Structural Characterization of the Extracellular Polysaccharide from *Vibrio cholerae* O1 El-Tor

Fitnat Yildiz1, Jiunn Fong1, Irina Sadovskaya3, Thierry Grard3, Evgeny Vinogradov2*

1 Department of Microbiology and Environmental Toxicology, University of California Santa Cruz, Santa Cruz, California, United States of America, 2 National Research Council, Ottawa, Ontario, Canada, 3 Université du Littoral-Côte d’Opale, Boulogne-sur-mer, France

Abstract

The ability to form biofilms is important for environmental survival, transmission, and infectivity of *Vibrio cholerae*, the causative agent of cholera in humans. To form biofilms, *V. cholerae* produces an extracellular matrix composed of proteins, nucleic acids and a glycoconjugate, termed *Vibrio* exopolysaccharide (VPS). Here, we present the data on isolation and characterization of the polysaccharide part of the VPS (VPS-PS), which has the following structure:

\[
\alpha-D-Glc \text{ partially (}-20\%) \text{ replaced with } \alpha-D-GlcNac. \quad \alpha-GulNacAld is an amide between 2-acetamido-2-deoxy-\alpha-guluronic acid and glycine. Apparently, the polysaccharide is bound to a yet unidentified component, which gives it high viscosity and completely suppresses any NMR signals belonging to the sugar chains of the VPS. The only reliable method to remove this component at present is a treatment of the whole glycoconjugate with concentrated hydrochloric acid.
\]

Introduction

The facultative human pathogen *Vibrio cholerae* is responsible for cholera, a significant disease in developing countries and areas impacted by natural and man-made disasters [1,2]. *V. cholerae* is able to form biofilms - *i.e.*, matrix-enclosed surface-associated communities, which is critical for it environmental survival, transmission and infectivity [3,4]. In aquatic environments, *V. cholerae* forms biofilms on surfaces of phytoplankton, zooplankton, aquatic plants, crustaceans, and insects [5–9]. In addition, surface waters of cholera endemic areas harbor *V. cholerae* as clumps or biofilm-like aggregates, and removal of particles >20 μm in diameter from water can decrease cholera incidence by half [10,11]. It is well accepted that biofilm formation in aquatic ecosystems both enhances survival and persistence of *V. cholerae*, by providing nutrients and protection from protozoan grazing and phage predation [12], and that it plays a critical role in the transmission of the pathogen.

*V. cholerae* also forms biofilms while inside infected individuals. Stool samples from cholera patients contain *V. cholerae* both in biofilm-like clumps and in a planktonic form [4]. The average infectivity of biofilm-like clumps is higher than that of planktonic cells, and growth in biofilm induces a hyper-infectious phenotype, suggesting that *V. cholerae* biofilms are important in the disease process [4,13].

Biofilm formation depends on production of a biofilm matrix *i.e.*, exopolysaccharides, proteins, and nucleic acids [14]. In many bacteria exopolysaccharides represent a major portion of the biofilm matrix; and mutants unable to produce exopolysaccharides are impaired in biofilm formation [15]. For example, the opportunistic human pathogen Pseudomonas aeruginosa has the capacity to produce several polysaccharides that contribute to biofilm formation. One of them is alginate, produced by mucoid *P. aeruginosa* strains isolated from patients with cystic fibrosis. Alginate is a partially O-acetylated unbranched heteropolymer of β-1–4 linked D-mannuronic acid and L-guluronic acid residues. *P. aeruginosa* strains with enhanced alginate production form biofilms that have highly structured architecture, and are resistant to the antibiotic tobramycin [16]. Non-mucoid *P. aeruginosa* strains produce Psl and/or Pel. Psl polysaccharide is consist of a branched pentasaccharide repeating unit composed of D-mannose, D-glucose and L-rhamnose residues [17]. Pel is a glucose-rich polysaccharide of unknown structure [18]. Pel polysaccharide is involved in maintaining cell-cell interactions in biofilms, while PsI appears to be the primary structural polysaccharide for maintenance of mature biofilm architecture [19].

Polysaccharide intercellular adhesin (PIA) or polymeric N-acetyl-glucosamine (PNAG), consisting of partially N-deacetylated poly-1,6-β-GlcNac, plays critical roles in *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilm development [20], [21]. PIA-like polymers also serve as adhesins that stabilize biofilms of *E. coli* [22].

The importance of exopolysaccharides in biofilm formation has also been recognized in *Vibrio* spp. *Vibrio fischeri* uses symbiosis polysaccharide biofilms, SyP, to colonize its host, the squid Euprymna scolopes [23]. *V. parahaemolyticus* polysaccharide CPSA contains approximately equal amounts of fucose, galactose,
glucose and N-acetylglucosamine and is critical for biofilm formation [24]. Structures of exopolysaccharides produced by these Vibrio species remains to be determined.

One of the major components of *V. cholerae* biofilm matrix is *Vibrio* exopolysaccharide (VPS) [25,26]. The VPS biosynthesis genes are found in two regions on the large chromosome of *V. cholerae* O1 El Tor [vpsU (VC0916), vpsA-K, VC0917-27 (vps-I cluster); vpsL-Q, VC0934-9 (vps-II cluster)] [26]. Systematic deletion of vps genes and phenotypic analysis of vps mutants has revealed that most of the genes of the vps clusters are required for VPS biosynthesis and biofilm formation [26]. Recent studies using a rabbit ileal loop model system have revealed that VPS is required for *in vivo* biofilm formation [27]. Infant mouse colonization assays have also revealed that strains unable to produce VPS exhibit a defect in intestinal colonization compared to the wild-type [26]. Taken together, these studies highlight the importance of the biofilm growth mode and VPS production in both the intestinal and transmission phases of *V. cholerae*’s life cycle.

We previously reported composition and linkage analysis of the crude VPS preparations from rugose variant of *V. cholerae* O1 El Tor [25]. However, the precise chemical structure of VPS was unknown. We report here, for the first time, the chemical structure of the polysaccharide part of VPS, produced by the *V. cholerae* O1 El Tor rugose variant, A1552R, secreting high levels of VPS [25]. Characterization of the major structural component of *V. cholerae* biofilm matrix sets the stage for further structure-function analysis of the biofilm matrix and will facilitate identification of targets to combat this deadly pathogen.

**Materials and Methods**

**Bacterial Strains and Growth Conditions**

Cells of the *Vibrio cholerae* rugose variant, A1552R (FY_VC_2), which produces high level of VPS [26] were routinely grown in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl) at 30°C. To isolate biofilm matrix, overnight-grown cultures were spread on cellulose dialysis membranes placed on the surface of M9 agar plates (150×15 mm) supplemented with glucose (10 mM), casamino acids (0.5%) and MEM vitamins and incubated for 48 h at 30°C. Bacteria from these plates are harvested and suspended in phosphate-buffered saline (PBS). The samples are shaken on a rotary shaker at 4°C for 24 h. During this time, the biofilm matrix...
Main structure

\(-4\)-\(\alpha\)-L-Gulp\(\alpha\)Ac\(\alpha\)Gly\(\alpha\)3OAc\(-1\)-\(\beta\)-D-Glc\(\beta\)\(-1\)-\(\alpha\)-D-Glc\(\alpha\)\(-1\)-\(\alpha\)-D-Gal\(\beta\)\(-1\)-

Minor variant (~20%)

\(-4\)-\(\alpha\)-L-Gulp\(\alpha\)Ac\(\alpha\)Gly\(\alpha\)3OAc\(-1\)-\(\beta\)-D-Glc\(\beta\)\(-1\)-\(\alpha\)-D-Glc\(\alpha\)\(-1\)-\(\alpha\)-D-Gal\(\beta\)\(-1\)-

Smith degradation product OS1

\(\alpha\)-L-Gulp\(\alpha\)Ac\(\alpha\)Gly\(-1\)-\(\beta\)-Ery-ol-1d

\(\beta\)

\[\text{HOOCCH}_2\text{NH} \quad \text{O} = \text{CMe} \quad \text{O} \quad \text{HNCMe} \quad \text{O} \]

\(\alpha\)-L-Gulp\(\alpha\)Ac\(\alpha\)Gly\(\alpha\)3OAc

Figure 2. Structures of the VPS-PS and its derivative.

doi:10.1371/journal.pone.0086751.g002

Figure 3. \(^1\)H-\(^13\)C HSQC spectrum of the VPS-PS with \(^1\)H NMR trace.

doi:10.1371/journal.pone.0086751.g003
Preparation of the VPS 

Biological cells were collected by centrifugation (5,000g, 4°C, 45 min). The supernatant was clarified with additional centrifugation (8,000g, 4°C, 45 min) and dialyzed for 2 days against distilled water containing 0.02% sodium azide. We used cellulose tubing MEWO 12–14 kDa, with repeated change of the water to be clear and not very viscous solution, which was lyophilized to give VPS. The yield from the material after enzymatic treatment was about 50%.

Preparation of the VPS-PS 

VPS (50 mg) was dissolved in 37% HCL (2 mL) to give the clear solution a violet color. It was kept for 15 min at room temperature, poured into 100 mL of water and lyophilized. Dried material was washed with water; the precipitate was removed by centrifugation; and the solution passed through a Biogel P6 column to produce polymeric material. This contained VPS-PS and a small amount of the O-polysaccharide, which was removed by anion-exchange chromatography on Hitrap Q column in a gradient of NaCl from 0 to 1 M in 1 h. The VPS-PS was eluted as a broad peak over 10 min and collected in 3 parts, each of which had the same purity and structure. The yield of the VPS-PS from VPS was about 50–70%. A small amount of water-insoluble material was obtained, which we were able to partially dissolve in chloroform, with the rest soluble in DMSO.

General and Analytical Methods 

NMR experiments were carried out on a Varian INOVA 500 MHz (1H) spectrometer with 3 mm gradient probe at 25–50°C with acetone internal reference (2.225 ppm for 1H and 31.45 ppm for 13C), using standard pulse sequences gCOSY (gradient COrelation Spectroscopy), TOCSY (Total Correlation Spectroscopy) (mixing time 120 ms), NOESY (Nuclear Overhauser Effect Spectroscopy) (mixing time 300 ms), gHSQC (gradient Heteronuclear Single Quantum Coherence), and gHMBC (gradient Heteronuclear Multiple Bond Coherence) (100 ms long range transfer delay). AQ time was kept at 0.8–1 sec for H-H correlations and 0.25 sec for HSQC. 256 increments were acquired for t1 in all 2D spectra, except 512 for gCOSY.

Chromatography 

Gel chromatography was performed on a Sephadex G-15 column (1.5×60 cm) or a Biogel P6 column (2.5×60 cm) in pyrindine-acetic acid buffer (4 mL:10 mL:1 L water), and monitored by refractive index detector (Gibson). Anion exchange chromatography was done on an Hitrap Q column (2×5 mL size, Amersham), with UV monitoring at 220 nm in a linear gradient of NaCl (0–1 M, 1 h) at the 3 mL/min. Fractions of 0.25 mL were collected and additionally tested for carbohydrates, by spotting on an SiO2 TLC plate, dipping them in 5% H2SO4 in EtOH and heating with a heat-gun. All fractions of interest were dried in a Savant drying centrifuge and 1H spectra were recorded for each fraction without desalting. For 2D NMR, desalting was performed on a Sephadex G15 column.

Monosaccharide Analysis 

Samples with added inositol standard were hydrolyzed with 3 M TFA at 120°C. Monosaccharides were converted to alditol acetates by conventional methods and identified by GC-MS on a Trace GC Ultra apparatus, coupled with a DSQ II Mass spectrometer (Thermo Scientific), and equipped with a TR-5MS capillary column (30 m×0.25 mm ID×0.25 μm film) with helium carrier gas, using a temperature gradient 170°C (3 min), 250°C at 5°C·min⁻¹.

Methanolation 

A sample (<0.5 mg) in methanol (0.5 mL) was cooled in dry ice and 0.05 mL of acetyl chloride was added, the vial closed and heated at 90°C for 3 h, dried and acetylated with 0.3 mL Ac2O - 0.3 mL pyridine (30 min, 90°C), dried again using a stream of air, and analyzed by GC-MS on a Varian Saturn 2000 instrument (EI ion trap) on a DB-17 capillary column, at a temperature gradient of 170–270°C at 4°C/min.

Determination of Absolute Configurations of Monosaccharides 

To the polysaccharide sample (0.2 mg) (R)-2-BuOH (0.2 mL) and acetyl chloride (0.02 mL) were added at room temperature, heated at 90°C for 2 h, dried by air stream, acetylated, analyzed by GC-MS as described above. Standards were prepared from

| Table 1. 1H and 13C chemical shifts of the VPS-PS and its derivatives. |
|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
| H/C-1 | H/C-2 | H/C-3 | H/C-4 | H/C-5 | H/C-6 |
|---|---|---|---|---|---|
| α-Gal A | 5.19 | 3.91 | 3.93 | 4.06 | 3.77 | 3.77 | 3.77 | 3.77 | 3.77 | 3.77 |
| C | 97.9 | 69.6 | 70.3 | 80.2 | 72.5 | 61.4 |  |
| α-Gal A' | 5.20 | 3.91 | 3.98 | 4.01 | 3.77 | 3.77 | 3.77 | 3.77 | 3.77 | 3.77 |
| C | 99.5 | 69.6 | 70.0 | 78.8 | 72.5 | 61.4 |  |
| B | 5.11 | 4.68 | 5.21 | 4.27 | 5.08 |  |
| C | 98.5 | 46.1 | 68.2 | 71.3 | 68.6 | 172.2 |  |
| O-Deacylated B | 5.09 | 4.44 | 4.15 | 4.22 | 5.10 |  |
| C | 99.0 | 47.1 | 65.6 | 73.9 | 67.8 |  |
| α-Glc F | 4.93 | 3.95 | 3.93 | 3.76 | 4.28 | 3.84 | 3.91 |  |
| C | 99.6 | 55.4 | 70.3 | 80.6 | 72.2 | 61.4 |  |
| α-Glc D | 4.97 | 3.60 | 3.86 | 3.68 | 4.21 | 3.85 | 3.89 |  |
| C | 101.5 | 73.2 | 72.9 | 80.1 | 72.3 | 61.4 |  |
| β-Glc E | 4.53 | 3.35 | 3.66 | 3.64 | 3.70 | 3.87 |  |
| C | 103.8 | 75.4 | 76.1 | 78.1 | 76.7 | 61.5 |  |
| Gly | 3.94 | 4.10 |  |
| C | 175.5 | 43.0 |  |
| B, OS1 | 5.18 | 4.34 | 3.97 | 4.18 | 4.82 |  |
| C | 99.3 | 47.2 | 69.9 | 70.5 | 68.3 |  |
| Gly, OS1 | 3.91 | 3.99 |  |
| C | 43.4 |  |  |

Spectra of the polysaccharide were recorded at 500 MHz, at 30°C in D2O, OS1 at 25°C. Residue A* belongs to the structure with GlcNAc F, instead of Glc D. NAc signal in VPS-PS at 2.04/23.3 ppm, OAc at 2.19/22.0 ppm (N/C).

doi:10.1371/journal.pone.0086751.t001

separates from the cells and crude biofilm matrix was isolated and processed as described below.
monosaccharides of known configuration with (R)- and (S)-2-
BuOH.

Periodate Oxidation

VPS-PS (10 mg) was dissolved in water (2 mL) and NaIO₄ (20 mg) was added. This solution was kept for 24 h. Ethylene
glycol (0.2 mL) and the excess NaBD₄ were added, with the
solution kept for 1 h, treated with 0.2 mL of AcOH, and desalted
on a Sephadex G-15 column. The product was hydrolyzed with
2% AcOH 2 h at 100°C, and desalted on a Sephadex G-15
column to give OS1.

Results

Preparation and Properties of the VPS and its
Polysaccharide Part, VPS-PS

VPS was first identified and characterized in a rugose variant of
V. cholerae A1552R [25]. This rugose variant has enhanced
capacity to produce VPS and we have utilized A1552R to gain
a better understanding of the chemical structure of the VPS.

The Vibrio cholerae rugose variant and an isogenic strain lacking
vps gene clusters vps-I and vps-II (Δ-VPS) were grown in a
chemically defined medium which did not contain HMW
carbohydrates. The crude extracellular (EC) material was recov-
Figure 5. Mass spectra of acetylated methyl glycosides methyl esters of 2-aminouronic acids obtained by methanolysis from VPS-PS and ECA (contains ManNAcA).
doi:10.1371/journal.pone.0086751.g005

Figure 6. $^1$H NMR spectrum of the O-deacetylated VPS-PS.
doi:10.1371/journal.pone.0086751.g006
Figure 7. Positive mode MS-MS spectra of the VPS-PS (top) and O-deacylated VPS-PS (bottom).

doi:10.1371/journal.pone.0086751.g007
ered from the biofilm matrix. Aqueous solutions of the crude EC material of the rugose strain were very viscous, 100 mg of the material dissolved in 50 mL of water produced gel-like solution. The viscosity of a corresponding sample from the Δ-VPS strain was much less pronounced. Crude r-VPS had a higher carbohydrate/protein ratio than the corresponding Δ-VPS sample (~20 and ~2 μg carbohydrate per 1 μg of protein (in IgG/BSA equivalents), respectively). UV measurements (260 and 290 nm) of the non-dialyzable extracellular material indicated that the protein-nucleic acid content was about 30%. NMR spectra of the whole high-molecular mass extracellular material showed signals characteristic for proteins, lipids, and nucleic acids. No significant signals of the anomeric protons of sugars (4.5–6 ppm) were observed in 1H NMR spectra at neutral, acidic or basic pH (Fig. 1).

Monosaccharide analysis showed the presence of ~20% glucose and 10–15% galactose, as well as small amounts of LD-hepseose (indicating the presence of LPS), glucosamine, and perosamine. To remove nucleic acids and proteins, a dialyzed extracellular material was treated with DNase, RNase, and then Proteinase K. LPS was removed by ultracentrifugation, the transparent and colorless solution was dialyzed and hophylized, with the overall yield of the VPS at about 50 to 80% of the starting crude material.

The resulting VPS material showed no sugar signals in 1H NMR spectra in D$_2$O at temperatures up to 50°C (Fig. 1). NMR spectra of the whole high-molecular mass extracellular material contained a complex mixture of signals, but clearly did not include sugar anomeric signals. The DMSO solution was extremely viscous, and also showed no meaningful NMR signals.

Several methods (ultracentrifugation, gel chromatography, anion-exchange chromatography, mild acid or base hydrolysis, 48% HF, EDTA treatment) were tried in order to isolate the polysaccharide part of the VPS so that it could be used for further analysis, all with no success. Alkaline treatment produced a small amount of partially depolymerized VPS-PS (polysaccharide part of VPS), which was initially used for the structural analysis. Occasionally, it was found that treatment of the VPS with 37% HCl at room temperature for a short time releases a polysaccharide VPS-PS, suitable for NMR analysis.

VPS-PS was additionally purified by anion-exchange chromatography to remove minor impurities of the typical V. cholerae O1 LPS O-chain. VPS-PS was eluted along a NaCl gradient, resulting in several broad peaks. Three fractions were collected. Each of the fractions had identical NMR spectra, and probably differed in molecular mass. Monosaccharide analysis of the VPS-PS showed the presence of glucose, galactose, and a smaller amount of glucosamine. All these monosaccharides had a D-configuration, determined by GC of 2-butyldglycoside acetates.

VPS-PS structure was analyzed using 2D NMR spectroscopy. Four major monosaccharide spin-systems were identified in the spectra. Three of them belonged to α-gal, α-glc and β-glc (Figs. 1–3, Table 1). The fourth monosaccharide had an aminogroup at position 2 (C-2 signal at 46.1 ppm) and no H-6 protons. In HMBC spectrum its H-4 and H-5 showed correlations with a carbonyl carbon signal (Table 1), indicating that it was an uronic acid. Intraring vicinal proton coupling constants were all <3 Hz in this monosaccharide. High-field position of the C-2 signal together with all small proton couplings indicated that it was a 2-acetamido-2-deoxy-α-guluronic acid. There was no standard available for the direct identification of this monosaccharide, however, it was shown to be different from mannosaminouronic acid (from enterobacterial common antigen, ECA, [29]) and galactosaminouronic acid [from P. aeruginosa PA14 O-specific polysaccharide [29]] by GC of acetylated methanolysis products (Fig. 4). Mass spectra of the derivatives of all aminouronic acids were identical (Fig. 5).

The H-3 signal of the aminoguluronic acid residue B was shifted to the low-field (5.21 ppm), due to the O-acetylation at O-3. A minor variant of the non-O-acetylated residue B was also observed in the spectra. The O-acetyl signal was observed at 2.19/22.0 ppm (H/C).

The HSQC spectrum of the VPS-PS contained a set of non-sugar signals at 3.9–4.4 ppm (H/43.4 ppm (C), correlating to a carbonyl signal at 175.5 ppm. Position of these signals indicate a glycene spin system. Glycine was linked to GulNAcA by the amide linkage between GulNAcA C-6 and Gly N-2, which was confirmed by the observation of the HMBC correlation between Gly H-2 and GulNAcA C-6 at 172.2 ppm. The presence of an amide was also confirmed by the recording of the HSQC spectra at different pH (3, 5 and 9), which showed that GulNAcA signals were not sensitive to acidity and, consequently, its carbonyl group cannot ionize due to amiation.

The sequence of the monosaccharides was determined using NOESY data, where the following correlations were observed: A1:B4; B1:E3;4,5; D1:A4; E1:D4, indicating the sequence as shown on Fig. 2. The β-Glc residue E had overlapping protons 3,4,5; thus, NOE or HMBC could not be used to determine its substitution. Selection between possible substitution at O-3 or O-4 was based on the fact that a non-substituted C-4 signal of β-Glc should be around 70 ppm, and there was no such signal in the spectra. Consequently, β-Glc E was substituted at O-4.

The 1H NMR spectra of VPS-PS contained several small signals in the anemic region (Fig. 3). Also, several smaller O-and N-acetate signals were visible (not shown). Deacylation of the VPS-PS with ammonia (24% NH$_4$OH, 25°C, 16 h) led to the disappearance of nearly all minor signals (Fig. 6). However, some signals remained and were identified as belonging to a structural variant, in which α-Glc D was replaced by α-GlcNAc F. This variant constituted about 20% of the total structure.

Positive and negative mode ESI-MS analysis of the whole VPS-PS showed no peaks, indicating the polymeric nature of VPS-PS. In the high orifice voltage MS-MS spectrum of VPS-PS (Fig. 7) a major peak of HexNAcAGlyOAc at m/z 317.4 was observed. A series of higher mass signals was observed which corresponded to an addition to HexNAcAGlyOAc of a hexose, and then another hexose or N-acetylatedhexosamine (continuing with variants with Glc D or GlcNAc F), continuing with another hexose. The same sequence was observed in the MS-MS spectrum of the O-deacetylated VPS-PS, except that it lacked the O-acete (HexNAcAGly at m/z 275.4).

Periodate oxidation of the VPS-PS produced a glycoside OS1 (Fig. 2), containing α-GulNAcGly and erythrothol (NMR data in Table 1). O-Acetate was mostly lost during the treatment.

Assignment of the signals was confirmed by $^{13}$C spectra calculations [30], with α-D-GalNAcA or α-L-GalNAcA used, instead of α-GulNAcA. Chemical shifts for units A, D, and E showed good agreement with experimental data when α-L-GalNAcA was used, except for C-1 of α-Gal, which had an experimental chemical shift of 97.9 ppm and calculated chemical shift at 100.1 ppm (2.2 ppm deviation). With the use of α-D-GalNAcA for calculation this deviation increased to 3.6 ppm. Furthermore, the use of α-D-GalNAcA produced a large error for E-4, which was 2 ppm off. This points to a most probable L-configuration of GulNAcA, which would be also expected considering possible biosynthesis of L-GulNAcA from D-ManNAc by inversion of its C-5.

Cleavage of the VPS using concentrated HCl produced a minor amount of the precipitate, which was partially dissolved in
V. cholerae forms biofilms during aquatic and intestinal stages of its life cycles. Biofilms gain their structure and integrity, in part, from the exopolysaccharide VPS. We have previously isolated VPS, performed initial glycosyl composition and linkage analyses, and identified the gene clusters required for VPS biosynthesis. However, chemical structure of VPS has remained elusive due to difficulties in obtaining its NMR spectra. In this study, we report the chemical structure of VPS, and describe a reliable method for the isolation of VPS from the extracellular material produced by V. cholerae.

While V. cholerae biofilm matrix contains proteins, nucleic acids, lipids, and LPS, VPS-PS is clearly its main component, constituting more than 50% of the material by mass. Lipids released from VPS by HCl treatment were obtained in small amounts. VPS-PS showed no signals in the NMR spectra, until its release with HCl. What actually happens during HCl treatment remains unclear, as well as the nature of the non-carbohydrate part of the VPS and its bond to the VPS-PS. All fractions of size-exclusion chromatography separation of HCl-treatment products were tested by NMR and no components that could be released from the VPS-PS was found. VPS-PS spectra contained no visible signals of reducing monosaccharides, thus it was not significantly depolymerized. Although it seems a harsh treatment, concentrated HCl caused no observable degradation of the polysaccharide.

VPS-PS contains a novel monosaccharide, 2-acetamido-2-deoxy-L-guluronic acid, which is present in the polymer as its amide with glycine - another unusual component of natural polysaccharides. 2-Acetamido-2-deoxy-L-guluronic acid could be biosynthesized from 2-acetamido-2-deoxy-D-mannuronic acid by the enzymatic inversion of C-5 configuration, like it happens in the conversion of D-mannuronic acid into L-guluronic acid in alginate biosynthesis [31], or D-gluconic conversion to L-iduronic in heparin-like polymers [32]. Interestingly, one of the vps gene cluster genes (vpsB/VC0918) encodes a protein predicted to function as UDP-glucose/GDP-mannose dehydrogenase, which catalyzes formation of UDP-glucuronic acid or GDP-mannuronic acid. This is in good agreement with the presence of the acidic sugar GloNAcA in the VPS structure. Two genes involved in globiosynthesis operon - vpsC (VC0919) and vpsG (VC0923) - are predicted to encode serine acetyltansferase-related proteins that may be involved in VPS O-acetylation. The presence of GloNAc is consistent with our previous report showing that VPS could be stained with a Cy3-labeled wheat germ agglutinin, which recognizes GloNAc [33].

Different structures of capsular polysaccharides have been reported for two strains of V. cholerae: O139 ([34–36] and NRT368 [37]. Of these two examples, O139 CPS consists of the components of the LPS O-chain, but NRT368 CPS has some properties common with VPS-PS, in that it contains uronic acids and is O-acetylated.

Humans ingest V. cholerae biofilms as part of the pathogen’s normal transmission route. The presence of the VPS in these biofilms may influence progression of the disease, and/or development of an immune response against V. cholerae. Thus, characterization of the complete chemical structure of VPS and better understanding of VPS biosynthesis could lead to development of inhibitors of biofilm formation and, thus, reduce transmission of the pathogen.

Acknowledgments
Authors thank mass spectrometry group of NRC for obtaining mass spectra.

Author Contributions
Conceived and designed the experiments: EV FHY JF IS. Performed the experiments: EV JF IS. Analyzed the data: FHY EV JF IS. Contributed reagents/materials/analysis tools: TG. Wrote the paper: EV FHY JF IS.

References
1. Faruque SM, Albert MJ and Mkraliano, JJ. (1998) Epidemiology, genetics, and ecology of toxigenic Vibrio cholerae. Microbiology and molecular biology reviews : MMIR 62: 1301–1314.
2. Kaper JB, Morris JG, Jr., Levine MM (1995) Cholera. Clin Microbiol Rev 8: 48–86.
3. Alam M, Sultana M, Nair GB, Sack RB, Sack DA, et al. (2006) Toxicogenic Vibrio cholerae in the aquatic environment of Mathbaria, Bangladesh. Appl Environ Microbiol 72: 2849–2853.
4. Faruque SM, Biswas K, Uddin SM, Ahmad QS, Sack DA, et al. (2006) Transmissibility of cholera: in vivo-formed biofilms and their relationship to infectivity and persistence in the environment. Proc Natl Acad Sci U S A 103: 6350–6355.
5. Broza M, Gancz H, Halpern M, Kashi Y (2005) Adult non-biting midges, possible windborne carriers of Vibrio cholerae non-O1 non-O139. Environmental Microbiology 7: 576–585.
6. Halpern M, Broza-H, Mittler S, Azakasa E and Broza M (2004) Chironomid egg masses as a natural reservoir of Vibrio cholerae non-O1 and non-O139 in freshwater habitats. Microbial Ecology 47: 341–349.
7. Huq A, Colwell RR, Chowdhury MA, Xu B, Moniruzzaman SM, et al. (1995) Coexistence of Vibrio cholerae O1 and O139 in Bengal in plankton in Bangladesh. Lancet 345: 1249.
8. Huq A, Huq SA, Grimes DJ, O’Brien M, Chu KH, et al. (1986) Colonization of the gut of the blue crab (Callinectes sapidus) by Vibrio cholerae. Appl Environ Microbiol 52: 586–588.
9. Huq A, Small EB, West PA, Huq MI, Rahman R, et al. (1983) Ecological relationships between Vibrio cholerae and planktonic crustacean copepods. Appl Environ Microbiol 45: 275–280.
10. Huq A, Xu B, Chowdhury MA, Islam MS, Montilla R, et al. (1996) A simple filtration method to remove plankton-associated Vibrio cholerae in raw water supplies in developing countries. Appl Environ Microbiol 62: 2508–2512.
11. Colwell RR, Huq A, Islam MS, Aziz KM, Yunus M, et al. (2003) Reduction of cholera in Bangladesh villages by simple filtration. Proc Natl Acad Sci U S A 100: 1051–1055.
12. Matz C, McDougald D, Moreno AM, Yung PY, Yildiz FH, et al. (2005) Biofilm formation and phenotypic variation enhance predation-driven persistence of Vibrio cholerae. Proc Natl Acad Sci U S A 102: 16019–16024.
13. Tamayo R, Patimmalla B, Camilli A (2010) Growth in a biofilm induces a hyperinfectious phenotype in Vibrio cholerae. Infect Immun 78: 3560–3569.
14. Yildiz FH, Visack KL (2009) Vibrio biofilms: so much the same yet so different. Trends Microbiol 17: 109–119.
15. Fleming HC, Wingender J (2010) The biofilm matrix. Nature Reviews Microbiology 8: 623–633.
16. Heurteb M, Titzel GM, Balzer GJ, Heydorn A, Molin S, et al. (2001) Alginate synthesis in Vibrio cholerae O1: extracellular polysaccharide synthesis enzymes in Escherichia coli. J Bacteriol 183: 5395–5401.
17. Byrd MS, Sadowskayia I, Vinogradov E, Lu H, Sprinkle AB, et al. (2009) Genetic and biochemical analyses of the Pseudomonas aeruginosa Psl exopolysaccharide reveal overlapping roles for polysaccharide synthesis enzymes in Psl and LPS production. Mol Microbiol 73: 622–638.
18. Friedman L, Kolter R (2004) Two genetic loci produce distinct carbohydrate-rich structural components of the Pseudomonas aeruginosa biofilm matrix. J Bacteriol 186: 4457–4465.

19. Colvin KM, Gordon VD, Murakami K, Borlee BR, Wozniak DJ, et al. (2011) The pel polysaccharide can serve a structural and protective role in the biofilm matrix of Pseudomonas aeruginosa. PLoS pathogens 7: e1001264.

20. O’Gara JP (2007) ica and beyond: biofilm mechanisms and regulation in Staphylococcus epidermidis and Staphylococcus aurous. FEMS Microbiology Lett 270: 179–189.

21. Mack D, Fischer W, Krookotsh A, Lepold K, Hartmann R, et al. (1996) The intercellular adhesion involved in biofilm accumulation of Staphylococcus epidermidis is a linear beta-1,6-linked glucosaminoglycan: purification and structural analysis. J Bacteriol 178: 173–183.

22. Wang X, Preston JF, 3rd, Romans T (2004) The pgaABCD locus of Escherichia coli promotes the synthesis of a polysaccharide adhesin required for biofilm formation. J Bacteriol 186: 2724–2734.

23. Yip ES, Gravellini K, DeLoney-Marino CR, Visick KL (2006) The symbiosis regulator rscS controls the syp gene locus, biofilm formation and symbiotic aggregation by Vibrio fischeri. Mol Microbiol 62: 1596–1600.

24. Enos-Berlage JL, McCarter LL (2000) Relation of capsular polysaccharide production and colonial cell organization to colony morphology in Vibrio parahaemolyticus. J Bacteriol 182: 5513–5520.

25. Yildiz FH, Schoolnik GK (1999) Vibrio cholerae O1 El Tor: identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorene resistance, and biofilm formation. Proc Nail Acad Sci U S A 96: 4020–4033.

26. Fong JC, Syed KA, Klow KE, Yildiz FH (2010) Role of Vibrio polysaccharide production and colonial cell organization to colony morphology in Vibrio parahaemolyticus. J Bacteriol 182: 5513–5520.

27. Kamruzzaman M, Udden SM, Cameron DE, Calderwood SB, Nair GB, et al. (2010) Quorum-regulated biofilms enhance the development of conditionally viable, environmental Vibrio cholerae. Proc Nail Acad Sci U S A 107: 1580–1583.