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Bacteriophage 933W encodes a functional esterase downstream of the Shiga toxin 2a operon

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ABSTRACT

In this study, the 1938 bp open reading frame z1466, which is encoded directly downstream the Shiga toxin 2a (Stx2a) operon in *E. coli* O157:H7 phage 933W was cloned and expressed recombinantly. Purification with Ni-NTA agarose beads with subsequent SDS-PAGE revealed a 68 kDa protein, designated 933Wp42-His. Analysis of 933Wp42-His demonstrated an esterase activity by activity staining of native gels using triacetin as a substrate. Purified 933Wp42-His demonstrated a K_m value of about 10 mM and a V_{max} value of 1.667 nkat/ml for 4-methylumbelliferyl-acetate (4-MUF-Ac) as a substrate. The enzyme was most active in the pH-range of 7.0–8.0, and at 50 °C. Furthermore, 933Wp42-His was able to hydrolyze acetic acid from mucin, and 5-N-acetyl-9-O-acetyl neuraminic acid (Neu5,9Ac_2). This is the first description of an enzymatic activity of the Stx-phage-encoded protein 933Wp42. Its role in substrate utilization during colonization and human infection is discussed.

Introduction

The production of one or more Shiga toxins (Stx) is considered to be the major pathogenicity factor of enterohemorrhagic *Escherichia coli* (EHEC) (Karch et al., 2005). Besides the major groups, Stx1 and Stx2, a plethora of different Stx has been described, the nomenclature of which has recently been harmonized (Scheutz et al., 2012). In this recommendation, Shiga toxins are divided in types, subtypes and variants. According to this, Stx2 of *E. coli* O157:H7 strain EDL933 has been reclassified as Stx2a (Scheutz et al., 2012). The genetic information for Shiga toxin is generally located in the genome of lambdoid prophages (Allison, 2007; Unkmeir and Schmidt, 2000). Recently, it has been shown that induction of the 933W prophage is essential for developing renal disease in a mouse model (Tyler et al., 2013).

The position of *stx* genes in the prophage region between the late antiterminator gene Q and lysis gene S allows *stx* to be co-transcribed with the lytic phage cycle genes upon induction (Neely and Friedman, 1998). Whereas this region is about 500 bp in phage lambda, its size is about 5 kb in *E. coli* O157:H7 phage 933W and of similar size in other Stx-prophages (Plunkett et al., 1999; Unkmeir and Schmidt, 2000).

Detailed analysis of this region in Stx2-converting phage 933W shows that it contains genes for three rare tRNAs, the stx_{2a} operon, and a large open reading frame (orf) with a size of 1938 bp, which has been designated z1466 (L0105) (Plunkett et al., 1999). This orf encodes a putative 645 amino acid protein designated 933Wp42 (accession no. NP_049502). Interestingly, z1466 (L0105)-homologues genes are present in many Stx2-encoding phages but not frequently in Stx1-encoding phages (Unkmeir and Schmidt, 2000). In previous work, it could be shown, that upon induction with norfloxacin, the whole *stx*-region, including the z1466 gene, is strongly expressed in prophage 933W (Herold et al., 2005). Moreover, a more than 40-fold expression of the corresponding protein 933Wp42 could be shown under aerobic growth conditions in simulated colon environmental medium (Polzin et al., 2013). The 645 amino acid protein 933Wp42 is homologous to NanS and YjhS of *Escherichia coli*, which are encoded in the bacterial chromosome (Plunkett et al., 1999). Interestingly, *nanS* is only

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981 bp in size, whereas the length of z1466 is 1938 bp (Plunkett et al., 1999; Blattner et al., 1997). The *yjhs* gene of *E. coli* belongs to an operon, which is involved in the catabolism of sialic acids (Steenbergen et al., 2009). NanS was characterized intensively and it has been shown that it deacetylates the 9 position of 9-O-sialic acid (Rangarajan et al., 2011). Sialic acids constitute a family of more than 40 naturally occurring 9-carbon keto sugar acids, occurring in a number of living organisms including pathogenic bacteria (Lamari and Karamanos, 2002; Vimr et al., 2004; Vimr, 2013). Most commensals and pathogens are able to use sialic acid esters as a carbon source or sialic acid may be involved in host cell signaling (Vimr et al., 2004). It could be shown that sialic acids are present in the human intestinal tract building a gradient from the ileum to the rectum in different amounts. Therefore, esterases may be very useful for growth, especially when particular sialic acids can be catabolized and enable the pathogens to grow under adverse environmental conditions with limiting amounts of carbon sources.

The aim of this study was to clone and express the z1466 gene of phage 933W and to characterize the corresponding protein 933Wp42 biochemically.

Materials and methods

Bacterial strains, plasmids, and growth conditions

E. coli strain BL21(DE3) [F–*ompT hsdSB*(rB^-, mB^-) *gal dcm* (DE3)] (Studier and Moffatt, 1986), was used as a host for the multicopy His6-Tag fusion vector pET-22b(+) (Novagen®). The latter contains a T7 promoter in its multiple cloning site as well as an ampicillin resistance gene. *E. coli* C600/933W harbors the Shiga toxin-encoding phage 933W (O'Brien et al., 1984) and was used as a source for the amplification of z1466.

Strains were routinely cultivated in Luria-Bertani (LB) broth composed of 10 g/l Bacto™ tryptone, 5 g/l yeast extract, and 10 g/l NaCl, pH 7.5 under aeration at 30 °C or 37 °C with 180 rpm unless otherwise mentioned. For solid media, the broth was mixed with 1.5% (w/v) Bacto™ agar. Ampicillin and kanamycin were added when needed in final concentrations of 100 μg/ml and 50 μg/ml, respectively. SOC-Medium contained 20 g/l Bacto™ tryptone, 5 g/l yeast extract, 0.5 g/l NaCl and 2.5 mM KCl, pH 7. After autoclaving, 5 mM $MgCl_2$, 5 mM $MgSO_4$, and 20 mM glucose (sterile filtrated) were added.

Cloning, over expression and purification of 933Wp42

The open reading frame of z1466 of prophage 933W in laboratory strain *E. coli* C600/933W (Perna et al., 2001; O'Brien et al., 1984) was amplified by PCR with primers p42-NdeI-for (5′-ccc CAT ATG GCA TTT AAA CAC TAT G-3′) and p42-XhoI-rev (5′-ggg CTC GAG TGC CGC AGT GTC TGT GCT G-3′). Underlined letters indicate NdeI and XhoI recognition sites, respectively. The resulting PCR product was digested with NdeI and XhoI, and then ligated with plasmid pET-22b(+) (Novagen®, Merck), digested with the same enzymes. The resulting recombinant plasmid was designated as pET-z1466-his. It was then transformed into electrocompetent *E. coli* BL21(DE3) by standard protocols (Studier and Moffatt, 1986). Twenty-five ml LB broth containing 100 μg/ml ampicillin were inoculated with a single colony of *E. coli* BL21(DE3)/pET-z1466-his. The culture was incubated in a rotary shaker with 180 rounds per minute (rpm) overnight at 37 °C. Bacterial cells were harvested by centrifugation at 4,000 × *g* at room temperature for 10 min. The cell pellet was resuspended in 25 ml LB broth and diluted 1:100 in 200 ml LB broth supplemented with 100 μg/ml ampicillin. The culture was grown at 37 °C with 180 rpm until an OD_{600} of 0.6 was achieved and then induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Roth, Germany). After that treatment, the culture was incubated at 37 °C with 180 rpm for 4 h. *E. coli* BL21(DE3)/pET-22b(+) served as a negative control. Cells were harvested by centrifugation at 4,000 × *g* at 4 °C for 10 min, resuspended in His-lysis-buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8) and sonicated with a Branson Sonifier with a microtip for 6 × 10 s on ice. The cell lysate was centrifuged at 25,000 × *g* for 20 min at 4 °C. Purification of the His-tagged protein 933Wp42-His was carried out by nickel affinity chromatography with Ni-nitrilotriacetic acid (NTA) agarose beads according to the manufacturers' recommendation (Qiagen GmbH, Germany). After washing with wash-buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8) the bound protein was eluted in three consecutive steps using 2 ml, 1 ml and 0.5 ml 250 mM imidazole in the same buffer. Crude extracts and purified proteins were assayed by SDS-PAGE and stained with Coomassie brilliant blue R-250. The protein concentration was determined with a Bradford protein assay using bovine serum albumin (Roth, Germany) as a standard. Diluted protein solutions (2.5 μg) were subjected to gel electrophoresis on a 12% SDS-PAGE gel and blotted onto a Whatman®, Protran nitrocellulose transfer membrane (GE-Healthcare, Germany) at 100 mA for 1 h. After being blocked in TBS-buffer containing 0.1% (w/v) Tween 20 and 5% (w/v) skimmed milk at room temperature for 1 h, the membranes were incubated for 1 h at room temperature with an anti-mouse-His_6 antibody (GE Healthcare, Munich, Germany) (1:3000), and a goat-anti-mouse-lgG HRP antibody (Santa Cruz, Heidelberg, Germany) (1:5000), respectively. Antibody–antigen complexes were treated with ECL-solution (0.1 M Tris/HCl, pH 9, 1.25 mM Luminole, 0.2 mM p-Coumaric acid, H_2O_2) and chemiluminescence was detected with a Molecular Imager®, ChemiDoc™ XRS+ System (Bio-Rad Laboratories GmbH, Munich, Germany).

DNA sequencing

Double-stranded DNA sequence analysis of recombinant z1466 in plasmid pET-22b(+) was performed in a Beckman CEQ 8000 capillary sequencer (Beckman Coulter, Germany) using 10 nucleotide primers targeting all regions of the gene and the junctions to the vector as described earlier (Creuzburg et al., 2011).

Native Polyacrylamide Gel Electrophoresis (PAGE) and activity staining

Native PAGE and activity staining was conducted following the protocols of Singh et al. (2006). Briefly, chromogenic substrate plates were prepared by using phenol red (0.01%) along with 1% (w/v) triacetin (Sigma–Aldrich, Germany) as a substrate, 2% (w/v) agarose and some drops Triton X-100. The pH-value was adjusted to 7.3–7.4 with 1 M NaOH. Native PAGE was performed using a Mini-PROTEAN 3 chamber and a Mini-PROTEAN TGX™ any kD™ gel (Bio-Rad Laboratories GmbH, Munich, Germany). After running the PAGE, the gel was rinsed three times with distilled water and equilibrated in 50 mM Tris/HCl (pH 7.8) for 30 min at room temperature. The gel was overlaid with the molten chromogenic substrate (at 40 °C), which was then allowed to solidify and incubated at room temperature. Depending on esterase activity, a yellow band over a pink colored background should be observed within 15 min.

Standard esterase activity assay and substrate specificity

Esterase activity was investigated by measuring hydrolysis of 4-methylumbelliferyl acetate (4-MUF-Ac) (Sigma–Aldrich, Germany). The reaction was carried out in a final volume of 290 μl. The standard reaction mixture contained 176 μl Tris/HCl buffer (50 mM, pH 7.0), 40 μl enzyme preparation and 24 μl substrate solution. Enzymatic hydrolysis was initiated after preincubation at