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# The effect of *waaL* genes deletion from *Yersinia enterocolitica* O:3 genome on bacteria LPS' phenotype

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***Aim***. *To estimate WaaL ligase contribution in the lipopolysaccharide (LPS) phenotype profile formation of Y. enterocolitica O:3 (YeO3) bacteria.* ***Methods.*** *The waaL-knock-out mutants were created by an allelic exchange strategy. The LPS phenotypes of created mutants were visualized by silver-stained DOC-PAGE and immunoblotting with specific outer core (core oligosaccharide, hexasaccharide, OC) and O-polysaccharide (OPS or O-Ag) monoclonal antibodies.* ***Results***. *Deletion of $waaL_{OS}$ gene from YeO3 genome has a marked effect on OC ligation in either single or double mutants. The $waaL_{PS}$ deletion has an opposite effect on the OPS ligation – barely detected increasing of OPS bands.* ***Conclusions***. *The LPS ligases of YeO3 exhibit relaxed donor substrate specificity. Under given conditions the effect of $WaaL_{OS}$ ligase is more significant for OC and OPS ligation onto lipid A than that of $WaaL_{PS}$.*

*Keywords: WaaL ligase, LPS, Yersinia enterocolitica, DOC-PAGE.*

**Introduction**. In Europe, yersiniosis is the third most common bacterial zoonosis after campylobacteriosis and salmonellosis [1]. *Y. enterocolitica* is a well known human and animal pathogen. Among humans, the pathway of *Y. enterocolitica* is associated with intestinal disease, such as enterocolitis, with inflammatory diarrhea, ileitis, mesenteric appendicitis and gastroenteritis. A diarrheal disease is sometimes followed by post-infectious reactive arthritis.

It is well known that all Gram-negative bacteria contain an outer leaflet with a large amount of lipopolysaccharide (LPS). LPS is a glycolipid consisting of three domains: the lipid A moiety, the core and the distal O-polysaccharide (OPS or O-Ag). The homopolymeric O-Ag is composed of β1,2-linked 6-deoxy-L-altrose residues. Together with the hexasaccharide core, the O-Ag is linked to the inner core (IC) of LPS to form a branched structure [2]. The antigenic variations of OPS in the *Y. enterocolitica* isolates are distinguished serologically. Nowadays, more than 50 serotypes are known, of which O:3, O:5, 27, O:8 and O:9 are pathogenic [3].



The LPS biosynthesis is a complex process that includes the stepwise transformation of the primary substrate under enzymatic treatment. The WaaL proteins are involved in the ligation of OC and O-Ag onto the lipid A core. According to the *in silico* investigations, the *Y. enterocolitica* O:3 (YeO3) genome contains at least three genes responsible for the WaaL proteins expression. *Yersinia pestis* and *Yersinia pseudotuberculosis*, however, carry only the $waaL_{PS}$ gene, whereas either $waaL_{OS}$ or $waaL_{XS}$ or both are additionally present in other *Yersinia* species.

It was shown that deletion of $waaL_{OS}$ and $waaL_{PS}$ genes correlate with the OPS and the OC expression. For this purpose LPS ligases were named as $WaaL_{PS}$ and $WaaL_{OS}$ respectively. The third ligase named as $WaaL_{XS}$ was not involved in the LPS or ESA biosynthesis [4].

The current study is aimed at the estimation of a role of the ligases in the *Y. enterocolitica*' LPS phenotype profile formation. The *waaL*-knock-out mutants of YeO3

*Bacterial strains*

| Strain | Genotype | Reference |
|---|---|---|
| | *Yersinia enterocolitica* | |
| 6471/76 | YeO3 wild type strain, patient isolate | [6] |
| 6471/76-c | YeO3-c virulence plasmid cured derivative of 6471/76 | [6] |
| YeO3_Δos | $waaL_{OS}$::pSW23Tlig1727su | This work |
| YeO3_Δps | $waaL_{PS}$::pSW29-lig532del, $Km^R$ | This work |
| YeO3_Δos_Δps | $waaL_{OS}$::pSW23T-lig1727su $waaL_{PS}$::pSW29-lig532del, $Km^R$ | This work |
| | *Escherichia coli* | |
| ω7249 | B2163 Λnic35, *E. coli* strain for suiside vector delivery, requirement for diaminopimelic acid 0.3 mM, $Km^R$ | [7] |
| S17-1λpir | A-pir lysogen of S17-1, *E. coli* strain for suiside vector delivery | [8] |
| DH10B | F-mcrA Δ(mrr-hsdRMS-mcrBC), Φ80lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ-rpsL nupG tonA | Life Technologies |

were created by the allelic exchange strategy. Phenotypes of created mutants were visualized by silver-stained DOC-PAGE and immunoblotting with specific OC and O-Ag monoclonal antibodies.

**Materials and methods**. *Bacterial strains and culture conditions*. Bacterial strains are listed in Table. *Yersinia* strains were grown at 22–25 °C (RT) and *Escherichia coli* strains at 37 °C in Luria Broth (LB) media. LB supplemented with 1.5 % Bacto Agar was used for all solid cultures. As a selective medium CIN agar supplemented with appropriate antibiotics was used. When appropriate, the antibiotics were added to the media at the following concentrations: kanamycin (Km), 100 μg/ml in agar plates and 20 μg/ml in broth; chloramphenicol (Clm), 20 μg/ml.

*General DNA techniques*. Isolation of plasmids and genomic DNA was done with kits. All enzymes were used according to the supplier's specifications. Small-scale plasmid DNA preparations were carried out using plasmid mini prep kits. Plasmid DNA was moved by electroporation into *Y. enterocolitica* or heat shock transformation. Recombinant plasmids were mobilized from *E. coli* strains to *Y. enterocolitica* by conjugation.

*Mutants construction*. The $waaL_{OS}$ and $waaL_{PS}$ genes were amplified by PCR with primer pairs O3lig YE1727F5 & O3ligYE1727R5 and O3ligYE532F2 & O3ligYE532R2 with DyNAzyme DNA-polymerase («Thermo Scientific», USA) from isolated genomical DNA of YeO3. Amplified DNA was purified with Kit method and digested with NsiI (Mph 1103I) for the $waaL_{OS}$ gene and PstI for $waaL_{PS}$. The digested and purified fragments were cloned into the PstI digested suicide vector *pSW23T* and the constructed plasmids were named as *pSW23T-*$waaL_{OS}$ and *pSW23T-*$waaL_{PS}$ respectively. The constructions were mobilized from *E. coli* ω7249 into YeO3 strains by conjugation as described earlier [5]. For elimination of suicide vector and the wild-type genes, the optimized cycloserine enrichment method was used [5].

For large-scale screening of knock-out mutants among Clm sensitive bacteria ($Clm^S$) colonies we used Colony hybridization kit method («Roche», France). Isolated genomical DNA from negative colonies were diluted and used as a template for PCR with different primer pairs. DNA of wild-type strain YeO3 was used as a control.

*Complementation*. The $waaL_{OS}$ and $waaL_{PS}$ genes were amplified with Phusion DNA polymerase from YeO3-c with O3ligYe1727f & O3ligYe1727r, O3lig Ye532f & O3ligYe532r primer pairs. PCR fragments were phosphorylated with polynucleotide kinase in the presence of 10 mM ATP, digested with EcoRI and ligated with EcoRI and ScaI digested, SAP-treated pTM100. The constructed plasmids were named pEPlig1727 & pEPlig532 and electroporated into S17-1λ pir with further mobilization into YeO3 ligase mutants by conjugation. Obtained colonies were screened on appropriate antibiotic plates with CIN agar [4].

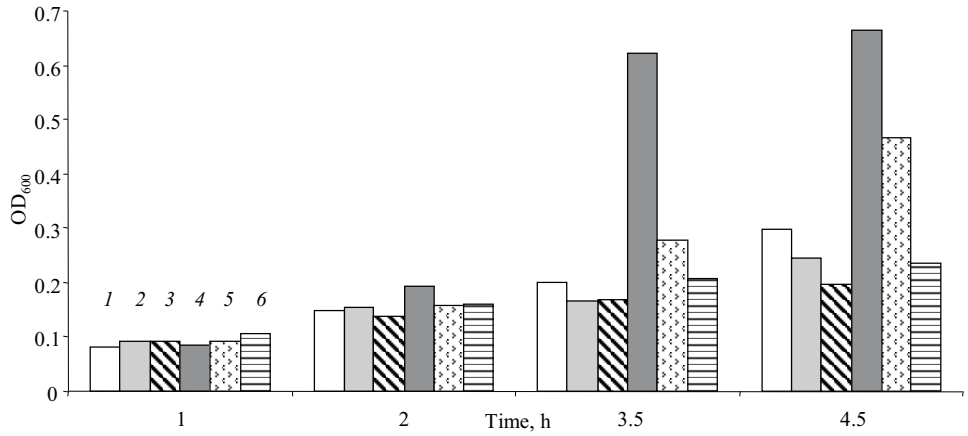


Fig. 1. Optimization of cycloserine enrichment method: *1* – YeO3 wild type (WT), 2.5 µl v/v; *2* – WT, 5 µl v/v; *3* – WT, 10 µl v/v; *4* – merodiploids (MD), 2.5 µl v/v; *5* – MD, 5 µl v/v; *6* – MD, 10 µl v/v

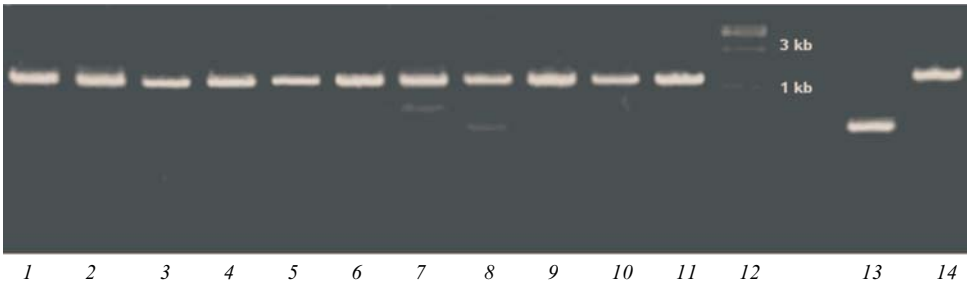


Fig. 2. Amplification of $waaL_{OS}$ gene by PCR with pair of primers O3lig Ye1727f & O3ligYe1727r: *1–11* – checking colonies; *12* – gene ruler; *13* – $waaL_{OS}$-knock-out mutant; *14* – YeO3 wild type control

*DOC-PAGE analysis*. The bacteria were grown 16–20 h at RT in 5 ml of LB medium with appropriate antibiotics. The exact optical density of the cultures was measured at 600 nm ($OD_{600}$), 3 ml of the cultures were centrifuged and the pellets were resuspended in deoxycholate lysis buffer (2 % DOC, 4 % 2-mercaptoethanol, 10 % glycerol and 0.002 % bromophenol blue in 1 M Tris-HCl buffer, pH 6.8) in a volume adjusted according to density of the culture (100 µl/$OD_{600}$ ~ 1). Lypopolysaccaride phenotypes were analyzed by silver stained DOC-PAGE with previous proteinase K treated whole cell lysates [4]. The Western blotting O-polysaccharide, outer core and inner core expression were detected by O:3 specific mAbs 2B5 and TomA6.

**Results and discussion**. *Mutants construction*. The $waaL_{OS}$ and $waaL_{PS}$ mutants of YeO3 were constructed from fully virulent serotype O:3 patient isolate expressing complete LPS. With help of the allelic exchange strategy we managed to inactivate $waaL_{OS}$ and $waaL_{PS}$ encoding regions in the YeO3 genome. The constructed single and double mutants were complemented with the pEPlig1727 & pEPlig532 plasmids, which were supplemented with the functional *waaL* gene.

Traditional cycloserine enrichment method was additionally optimized [5]. The possibility was considered that constructed merodiploids (MD) are not fully resistant to Clm, as it should be (weak operon, *etc*.). To examine this possibility we tested several lines of conditions (concentration of Clm, incubation time, density of bacteria, *etc*.). According to these experimental data, the next improvements were done: reducing the Clm concentration in media to 2.5 µl v/v (instead of 10 µl v/v) and prolongation of incubation to 4–5 h (instead of 2–3 h) before D-cycloserition solution was added. A current modification of the method allowed us to pick up only bacteria after second crossing over (Fig. 1).

The colony hybridization method was used for specific detection of deletion in the *waaL* gene among $Clm^S$. Further justifications of deletion were performed by PCR (Fig. 2).

*Phenotype analysis*. We used two approaches to analyze the difference in the expression of OC and O-Ag: 1) Silver staining of DOC-PAGE; 2) Immunoblotting with OC and O-Ag specific antibodies.

It is noticeable from silver staining of DOC-PAGE that deletion in the $waaL_{OS}$ gene leads to a dramatic decrease in the OC expression and appearance of strong IC band (Fig. 3). However, the level of O-Ag expression reduced, as well, compared to the mutant with deletion in the $waaL_{PS}$ gene. The deletion in the $waaL_{PS}$ gene

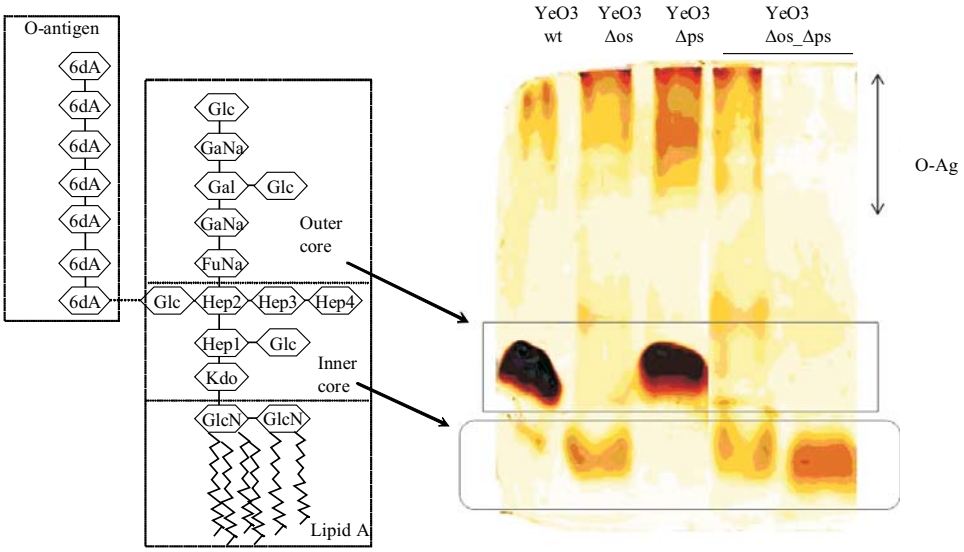


Fig. 3. Silver-stained DOC-PAGE analysis of LPS phenotypes of different ligase mutants

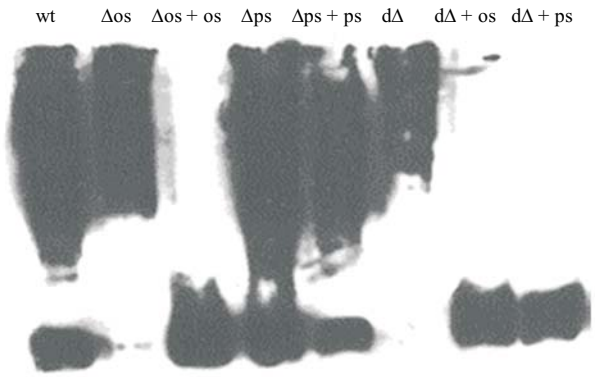


Fig. 4. Immunoblot analysis of LPS phenotypes of *waaL* mutants of YeO3. DOC-PAGE membrane was probed with mAb 2B5 and TomA6

seems to be insignificant for the OC expression and even to stimulate the OPS formation. In case of the double YeO3_Δos_Δps mutants, it is noticeable a strong expression of IC bands, the absence of OC in both variants and the absence of O-Ag expression in one variant.

Western blotting analysis of LPS samples of the YeO3 ligase mutants and their complementation of single and double mutants were performed with the OC-specific mAb 2B5 and O-Ag-specific mAb TomA6 (Fig. 4). The deletion in the $waaL_{OS}$ gene resulted in reduction of the OC expression, as it was shown with silver staining of DOC-PAGE. Also, the $waaL_{OS}$-knockout mutant complemented with the functional $waaL_{OS}$ gene showed full recovery of OC and the decreasing of O-Ag expression at the same time.

Similar results were obtained with the double mutant and complementation variants. The LPS profile of the double YeO3_Δos_Δps mutant in immunoblotting was similar to that with silver staining where only the O-Ag expression took place. The complementation with the functional $waaL_{OS}$ and $waaL_{PS}$ genes showed inhibition of O-Ag expression and full recovery of OC in both cases. Disruption in the $waaL_{PS}$ gene as a single mutation leads to hardly noticeable stimulation of the O-Ag expression.

Thereby, the data obtained from silver staining and immunoblotting of DOC-PAGE evidence the participation of WaaL ligase in the LPS phenotype creation. However, strict substrate specificity of the LPS ligase in YeO3 was not detected.

**Conclusions**. Summarizing the obtained data we can conclude that the LPS ligases of YeO3 exhibit relaxed donor substrate specificity. It has been established that under given conditions the effect of the $WaaL_{OS}$ ligase is more significant for the OC and OPS ligation onto lipid A than for the $WaaL_{PS}$ ligase. It is possible that deletion of the $waaL_{OS}$ and $waaL_{PS}$ genes and changes in the OC or O-Ag moieties of LPS enhance the ability of pathogen to evade host defenses. Further work is required to elucidate the biological significance of these different settings.

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Вплив делецій генів лігаз *waaL* на фенотип ліпополісахаридів у бактерій *Yersinia enterocolitica* O:3

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Резюме

***Мета**. Дослідити участь лігаз WaaL у формуванні фенотипу ліпополісахариду (LPS) серед бактерій Y. enterocolitica O:3 (YeO3). **Методи**. Нокаутні мутанти по генах лігаз waaL створено внаслідок обміну алелями. Фенотипи LPS отриманих мутантів візуалізували, забарвлюючи сріблом гель DOC-PAGE, а також використовували імуноблот зі специфічними моноклональними антитілами до кору (корового олігосахариду, гексасахариду, ОС) та О-полісахариду (OPS, O-Ag). **Результати**. Делеція гена лігази $waaL_{OS}$ з геному бактерій YeO3 чинить помітний вплив на лігування ОС як в одиночних, так і в подвійних мутантах. Проте маніпуляції з геном лігази waaLPS призводять до ледь помітної стимуляції утворення OPS. **Висновки**. Лігази LPS бактерій YeO3 демонструють низьку субстратну специфічність. Участь лігази $WaaL_{OS}$ у формуванні повноцінної структури LPS є суттєвішою, аніж WaaLPS, за даних умов.*

*Ключові слова: лігази WaaL, LPS, Yersinia enterocolitica, DOC-PAGE.*

Влияние делеций генов лигаз *waaL* на фенотип липополисахаридов у бактерий *Yersinia enterocolitica* O:3

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Резюме

***Цель**. Исследовать участие лигаз WaaL в формировании фенотипа липополисахарида (LPS) среди бактерий Y. enterocolitica O:3 (YeO3). **Методы**. Нокаутные мутанты по генам лигаз waaL созданы вследствие обмена аллелями. Фенотипы LPS полученных мутантов визуализировали, окрашивая серебром гель DOC-PAGE, а также с использованием иммуноблота со специфическими моноклональными антителами к кору (коровому олигосахариду, гексасахариду, ОС) и О-полисахариду (OPS, O-Ag). **Результаты**. Делеция гена лигазы $waaL_{OS}$ из генома бактерий YeO3 оказывает заметное влияние на лигирование ОС как в одиночных, так и двойных мутантах. Однако манипуляции с геном лигаз $waaL_{PS}$ приводят к едва заметной стимуляции образования OPS. **Выводы**. Лигазы LPS бактерий YeO3 демонстрируют низкую субстратную специфичность. Участие лигазы $WaaL_{OS}$ в образовании полноценной структуры LPS является более существенным, чем WaaLPS, при данных условиях.*

*Ключевые слова: лигазы WaaL, LPS, Yersinia enterocolitica, DOC-PAGE.*

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