UDC 579.234

THE ROLE OF WAAL LIGASES OF YERSINIA ENTEROCOLITICA O:3 AND O:8 IN LIPOPOLYSACCHARIDE BIOSYNTHESIS AND STRESS ADAPTATION

J.I. SHEVCHENKO[1, 2], V.K. POZUR[1], M.SKURNIK[2]

[1] Taras Shevchenko National University of Kiev
Ukraine, 03022, Kiev, Akademika Hlushkova str., 2
[2] Haartman Institute, University of Helsinki
Finland, FIN-00014, Haartmaninkatu 3 (P.O. Box 21)
e-mail: julia.i.shevchenko@gmail.com

Aim. *The aim of current studies was to estimate participation of WaaL ligases in lipopolysaccharide (LPS) biosynthesis of Yersinia enterocolitica O:3 and O:8 (YeO3, YeO8) and determine the role of ligases for bacterial growth in conditions of osmotic pressure.* ***Methods***. *The single and double waaL mutants were created by allelic exchange strategy. Phenotypes of created mutants were visualized by silver-stained DOC-PAGE and immunoblotting with specific OC and O-Ag monoclonal antibodies. The growth media with 3,5 % NaCl was used as a sensitivity assay.* ***Results***. *Deletion of $waaL_{OS}$ gene from YeO3 genome has a marked effect on OC ligation either in single or double mutants. The $waaL_{PS}$ deletion has an opposite effect on the OPS ligation – barely detected increasing of OPS bands. A dramatic decreasing of growth potential in hypertonic medium was observed only in YeO3 double ligase mutant.* ***Conclusions***. *The importance of WaaL ligase for environmental stress resistance was detected only for YeO3 double mutant. Single ligase gene deletions not affect the osmotic resistance of bacteria, which could be explained as compensation of damaged ligase function by another ligase.*

Keywords: *Yersinia enterocolitica, LPS, WaaL ligases, osmotic pressure.*

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

As all Gram-negative bacteria, *Y. enterocolitica* contains an outer leaflet with a large number of LPS. It is a glycolipid consists of three domains: lipid A moiety, the core and the distal O-polysaccharide. The antigenic variation of OPS in 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 [2]. 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 of LPS to form a branched structure in YeO3 [3].

LPS biosynthesis is a complex process that includes the stepwise transformation of the primary substrate under enzymatic treatment. The WaaL proteins involved in the ligation of OC and O-Ag onto the lipid A core. Based on *in silico* investigations, *Y. enterocolitica* genome contains at least three gens responsible for the WaaL proteins expression [4].

Yersinia pestis and *Yersinia pseudotuberculosis*, however, carry only the one gene. Also, in the other bacteria studied this far, such as *E. coli* or *Salmonella* only one ligase gene has been described as a responsible for the ligation of LPS moieties.

In this work, we characterized the LPS phenotype formation in *waaL* mutants of YeO3 and YeO8 and analyzed the role of WaaL ligases in environmental stress resistance. As a stress agent hypertonic growth medium was used.

Materials and methods

Bacterial strains and culture conditions. The bacterial strains and plasmids are listed in Table 1. *Y. enterocolitica* strains were grown at 22–25 ˚C (RT) and *E. coli* strains at 37 ˚C in Luria Broth (LB) media. LB supplemented with 1.5 % Bacto Agar was used for all solid cultures. CIN agar was

Table 1. Bacterial strains and plasmids

	Strain	Genotype	Reference
Yersinia enterocolitica	6471/76	YeO3 wild type strain, patient isolate	[7]
	6471/76-c	YeO3-c virulence plasmid cured derivative of 6471/76	[7]
	YeO3_Δos	waaLos::pSW23Tlig1727su	This work
	YeO3_Δps	waaLps::pSW29-lig532del, KmR	This work
	YeO3_Δos_Δps	waaLos::pSW23T-lig1727su waaLps::pSW29-lig532del, KmR	This work
	8081	YeO8 wild type strain, patient isolate	[8]
	8081-L2	R^-M^+ derivative of wild-type strain 8081; serotype O:8; pYV+	[9]
	8081-Res	R^-M^+ derivative of 8081-c; serotype O:8; the pYV-cured derivative of 8081	[10]
	YeO8_Δos	waaLos::pSW23T-lig1727su, pYV+, ClmR	This work
	YeO8_Δps	waaLps::pSW29-lig532del, KmR	This work
	YeO8_Δos_Δps	waaLos::pSW23T-lig1727su waaLps::pSW29-lig532del, ClmR, KmR	This work
Escherichia coli	ω7249	B2163Λnic35, *E. coli* strain for suiside vector delivery, requirement for diaminopimelic acid 0.3mM, KmR	[11]
	S17-1λ pir	A-pir lysogen of S17-1, E. coli strain for suiside vector delivery	[12]
	DH10B	F- mcrA Δ(mrr-hsdRMS-mcrBC), Φ80lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ-rpsL nupG tonA	Life Technologies
	CLM24	D*waaL* derivative of W3110	[13]
Plasmids			
pSW23T		Suicide vector, ClmR	[14]
pTM100		Mobilizable cloning vector, ClmR TetR	[15]
pLos		$waaL_{OS}$ of YeO3 cloned in pTM100, TetR	[4]
pLps		$waaL_{PS}$ of YeO3 cloned in pTM100, TetR	[4]

used for selecting transconjugants following mating experiments. When appropriate, 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) and tetracycline (Tet), 20 μg/ml.

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

Construction of mutants. The $waaL_{OS}$ and $waaL_{PS}$ genes were amplified by PCR with the primer pairs O3ligYE1727F5 & O3ligYE1727R5 and O3ligYE532F2 & O3ligYE532R2 using the Dynazyme II DNA-polymerases (Thermo Scientific) and genomic DNA of *Y. enterocolitica* O:3 as a template. Amplified DNA was purified with Kit method and digested with NsiI (Mph 1103I) for $waaL_{OS}$ gene and PstI for $waaL_{PS}$. Digested and purified fragments were cloned into 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 *Y. enterocolitica* O:3 and O:8 strains by conjugation as described earlier [4]. For elimination suicide vector and the wild-type genes were used optimized cycloserine enrichment method previously described by Biedzka-Sarek [5]. For large-scale screening of knock-out mutants among Clm^S colonies we were used Colony hybridization kit method (Roche). Isolated genomic DNA from negative colonies were diluted and used as a template for PCR with different primer pairs. DNA from the wild-type bacteria 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, O3ligYe532f & O3ligYe532r primer pairs. The PCR fragments were phosphorylated with polynucleotide kinase in the presence of 10mM ATP, digested with EcoRI and ligated with EcoRI and ScaI digested, SAP-treated pTM100. The constructed plasmids were electroporated into S17-1λ pir with further mobilization into YeO3 and YeO8 ligase mutants by conjugation. Obtained colonies were screened on appropriate antibiotic plates with CIN agar [4].

SDS-PAGE analysis and Western immunoblotting. To detect OC and OPS expression in obtained mutants, bacteria were grown overnight at RT in 5 ml of LB with an appropriate antibiotics. The wholecell lysates were prepared from 1 ml of bacterial cultures (OD_{600} adjusted to 0.2). The cultures were centrifuged for 15 min (1,500 x g), and pellets were resuspended in 100 μl of Laemmli sample buffer. The mixtures were heated at 95–99 °C for 10 min before being loaded onto sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). The Western blotting O-polysaccharide, outer core and inner core expression were detected by O:3 specific 2B5 & TomA6 and O:8 specific 1F1 monoclonal antibodies [6]. The secondary antibody was rabbit anti-mouse peroxidase-conjugated immunoglobulins (P0260; dilution 1:2,000). Antibody binding was detected by chemiluminescence using the ECL Western blotting detection reagents (Amersham Pharmacia Biotech) according to the manufacturer's instructions [5].

Sensitivity assay

The bacterial strains were grown overnight at RT in 5 ml of LB with an appropriate antibiotics. Salt endurance of strains was measured in LB medium containing 3.5%

NaCl. The strains were washed and inoculated into LB, followed by measuring the OD at 600 nm at regular time interval [16]. For the measuring of bacterial growth the Bioscreen system was used (5101370 Bioscreen Analyzer 220.110 V). During growing the microorganisms increase the turbidity of their growth medium. By measuring the turbidity of the medium over time, an optical density (OD) curve can be generated. Turbidometric measurements are made kinetically during the course of the run.

Results and discussion

Construction of mutants. To characterize the role of WaaL ligase in the LPS biosynthesis we constructed single and double mutants from fully virulent *Y. enterocolitica* O:3 and O:8 bacteria. To simplify the recognition of the mutants, the strains were named as YeO3_os and YeO8_os for $waaL_{OS}$, YeO3_ps and YeO8_ps for $waaL_{PS}$ and YeO3_os_ps and YeO8_os_ps for double mutants.

In two steps, with help of allelic exchange strategy, we managed to inactivate the $waaL_{OS}$ and $waaL_{PS}$ encoding regions in bacterial genome. Firstly, the merodiploids were obtained from transconjugants, which contained the suicide vector pSW23T. Secondly, cycloserine was used to enrich colonies in which the second crossing over had excluded the suicide vector and the *waaL* gene. The previously described cycloserine enrichment method was additionally optimized [5]. It was considered the possibility that constructed merodiploids (MD) not fully resistant to Clm, as it should be. To examine this possibility we tested different conditions (concentration of Clm, incubation time, density of bacteria, etc.) (data not shown).

Obtained mutants were confirmed by colony hybridization method, which was used for specific detection of the deletion in the *waaL* gene among chloramphenicol sensitive bacteria (Clm^S). Further verification of deletion was performed by PCR and Southern blot. The LPS phenotype was visualized with help of DOC-PAGE & silver staining and immunoblotting with specific OC and O-Ag monoclonal antibodies.

SDS-PAGE analysis and Western immunoblotting. We used two approaches to visualize the LPS phenotype expression: 1) Silver staining of DOC-PAGE; 2) Immunoblotting with OC and O-Ag specific antibodies.

It is noticeable from the silver staining of DOC-PAGE that deletion in the $waaL_{OS}$ gene leads to dramatic decreasing of OC expression and appearing of strong inner core (IC) bands in YeO3 and YeO8 ligase mutants (Fig.1, 2). However, a level of O-Ag expression reduced, as well, compare to mutant with deletion in $waaL_{PS}$ gene. The deletion in the $waaL_{PS}$ gene seems to be not significant for OC expression and stimulation of OPS ligation was observed. In case of double YeO3_os_ps mutants, it is noticeable a strong IC band and absence of OC expression (Fig. 1).

Western blotting analysis of YeO3 ligase mutants and their complementation of single and double mutants were performed with the OC-specific mAb 2B5 and O-Ag-

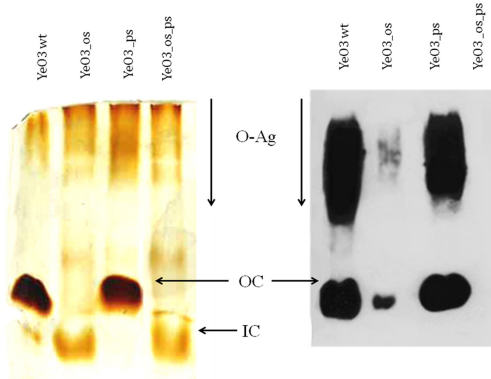

Fig. 1. Silver stained DOC-PAGE (left) and Immunoblotting with mAb TomA6 and 2B5 (right) of YeO3 strains

specific mAb TomA6 (Fig. 1). The deletion in the *waaL*$_{OS}$ gene resulted in reduction of OC expression, as it was shown with silver stained DOC-PAGE. Also, *waaL*$_{OS}$-knock-out mutant complemented with functional *waaL*$_{OS}$ gene showed full recovery of OC and decreasing of O-Ag expression in the same time (data not shown).

Similar results were obtained with double mutants and variants with complementation (data not shown). The LPS profile of double YeO3_os_ps mutants in immunoblotting was not efficient. One could speculate that deletion of both ligases leads to unrecognizable changes in LPS structure. The complementation with 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 OPS expression.

Immunoblotting with YeO8 strains was performed with YeO8' LPS specific monoclonal antibodies 1F1. The *waaL*$_{OS}$ gene deletion result in reduction of OC expression, as it was shown with silver stained DOC-PAGE with the same mutant. However, removing of *waaL*$_{PS}$ gene as a single mutation leads to barely detected stimulation of OPS expression and induction of IC creation. For both YeO8_os and YeO8_ps mutants the over expression of IC was observed. The YeO8_os_ps mutants didn't show clear LPS' phenotype difference in case of silver stained DOC-PAGE. The immunoblotting of double mutants with specific monoclonal antibodies didn't give any results. It was considered that LPS subunits were changed under mutagenesis which issued in not detectible structure (Fig. 2).

Sensitivity assay. Besides, the *waaL* deletions caused the alteration in the membrane profiles (Fig. 1, 2), it might have an impact on the structure and characteristics of the membrane. In stress response experiments among the *Yersinia enterocolitica*

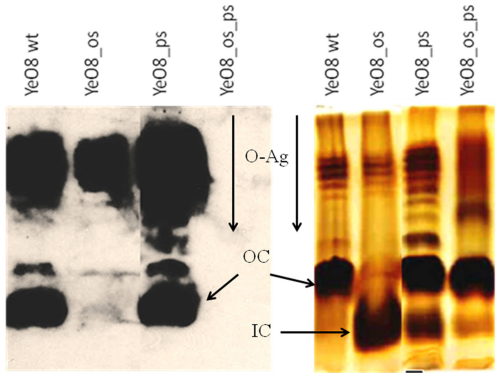

Fig. 2. Silver stained DOC-PAGE (right) and Immunoblotting with mAb 1F1 (left) of YeO8 strains.

O:3 and O:8 *waaL* mutants only the YeO3_os_ps double mutants showed high sensitivity to overdose NaCl concentration in the media (Fig. 3). However, the double ligase mutants of YeO8 showed significant stimulation of growth, compare to the wild type. The single ligase mutants of both serotypes showed similar results. The *waaL*$_{OS}$ ligase mutants behaved in the same way as the wild type bacteria. In contrast, YeO3_ps and YeO8_ps mutants showed slightly hire resistance to salted media then the wild type. The complementation of the ligase mutants with functional genes didn't show a proper filling of the lost function; ones were extremely sensitive to the salted media.

In conclusions, we have been able to show that deletion of *waaL* ligases from *Y. enterocolitica* genome affected creation of the LPS' phenotypes. To elucidate the biological significance of *waaL* genes we analyzed ligase mutants' growth curves in hypertonic media and found absence of growth among double ligase mutants of YeO3. All single mutants and double mutants of YeO8 demonstrated the growth potential closed to the wild type bacteria.

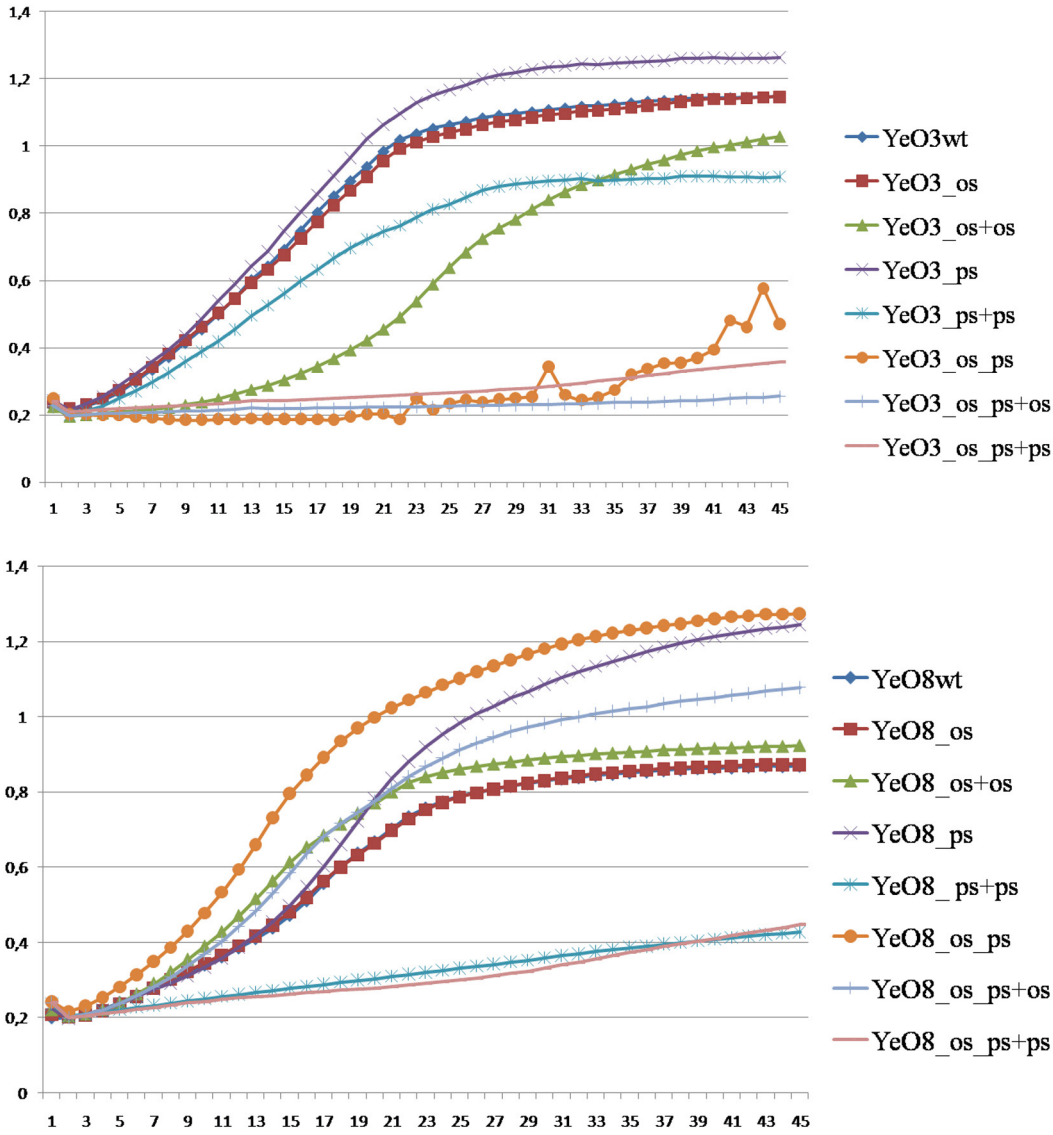

Fig. 3. The growth curves of *waaL* mutants of YeO3 and YeO8 in hypertonic medium.

Acknowledgments

This work was supported by grants from Center for International Mobility (CIMO), Finland TM-12-8286.

References

1. *Ortiz Martinez P., Mylona S., Drake I. et al*. Wide variety of bioserotypes of enteropathogenic *Yersinia* in tonsils of English pigs at slaughter // Int. J. Food Microbiol. – 2010. – Vol. 139, № 1–2. – P. 64–69.
2. *Sabina Y. 1., Rahman A., Ray R.C., Montet D. Yersinia enterocolitica:* Mode of transmission, molecular insights of virulence, and pathogenesis of infection // J. Pathog. – 2011. – Vol. 2011. – P. 429069.
3. *Skurnik M., Venho R., Toivanen P., Al-Hendy A*. A novel locus of *Yersinia enterocolitica* serotype

O:3 involved in lipopolysaccharide outer core biosynthesis // Mol. Microbiol. – 1995. – Vol. 17. – P. 575–594.
4. *Pinta E., Li Z., Batzilla J. et al*. Identification of three oligo-/polysaccharide-specific ligases in *Yersinia enterocolitica* // Molecular Microbiology. – 2012. – Vol. 83, № 1. – P. 125–136.
5. *Biedzka-Sarek M., Venho R., Skurnik M*. Role of YadA, Ail, and lipopolysaccharide in serum resistance of *Yersinia enterocolitica* serotype O:3 // Infect. Immun. – 2005. – Vol. 73, № 4. – P. 2232–2244.
6. *Bengoechea J.A., Najdenski H., Skurnik M*. Lipopolysaccharide O antigen status of *Yersinia enterocolitica O:8* is essential for virulence and absence of O antigen affects the expression of other *Yersinia* virulence factors // Mol. Microbiol. – 2004. – Vol. 52, № 2. – P. 451–469.
7. *Skurnik M*. Lack of correlation between the presence of plasmids and fimbriae in *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* // J. Appl. Bacteriol. – 1984. – Vol. 56. – P. 355–363.
8. *Portnoy D.A., Moseley S.L., Falkow S.* Characterization of plasmids and plasmid-associated determinants of *Yersinia enterocolitica* pathogenesis // Infection and Immunity. – 1981. – Vol. 31, № 2. – P. 775–782.
9. *Zhang L., Radziejewska-Lebrecht J., Krajewska-Pietrasik D. et al.* Molecular and chemical characterization of the lipopolysaccharide O-antigen and its role in the virulence of *Yersinia enterocolitica* serotype O:8 // Mol. Microbiol. – 1997. – Vol. 23, № 1. – P. 63–76.
10. *Zhang L., Skurnik M.* Isolation of an R- M+ mutant of *Yersinia enterocolitica* serotype O:8 and its application in construction of rough mutants utilizing mini-Tn5 derivatives and lipopolysaccharide-specific phage // J. Bacteriol. – 1994. – Vol. 176, № 6. – P. 1756–1760.
11. *Babic A., Guerout A.M., Mazel D.* Construction of an improved RP4 (RK2)-based conjugative system // Res. Microbiol. – 2008. – Vol. 159, № 7–8. – P. 545–549.
12. *Wilson K.J., Sessitsch A., Corbo J.C., Giller K.E., Akkermans A.D., Jefferson R.A.* beta-Glucuronidase (GUS) transposons for ecological and genetic studies of rhizobia and other gram-negative bacteria // Microbiology. – 1995. – Vol. 141, № 7. – P. 1691–1705.
13. *Feldman M.F., Wacker M., Hernandez M. et al.* Engineering N-linked protein glycosylation with diverse O antigen lipopolysaccharide structures in *Escherichia coli* // Proc. Natl. Acad. Sci. USA. – 2005. – Vol. 102, № 8. – P. 3016–3021.
14. *Demarre G., Guerout A.M., Matsumoto-Mashimo C. et al.* A new family of mobilizable suicide plasmids based on broad host range R388 plasmid (IncW) and RP4 plasmid (IncPalpha) conjugative machineries and their cognate *Escherichia coli* host strains // Res. Microbiol. – 2005. – Vol. 156, № 2. – P. 245–255.
15. *Michiels T., Cornelis G.R.* Secretion of hybrid proteins by the *Yersinia Yop* export system // J. Bacteriol. – 1991. – Vol. 173. – P. 1677–1685.
16. *Xu L., Wang Q., Xiao J. et al.* Characterization of *Edwardsiella tarda waaL:* roles in lipopolysaccharide biosynthesis, stress adaptation, and virulence toward fish // Arch. Microbiol. – 2010. – Vol. 192. – P. 1039–1047.

Presented by B.P. Matselyukh
Received 01.10.2014

РОЛЬ WAAL ЛІГАЗ В БІОСИНТЕЗІ ЛІПОПОЛІСАХАРИДУ ТА АДАПТАЦІЇ ДО СТРЕСУ У БАКТЕРІЙ *YERSINIA ENTEROCOLITICA* O:3 ТА O:8

Ю.І. Шевченко[1,2], В.К. Позур[1], М. Скурнік[2]

[1] Київський національний університет
імені Тараса Шевченка
Україна, 03022, Київ-022, вул. Академіка
Глушкова, 2
[2] Haartman Institute, University of Helsinki
Finland, FIN-00014, Haartmaninkatu 3
(P.O. Box 21)
e-mail: julia.i.shevchenko@gmail.com

Мета. Метою даних досліджень була оцінка участі WaaL лігаз в синтезі ліпополісахариду (ЛПС) бактерій *Yersinia enterocolitica* O:3 і O:8 (YeO3, YeO8) та визначення участі лігаз у формуванні ростового потенціалу бактерій в умовах осмотичного тиску. **Методи.** Одиночні та подвійні мутанти *waaL* були створені шляхом обміну алелями. Фенотипи створених мутантів візуалізували за допомогою гелів DOC-PAGE, забарвлених сріблом, та імуноблоту зі специфічними до кору і O-Ag моноклональними антитілами. Для аналізу стресостійкості мутантів використовували гіпертонічне живильне середовище, в якому аналізували ростові криві бактерій. **Результати.** Видалення гену $waaL_{OS}$ із геному бактерій YeO3 суттєво впливає на лігування кору як в одиничних, так і у подвійних мутантів. Делеція гену $waaL_{PS}$ має протилежний вплив на лігування OPS – відбувається ледь помітна стимуляція. Різке зниження ростово-

го потенціалу бактерій в гіпертонічному середовищі спостерігали тільки у подвійних мутантів бактерій YeO3. **Висновки.** Участь WaaL лігаз у формуванні стійкості бактерій YeO3 до гіпертонічного середовища виявлена тільки при делеції обох генів лігаз. Одиночні мутанти за генами лігаз не проявляють особливої чутливості до осмотичного тиску в середовищі, можливо спрацьовує компенсаторний механізм.

Ключові слова: *Yersinia enterocolitica*, ЛПС, WaaL лігази, осмотичний тиск.

РОЛЬ WAAL ЛИГАЗ В БИОСИНТЕЗЕ ЛИПОПОЛИСАХАРИДА И АДАПТАЦИИ К СТРЕССУ У БАКТЕРИЙ *YERSINIA ENTEROCOLITICA* O:3 И O:8

Ю.И. Шевченко[1,2], В.К. Позур[1], М. Скурник[2]

[1] Киевский национальный университет имени Тараса Шевченка
Украина, 03022, Киев-022, ул. Академика Глушкова, 2
[2] Haartman Institute, University of Helsinki
Finland, FIN-00014, Haartmaninkatu 3
(P.O. Box 21)
e-mail: julia.i.shevchenko@gmail.com

Цель. Целью данных исследований была оценка участия WaaL лигаз в синтезе липополисахарида (ЛПС) бактерий *Yersinia enterocolitica* O:3 и O:8 (YeO3, YeO8) и определение роли лигаз в росте бактерий в условиях осмотического давления. **Методы.** Одиночные и двойные мутанты *waaL* были созданы путем обмена аллелями. Фенотипы созданных мутантов визуализировали с помощью гелей DOC-PAGE, окрашенных серебром, и иммуноблота со специфическими к кору и O-Ag моноклональными антителами. Для анализа стрессоустойчивости мутантов использовали гипертоническую среду, в которой анализировали ростовые кривые бактерий. **Результаты.** Удаление гена $waaL_{OS}$ из генома бактерий YeO3 существенно влияет на лигирование кора как в случае одиночных, так и у двойных мутантов. Делеция гена $waaL_{PS}$ имеет противоположное влияние на лигирование OPS – происходит едва заметная стимуляция. Резкое снижение потенциала роста в гипертонической среде наблюдали только у двойных мутантов бактерий YeO3. **Выводы.** Участие WaaL лигаз в формировании устойчивости бактерий YeO3 к гипертонической среде было обнаружено только при делеции обоих генов лигаз. Одиночные мутанты по генам лигаз не проявляют особенной чувствительности к осмотическому давлению, возможно, срабатывает компенсаторный механизм.

Ключевые слова: *Yersinia enterocolitica*, ЛПС, WaaL лигазы, осмотическое давление.