors already described Vibrio-specific hemolysins. Because of the hemolytic phenotype only on washed blood agar plates, the heat sensitivity and the inability of the culture supernatant to affect Vero cells [1] we concluded the absence of a Tdh- or El Tor-related hemolysin [8]. The absence of the V. vulnificus hemolysin gene vvhA has been shown by earlier hybridization experiments [1]. Hybridization experiments using a polymerase chain reaction-generated 489-bp probe (using primers VaHF and VaHR) specific for the V. anguillarum hemolysin gene vah1 verified the absence of this gene in V. pommerensis sp. nov. as well (data not shown). These data show that the hemolysis-encoding genes from V. pommerensis sp. nov. might be different from those of related Vibrio species. In order to identify possible genetic markers for differentiation of the newly described V. pommerensis sp. nov. from closely related Vibrio species we decided to clone and characterize the hemolysis-encoding genes.

Fig. 1. Culture streak of the clones E. coli DH5α(pVH) (right) and E. coli DH5α(pUC19) (middle) as well as donor strain V. pommerensis sp. nov. CH-291 (left) on washed sheep blood agar plates.
Therefore we constructed a genomic library of DNA from strain CH-291 and screened the recombinant *E. coli* K12 DH5α transformants on washed blood agar dishes for colonies with a hemolytic phenotype in order to identify and characterize hemolysin-encoding genes in strain CH-291. The recipient strain *E. coli* DH5α does not show hemolysis, but harbors a cryptic cytolytin gene [9], which can be upregulated under certain conditions. However, the cryptic *E. coli* cytolytin works in a Ca²⁺-independent manner [10].

Out of about 10000 transformants screened, one clone harboring the plasmid pVH showed a clear hemolytic zone visible after 36 h of incubation (Fig. 1). Assuming a genome size of the *V. pommerensis* sp. nov. comparable to other *Vibrio* spp. ranging from 4 to 5 Mb [11], 10000 clones screened would result in a chance of >99% to represent a specific genomic sequence of the donor strain in the library [12]. *E. coli* DH5α (pVH) did not show hemolysis on conventional blood agar dishes, disproving an involvement of the cryptic *E. coli* K12 hemolysin, which is Ca²⁺-independent [10]. *E. coli* (pVH) was able to lyse erythrocytes from different animals like strain CH-291 with the exception of chicken erythrocytes which were not lysed. This could be explained by the existence of other hemolysins in CH-291, which were not detected in the genomic library.

Afterwards colinearity of the 15.6-kb recombinant DNA of plasmid pVH with the original donor DNA was verified using restriction fragment length polymorphism analysis (data not shown).

Since virulence factors like hemolysins can be plasmid-encoded [13], we investigated the localization of the cloned fragment in strain CH-291. Strain CH-291 contains a plasmid of a size of about 70 kb (pCH-291) [1]. We isolated plasmid pCH-291, restricted the DNA and performed a Southern blot using the labeled pVH as a probe. pCH-291 did not react with the probe and moreover did not share identical restriction fragments, indicating a chromosomal localization of the recombinant DNA of pVH in strain CH-291. This localization is not unusual, since hemolysins are more often located on chromosomes than on plasmids in *Vibrio* [8].

We then sequenced the recombinant DNA of the plasmid pVH. The sequence was deposited in the EMBL database (accession number AJ314791). Computer analysis identified 11 complete and one partial (orf 12) open reading frames with a size >300 bp (Fig. 2). A BLAST analysis [14] of the sequence obtained did not reveal any compelling similarities to known cytotoxins deposited in the database. Only orf 9 showed a slight similarity to a necrosis-inducing protein of *Bacillus halodurans* [15], which can be interpreted as a weak hint to the presence of a cell-damaging protein.

Because the results of the database search did not allow an identification of possible hemolysin genes on the cloned fragment of pVH, we further subcloned the fragment of

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**Fig. 2.** Recombinant DNA region of plasmid pVH with the generated subclones and their hemolysis-conferring properties after transformation into *E. coli* DH5α.
the plasmid pVH and tested the derived clones for hemolysis on washed blood agar plates in order to identify the hemolysin-encoding orfs. Additionally we disrupted the bacterial cells using ultrasound in order to release intracellular recombinant proteins and investigated their ability to lyse erythrocytes. The different subclones and their hemolytic features are depicted in Fig. 2. Supplemental information is provided in Table 2.

We identified two regions in the recombinant DNA of plasmid pVH, which conferred hemolysis on washed blood agar plates to recipient E. coli K12 strains. These regions were reflected by the partially overlapping plasmids pVHH and pVH2A. The plasmid pVHH conferred hemolysis only on washed blood agar plates, while the plasmid pVH2A conferred hemolysis to the cell lysate as well. A membrane-associated hemolysis as caused by clones harboring the plasmid pVHH was also reported for cloning of the hlx gene of Vibrio cholerae [16]. It was proposed that the recombinant hemolysin accumulated in the periplasmic space of the transformants. Overlapping plasmids pVH1H1 and pVH2P1 (Fig. 2), which do not harbor an intact orf 6, and did not cause hemolysis, indicated a possible role of this orf 6 as a hemolysin-encoding gene. Computer analysis of the deduced amino acid sequence revealed the presence of a lipoprotein binding site and a weak similarity to a putative E. coli outer membrane protein [17] arguing for a membrane-associated localization. Furthermore, the non-hemolytic properties of the cell lysate could be explained by this localization of the recombinant protein in the cell wall or the entrapment in the periplasmic space [5,18]. The centrifugation step after sonication could have eliminated the membrane-bound pro-

<table>
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<tr>
<th>Plasmid</th>
<th>Position of recombinant DNA in AJ314791</th>
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aValues represent the arithmetic average of two measurements at 540 nm.
bVector is pUC19; nd, not determined.
cVector is pBluescript II SK+.

Fig. 3. Dot-blot hybridization using the plasmid pVH as probe against DNA from several Vibrio and Aeromonas species. 1, V. navarrensis (CIP103381); 2, V. anguillarum (PT84057); 3, V. anguillarum (CH-350); 4, V. vulnificus (ATCC 27562); 5, V. vulnificus (ES71); 6, V. cincinnatiensis (CH-274); 7, V. mimicus (CH-386); 8, V. cholerae (CH-366); 9, V. cholerae (NIH 41); 10, V. cholerae (CH-174); 11, V. pomuerroris sp. nov. (CH-270); 12, V. pomuerroris sp. nov. (CH-271); 13, V. pommerensis sp. nov. (CH-280); 14, V. pomuerroris sp. nov. (CH-291); 15, V. cholerae (CH-355); 16, V. parahaemolyticus (CH-237); 17, V. metschnikowii (CH-1341); 18, V. furnissii (CH-1268); 19, V. alginolyticus (CH-1337); 20, V. fluvialis (CH-1253); 21, A. vibrio (CH-5701); 22, A. punctata (CH-5731).
tein encoded by orf 6 from the fraction used for the evaluation of the hemolysis. Another reason for the absence of a hemolytic activity of the lysate could be the formation of inclusion bodies of the hemolysin inside the cell, as reported by other authors [19]. Orf 6 encodes an 65-kDa protein with an estimated isoelectric point of 4.2. Other Vibrio-specific hemolysins have similar sizes [20–22]. In close proximity just upstream of orf 6 lies orf 5, whose deduced amino acid sequence shows compelling similarity to an outer membrane protein OmpA (REFSEQ accession number NP_231844) of V. cholerae [23]. The protein encoded by orf 5 could be involved in translocation of the hemolysin. For example, a hemolysin from Serratia marcescens has been reported to be dependent on an outer membrane protein for its translocation across the membrane, too. The gene encoding the outer membrane protein of S. marcescens is situated upstream of the hemolysin gene [19], also reflecting the hypothetical situation in strain CH-291. The exact role of orf 5 has to be evaluated in future experiments.

First experiments to identify the hemolysin-encoding gene of plasmid pVH2 (pVH2A) is a derivative of pVH2) by using transposon mutagenesis with Tn1725 did not allow us to determine the participation of single orfs in hemolysis. Tn1725 insertions were verified by EcoRI restriction (data not shown) and sequencing using the primers Tn1725/5’ and Tn1725/3’. Random mutagenesis destroyed orfs 8, 9, 11 and 12 (Fig. 2), but the cell lysates of the mutants were still hemolytic. The hemolytic phenotype on washed blood agar plates was reduced in all mutants in contrast to the clones bearing plasmid pVH2. A possible explanation for this may be the reduced copy number of the mutated plasmids because of the insertion of the 8-kb transposon. The mutagenesis experiments indicated that probably more than one orf of pVH2 contributed to the hemolysis caused by E. coli carrying pVH2. However, it is also possible that the hemolysins were encoded by genes which were not hit by the insertions of Tn1725. Further progress in the characterization of the region was achieved by additional subcloning experiments (Fig. 2). These experiments clearly showed the existence of two hemolysin-conferring regions on the plasmid pVH2A represented by the cloned fragments of plasmids pVH2H and pVH2E (Fig. 2). The existence of at least two hemolysin genes, which are located on an 8-kb cloned fragment, has been reported in other bacteria [24] and is therefore not unusual. The lysates of the clones bearing the plasmids pVH2H and pVH2E were hemolytic, while the intact bacteria did not show hemolysis on washed blood agar plates (Fig. 2, Table 2). The hemolytic phenotype conferred by pVH2A could be explained by a hemolytic effect of the two regions present on pVH2H and pVH2E (Table 2). Moreover, other proteins encoded on plasmid pVH2A and absent in single plasmids pVH2H or pVH2E could regulate or translocate hemolytic proteins. The BLAST search revealed an interesting orf (orf 9) on plasmid pVH2H, whose deduced amino acid sequence shows similarity to a necrosis-inducing protein of B. halodurans (60 of 244 amino acids are identical by BLASTX search). Orf 9 encodes a protein with a deduced molecular mass of 18 kDa and a theoretical isoelectric point of 8.1. The results of subcloning experiments and hemolysis assays pointed towards a role of this orf as a potential hemolysin gene.

We have not identified a potential hemolysin gene in plasmid pVH2E, since the small orfs within this plasmid have not been investigated. The smallest reported Vibrio hemolysin has a size of 92 amino acids [16], but other bacteria, like Staphylococcus aureus possess cytolysins of only 20–30 amino acids, the corresponding genes of which are very small [25]. In order to identify the responsible genes in plasmid pVH2E other mutagenesis methods directed against the small orfs have to be applied in order to characterize the recombinant DNA.

Another interesting feature of the recombinant DNA from plasmid pVH is the GC content, which varied considerably. After the first 8000 bp the GC content dropped from 50–60% to 25–35%. We determined the total GC content of strain CH-291 to be 50%. Moreover, orf 8, which is located at the border between the DNA regions with high and low GC content (Fig. 2), showed a weak similarity to a transposase of Xylella fastidiosa [26]. This orf seems to represent a cryptic insertion sequence (IS) element, because we have not detected an intact transposase gene and inverted repeats [27]. An integration of foreign DNA including the recombinant DNA of plasmid pVH2 via IS elements has probably taken place in the past. The hemolysin-conferring region thus has features of a genomic island. Other hemolysins of the genus Vibrio have also been reported to be flanked by IS elements [28]. The identification of the other end of the genomic island will be the focus of our future work.

3.2. Use of the hemolysin-conferring DNA region as a marker for the identification of the novel Vibrio pommerensis sp. nov.

The recently proposed species V. pommerensis sp. nov., which is represented by strain CH-291, seems to form a species [1] closely related to V. vulnificus and V. navarrensis. The occurrence of V. pommerensis sp. nov., as well as many other Vibrio in the Baltic Sea [1] necessitates the availability of markers for their identification and differentiation. In order to verify the applicability of the cloned hemolysis-conferring DNA region from CH-291 as a marker for this purpose, we used plasmid pVH as a probe and hybridized total DNA of different Vibrio and Aeromonas species against pVH (Fig. 3). The probe reacted only with V. pommerensis sp. nov. CH-270, CH-271, CH-280, and CH-291. Therefore, the recombinant DNA of plasmid pVH represents a suitable marker for differentiation and identification of V. pommerensis sp. nov.
References


