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Promotion of the Synthesis of a Concanavalin A-reactive Polysaccharide Upon Growth of *Escherichia coli* O157:H(-) on Solid Medium at 37°C

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Abstract

The polysaccharides exposed at the bacterial surface are involved in many processes related with the interaction of microorganisms with their environments, outside or inside the mammalian host. The aim of this study was to test the effects of growth temperatures (20oC and 37oC) on some characteristics of the surface polysaccharides of E. coli O157:H(-). The glycoconjugates were isolated by hot phenol-water extraction. The tests included ELISA, ELLA, immunoelectrophoresis (classical, or modified for application with concanavalin A /ConA/) and Western blot. It was shown that the water-phase fraction of the strain cultivated at 37oC was distinctive among the compared samples. It was poorly recognized by specific anti-E. coli O157 antiserum but, contrary to all the other fractions, interacted well with the lectin, ConA. A similar ConA-reactivity was demonstrated for the purified cyclic enterobacterial common antigen (ECACYC4). This implies that the ConA reactivity may likely be due to ECACYC. The promotion of the release of this ConA-reactive entity at 37oC, i.e., the temperature condition characteristic for host body, indicates its importance in the host-invader interplay.

Key words: *E. coli* O157, polysaccharides, ConA, ECA_{CYC}

Резюме

Полизахаридите, локализирани по клетъчната повърхност на бактериите, играят роля при множество процеси, свързани с взаимодействието между микробите и обкръжаващата ги среда извън или вътре в техния гостоприемник. Целта на изследването е да се изясни ефектът на растежната температура (20°C и 37°C) върху някои характеристики на повърхностните полизахариди на *E. coli* O157:H(-). Изолирането се осъществи чрез екстракция с воден разтвор на фенол при 60°C. Проведени са тестове ELISA, ELLA, имуноелектрофореза (класическа или модифицирана за работа с конканавалин А /ConA/) и имуноблот. Показано е, че фракцията от водна фаза от щама, култивиран при 37°C, се отличава съществено от останалите проби. Тя се разпознава много слабо от специфичен анти- *E. coli* O157 антисерум, но за разлика от всички останали фракции, се свързва с лектина КонА. Подобна КонА-реактивност се демонстрира и за проба от пречистен цикличен ентеробактериален общ антиген (ECA_{CYC4}). Това сочи, че КонА-реактивността на фракцията е възможно да с дължи на ECA_{CYC}. Стимулираното освобождаване на КонА-реактивните молекули при 37°C, т.е., температурата, характерна за тялото на гостоприемника, сочи тяхната важност при взаимоотношението бактерии-гостоприемник.

Introduction

Complex oligo- and polysaccharides located at the surfaces of bacterial, plant and animal cells can serve structural roles, mediate movement of glycoconjugates to the cell surface, act as markers that mediate cell-cell and cell-matrix recognition (Slifkin and Doyle, 1990), etc. In Gram-negative bacteria, the cell surface glycome is represented by lipopolysaccharides (LPS), capsules (where available) and exopolysaccharides, although gly-

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coproteins may also contribute. In addition, the Enterobacteriaceae produce a surface polysaccharide known as "enterobacterial common antigen" (ECA) (Erbel *et al*., 2003). The synthesis of these polysaccharides are regulated in response to environmental clues (Merino *et al*., 1992, Lerouge and Vanderleyden, 2001), hence the heterogeneity and dynamics of the bacterial glycome presents a major challenge for analysts.

The present study examines polysaccharides isolated from *Escherichia coli* O157:H(-). Strains with this O-serotype are often the agents of foodborne infections that may sometimes cause severe, even lethal complications (Kaper *et al*., 2004). The bacterial cell-surface polysaccharides are important participants in both the host-invader interplay and the survival of the bacteria in the environment (Greenfield and Whitfield, 2012). The transmission of *E. coli* O157:H(-) via its niches outside and inside the human body is accompanied with the respective temperature shifts. The question is: are these environmental shifts accompanied by changes in the glycome associated with the bacterial surface?

The present study combines the application of immunological approaches and glycan-binding proteins (lectins) to address the question whether the changes in cultivation temperature can influence the cell surface-associated glycome of the strain. The bacterial antigens were isolated by the most-widely applied method for LPS isolation, the method of Westphal and Jann (1965). It is based on the surface polysaccharide extraction with water phenol solution at 60°C followed by centrifugation upon which water and a phenol phases form. For most Gram-negative strains, the LPS is further isolated from the water-phase fraction. Unlike this, the O157 antigen is isolated from the phenol phase (Perry *et al*., 1986; Vinogradov *et al*., 1998). To our knowledge, water-phase polysaccharides of *E. coli* O157 have been examined earlier in two studies (Dodds *et al*., 1987 a, b). Using the immunoblot technique, the authors have shown cross-reactivity of these samples with heterologous antisera from *Brucella abortus*, *Yersinia enterocolitica* O9 and *Vibrio cholera*e. Later on, the water, but not the phenol phase, of the water-phenol extract has been shown to contain two cyclic forms of ECA, a tetramer and a pentamer (Fregolino *et al*., 2012). Bearing in mind that the growth temperature effects (if any) might be registered in either the water-, or the phenol-phase polysaccharides, or both, we compared these fractions isolated from bacteria grown at 20°C and 37°C.

Materials and Methods

Strain and cultivation

E. coli O157:H-, A2CK SS was used in the study. It is a $Stx1^-$, $Stx2^-$ strain. Overnight nutrient broth culture of the strain was used as inoculum. The bacteria were cultivated on nutrient agar for 24 h at 37°C or 48 h at 20°C.

Polysaccharides isolation

The method of Westphal and Jann (1965) was applied for isolation of the crude antigen fractions. The phenol-water extraction was performed for 1 h at 60oC with continuous stirring. The samples were centrifuged, the water and phenol phases were separated, dialysed extensively against running tap water for 4 days, and freeze-dried. ECACYC4 was purified as described earlier (Fregolino *et al.*, 2012). The polysaccharides were dissolved as 2 mg/ml stocks in double distilled, deionized water and kept frozen until use.

Reagents

Polyclonal rabbit diagnostic serum: anti-*E. coli* O157 (BulBio-NCIPD, Sofia, Bulgaria) was used as primary antibody. Anti-rabbit IgG-peroxidase (Sigma) was used as a secondary antibody. The following lectins were included: lectin from *Canavalia ensiformis* (concanavalin A, or ConA), lectin from *Triticum vulgaris*, or wheat-germ agglutinin (WGA), soybean agglutinin (SBA) *Ulex europaeus* agglutinin-I (UEA-I). Lectin-biotin and lectin-peroxidase conjugates were purchased from Vector Labs. Unlabeled ConA (Pharmacia) was applied for immunoelectrophoresis experiments. Avidin -peroxidase (Sigma) was used.

Dot blot

For dot-blot experiments, 1 mg/ml solutions of the polysaccharide samples were prepared in 0.2 M TBS (0.2 M Tris and 0.15 M NaCl), pH 7.3 with two further decimal dilutions. Samples of 5 µl were applied on nitrocellulose disks, allowed to dry, and the procedure was repeated so that the final amount of carbohydrate loaded to the disks was 10, 1, and 0.1 µg, respectively. Blocking of non-specific binding was by incubation for 1 hour in 5% BSA (Calbiochem). The disks were further incubated in ConA-peroxidase (1:100 in TBS containing 0.2 mM $CaCl_2$), or SBA-peroxidase, WGA-peroxidase and UEA-I-peroxidase (1:100 in TBS). Incubation in lectins was for 2 hours, followed by three washes in 0.05% Tween 20 in TBS, two washes in TBS, and one in distilled water. Controls included processing of disks devoid of antigen, or incubation in TBS instead of lectin. The peroxidase activity was demonstrated by short incubation in a mixture of

Na nitroprusside (0.4 g) and *o*-dianizidine dihydrochloride (0.04 g) in 100 ml of water to which 50 µl of 30% H_2O_2 was added.

Enzyme -linked immunosorbent assay (ELISA) and enzyme-linked lectin assay (ELLA)

For ELISA experiments, the aqueous stock solution of the polysaccharide samples was dissolved 1:10 in carbonate buffer, pH 9.6. The wells of 96-well flat-bottom microtitre plates were filled with 50 µl of the diluted antigen (final antigen amount of 5 µg) and loading was performed overnight at 4oC. Followed blocking with 2% BSA in PBS for 2 h at 37oC. Further protocols differed depending on the binding to be checked. To test the reactivity with the immune sera, 100 µl of serially diluted serum was applied to the wells and incubated for 1 h at 37oC. As a secondary antibody, peroxidase-conjugated anti-rabbit IgG was applied. To check for binding of the lectin ConA, ELLA test was applied. The wells were blocked with 5% BSA in PBS. Various amounts of ConA-biotin diluted in PBS containing 0.2 mM CaCl2 were applied onto the wells and incubated for 2 h at room temperature. After thorough washes followed incubation in avidin-peroxidase for 1 h. In both protocols controls with the omission of each of the labelling molecules were included. The ELLA test included also inhibition control in the presence of 0.2 M methyl mannoside. For both ELISA and ELLA experiments, the peroxidase activity was determined using a substrate solution of o-phenylene diamine and H2O2. The measurements were performed on a plate reader at 492 nm.

Immunoelectrophoresis

Immunoelectrophoresis was performed on 2% agarose gel in veronal buffer, pH 8.6, with the addition of 0.2 mM CaCl2 for the test with ConA. The polysaccharide stock solutions were applied into the pits and electrophoresis was done for 1 h at 120 V. Then throughs were cut into the gel and filled with either rabbit anti-E. coli O157 serum (1:100 in PBS), or 1 mg/ml ConA (Calbiochem), dissolved in TBS containing 0.2 mM CaCl2. The gel was placed in wet chamber and diffusion proceeded for 24 hours at room temperature. After an overnight wash in 0.9% NaCl, the gel was dried and coloured for 15 min in Coomasie brilliant blue R 250.

Wesrtern blot

Western blot was performed on a BioRad mini-gel equipment. Initially, SDS-PAGE electrophoresis of the samples was done on 12% separating acrylamide gel for 1 h at 180 V. Followed transfer onto nitrocellulose membrane for 1 h at 180 V. Blocking of non-specific binding was by incubation for 2 h in 1% BSA in PBS at room temperature. After extensive washes in PBS/BSA/0.01% Tween 20, the membrane was incubated overnight in rabbit anti-*E. coli* O157 serum (1:100 in PBS) at room temperature with continuous shaking. After three washes, followed 2 h incubation in the secondary antibody (anti-rabbit IgG-peroxidase, 1:400). Demonstration of the peroxidase activity was as in dot-blot.

Results

The dot-blot test applied with lectins showed positive reactivity only with ConA. This was registered for the water-phase fraction isolated from 37°C culture, as polysaccharide dose-dependent change in color intensity (data not shown). The other samples were negative. No reactivity with the other lectins was found.

The immunoreactivities of the fractions were examined by three approaches: ELISA, immunoelectrophoresis, and Western blot (Fig. 1). The three tests confirmed differences between the samples. Notably, the water-phase preparation of the bacteria cultivated at 37°C was characterized throughout by a low affinity for the polyclonal anti-*E. coli* O157 serum.

Bearing in mind the results from the dot-blot experiment, the further comparison between the fractions was performed with ConA. By both ELLA and immunoelectrophoresis, the water-phase fraction isolated from bacteria grown at 37°C was distinctive by its affinity for the lectin (Fig. 2). Upon immunoelectrophoresis, this sample produced two distinct precipitation bands with Con A. Some lower affinity for the lectin was registered for the water-phase fraction isolated from the strain grown at 20°C.

We have previously shown that ECA_{CYC} purified from the water phase phenol-water extract of the strain was non-reactive with anti-*E. coli* O157 specific antiserum, but reacted well with the C-type, mannan-binding lectin (Paunova-Krasteva *et al*., 2014). Therefore, the next question we tried to answer here was whether the purified ECA_{CYC4} could react with the here tested C-type lectin, ConA. The ELLA test performed with purified ECA_{CYC4} showed a well-expressed ConA-binding affinity (Fig. 3) which implies a similarity of this purified antigen with the water-phase crude fraction, 37°C (Fig. 2A).

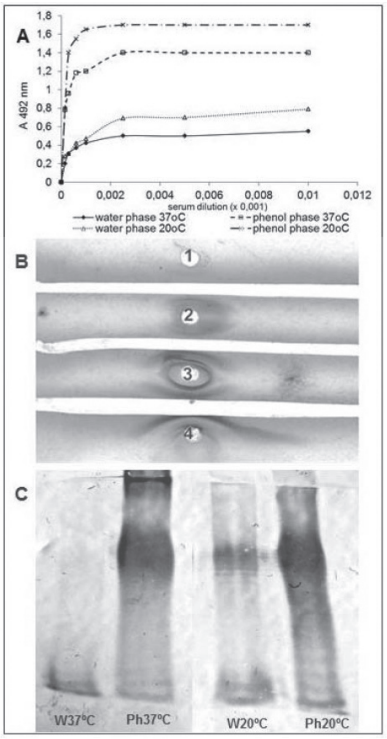

Fig. 1. Comparison of the reactivity of the water- and phenol-phase polysaccharides with polyclonal diagnostic rabbit anti-E. coli O157 serum. A, ELISA; B, immunoelectrophoresis: (1) water phase polysaccharide, 37°C, (2) water phase polysaccharide, 20°C, (3) phenol phase polysaccharide, 37°C, (4) phenol phase polysaccharide, 20°C; C, Western blot of the fractions.

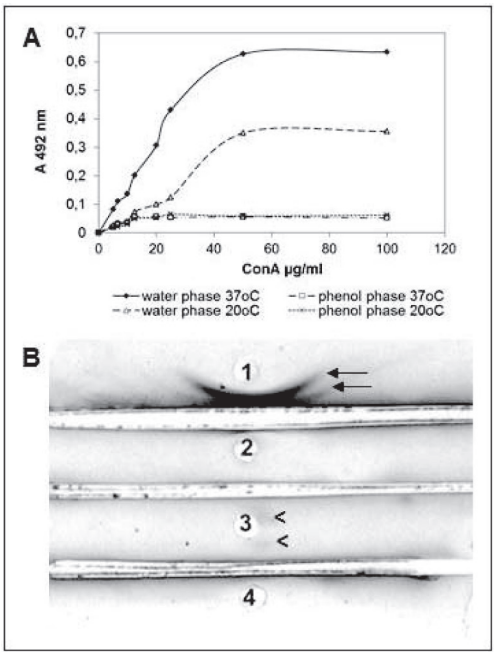

Fig. 2. Comparison of the reactivity of the water- and phenol-phase polysaccharides with ConA. A, ELLA; B, immunoelectrophoresis: (1) water phase polysaccharide, 37oC, (2) phenol phase polysaccharide, 37oC, (3) water phase polysaccharide, 20oC, (4) phenol phase polysaccharide, 20oC. In sample (1), two distinct precipitation bands are formed (arrows); in sample (3), more obscure areas slightly colored with Coomasie are registered (arrowheads).

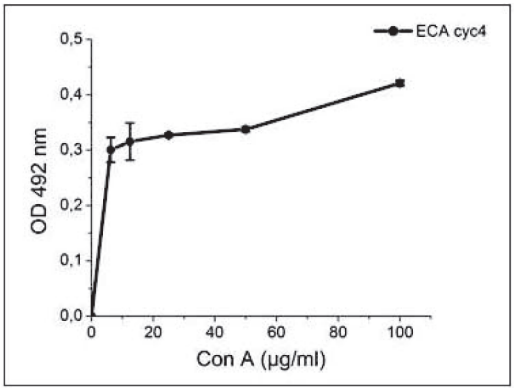

Fig. 3. ELLA test showing the ConA reactivity of purified ECA_{CYC4}.

Discussion

The present results show that the water-phase polysaccharide fraction of the strain is peculiar for its lack of immunoreactivity and the high affinity for ConA. In its low immunoreactivity, our data on this ConA - positive fraction is alike our previous results on ECA_{CYC} (Paunova-Krasteva *et al*., 2014). What is more, here we demonstrated the affinity of purified ECA_{CYC} for ConA. We can therefore conclude that the ConA reactivity of the water-phase crude extract from the strain grown at 37°C is due to ECA_{CYC}. Some, significantly less distinct ConA-reactivity was observed in the water-phase crude preparation of cells grown at 20°C. While this does not exclude the possibility that some amount of ECA_{CYC} may be produced also at this temperature, the results of the present study confirm a promotion of the synthesis of the ConA-reactive molecules when the strain was cultivated at 37°C.

ConA has long been known for its specific binding of mannosyl or glycosil non-reducing termini in glycoconjugates (Doyle, 1994). More recently however the multivalency of this lectin has attracted attention, based on newly synthesized multivalency glycoligands (Wittmann and Pieters, 2013; Ponader *et al*., 2014). The accommodation of such ligands is via the so-called "expanded binding site" which additionally strengthens the binding affinity via weak multi-locus molecular interactions (Moothoo and Naismith, 1998). Such type of reac-

tivity might expectedly be involved also in the system tested here.

The similarity in the ConA affinities of the crude water-phase fraction and the purified ECA_{CYC} is of special concern. We have previously shown that ECA_{CYC} may be considered as MAMP-ligands that interact with the host humoral mechanisms of non-self recognition (Paunova-Krasteva *et al*., 2014). The promotion of the release of the ConA-reactive entity (most likely - ECA_{CYC}) at 37°C, i.e., temperature condition characteristic for host body, indicates its importance in the host-invader interplay.

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