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ROLE OF WAAL LIGASES IN SERUM RESISTANCE OF *YERSINIA ENTEROCOLITICA* SEROTYPES O:3 AND O:8

*The **aim** of current study was to estimate WaaL ligase contribution in lipopolysaccharide (LPS) phenotype profile formation of Y. enterocolitica serotype O:3 (YeO3) and O:8 (YeO8) bacteria and its participation in serum killing protection. In lipopolysaccharide (LPS) biosynthesis of Gram-negative bacteria the waaL-encoded ligase joins O-polysaccharide (O-Ag) and outer core (OC) onto lipid A-core oligosaccharide. Three waaL genes named as $waaL_{OS}$, $waaL_{PS}$ and $waaL_{XS}$ were identified from Yersinia enterocolitica genome. **Methods**. The waaL-knock-out mutants were created by 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. To study the contribution of $WaaL_{OS}$ and $WaaL_{PS}$ to the survival of Yersinia bacteria in non-immune human serum, we constructed the series of single and double ligase mutants. Survival of bacteria was analyzed in normal serum (with functional classical, lectin, and alternative complement activation pathways) and EGTA-Mg-treated serum (only alternative pathway functional). **Results**. Our results demonstrated that WaaL ligases participate in the synthesis of proper LPS structure and play an important role in protection against serum killing. **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 then $WaaL_{PS}$ one.*

Key words: WaaL ligase, lipopolysaccharide, Yersinia enterocolitica, serum resistance.

Among humans, *Y. enterocolitica* causes intestinal disease, such as enterocolitis, with inflammatory diarrhea, ileitis, mesenteric appendicitis and gastroenteritis. The pathogen crosses the epithelial barrier through the M cells of Peyer's patches and infects the underlying tissues. Survival in deeper tissues and serum resistance depends on *Yersinia* virulence factors encoded by genes located on the chromosome (Ail and lipopolysaccharide O-Ag and OC and on the 70-kb virulence plasmid (YadA and Yop proteins) [2].

Activation of complement starts with activation of C3, then cleavage product of C3, C3b, deposits on microbial surface and bind complement component C5. C5b, the cleavage product of C5, subsequently initiates the cascade of interactions that lead to insertion of the membrane attack complex (MAC) (C5b-9), into the bacterial membrane. This event results in bacterial lysis and cell death. Many microbes, however, evolved mechanisms to evade this attack (thick peptidoglycan layer, long O-side chains, bacterial capsules, etc.) The C3b is the key protein of complement activation, which binds more strongly to YadA-negative bacteria grown at 37 °C. Only *Y. enterocolitica* bacteria grown at 37°C are resistant to killing by alternative pathway (AP), classical pathway (CP), or both (CP/AP) [2].

Y. enterocolitica genome contains the genes $waaL_{OS}$ and $waaL_{PS}$ encoding WaaL-ligases responsible for substituting lipidA with oligo- and polysaccharide moieties [2]. In this report, we analyzed serum resistance of *Y. enterocolitica* O:3 and *Y. enterocolitica* O:8 using single and double *waaL* mutants. The strains were analyzed for serum resistance in a killing assay in normal and EGTA [ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetra-acetic acid]-Mg serum.

Current investigations are important for evaluation of biological role and participation of WaaL ligases of *Yersinia enterocolitica* O:3 and O:8 in virulence realization. To this end we attempted serum-killing assay as a conventional system for estimating waaL-mutants pathogenicity. The obtained data indicated that the mutants with disrupted *waaL* genes were less resistant to serum killing.

Further investigations of WaaL ligases will concern importance of the ligases to the *Y. enterocolitica* pathogenesis. Knowledge of this pathway will enable the development of effective inhibitors and create new approaches to yersiniosis treatment.

Materials and Methods

Bacterial strains and culture conditions. Bacterial strains are listed in Table 1. For bactericidal assay, bacteria were grown to stationary phase overnight in 5 ml of Luria Broth (LB) media at 22–25 ºC (RT) for *Yersinia* and at 37 ºC for *E. coli* strains. To study serum resistance of *Yersinia* strains and their *waaL* mutants bacteria were grown at 37 °C. 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, 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 were 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.

Mutant construction. The $waaL_{OS}$ and $waaL_{PS}$ genes were amplified by PCR with primer pairs O3ligYE1727F5 & O3ligYE1727R5 and O3ligYE532F2 & O3ligYE532R2 using the Dynazyme II DNA-polymerases (Thermo Scientific) and genomic DNA of *Y. enterocolitica* O:3 as 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 (YeO3) and O:8 (YeO8) strains by conjugation as described earlier [1]. For elimination suicide vector and the wild-type genes was used optimized cycloserine enrichment method [1]. For large-scale screening of knock-out mutants among Clm^S colonies we 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 of wild-type strain YeO3 or YeO8 were used as a control.

Immunoblotting. To detect YadA expression in mutants, bacteria were grown overnight at 37 °C in 5 ml of LB with an appropriate antibiotics. 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 polyacrylamide gels. The separated samples were transferred to nitrocellulose membranes and nonspecific binding sites were blocked by immersing the membranes in a 5% skimmed milk–PBS solution (for 1h at RT). The membranes were incubated overnight at 4 °C with monoclonal antibody (mAb) specific for YadA (mAb 3G12), diluted 1:10. After four washes with PBS (each for 10 min), the membranes were incubated with peroxidase-conjugated rabbit anti-mouse immunoglobulins (P0260; dilution 1:2,000) for 1h at RT. Antibody binding was detected by chemiluminescence using the ECL Western blotting detection reagents (Amersham Pharmacia Biotech) according to the manufacturer's instructions [2].

Serum-killing assay. Normal human serum (NHS) was obtained from healthy human donors who were devoid of anti-*Yersinia* antibodies. Blood was allowed to clot for 15 min at room temperature and for 60 min at 4 °C. Following centrifugation (4 °C, 2,500 x g, 30 min), the sera were collected, pooled, and stored at – 70 °C in aliquots of 0.5 ml. Prior to use, the serum was allowed to thaw on ice. One-third of it was heat inactivated by incubation at 56 °C for 30 min (HIS). To block the CP activity, EGTA and $MgCl_2$ were added to another third to final concentrations of 10 and 5 mM, respectively. The last third represented the normal human serum [2]. Bacterial cultures were diluted appropriately to obtain 1.000–1300 bacteria in 10 µl. Triplicates of 10 µl of bacterial suspensions were incubated with 20 µl of NHS (final NHS concentration of 66.7%), 20 µl of HIS, or 20 µl of EGTA-Mg serum at 37 °C for 30 min. Before plating on LB plates with appropriate antibiotics, 70 µl of brain heart infusion broth (BHI) was added to each mixture to stop the complement function, and tubes were kept on ice. The serum bactericidal effect was calculated as

the survival percentage taking the bacterial counts obtained with bacteria incubated in HIS as 100%. The killing experiment was repeated three times for each strain.

Results and Discussion

The main goal was to characterize whether the absence of the WaaL ligase activity would influence the inherent serum resistance of pathogenic *Y. enterocolitica* serotype O:3 and O:8 bacteria. For this purpose we have constructed single and double *waaL*-knock-out mutants of YeO3 and YeO8 pathogenic strains.

Construction of *Y. enterocolitica* ligase mutants. Fully virulent *Y. enterocolitica* O:3 and O:8 strains were used for construction of *waaL* mutants. With help of allelic exchange strategy we managed to inactivate the $waaL_{OS}$ and $waaL_{PS}$ encoding regions. Obtained mutants were confirmed by colony hybridization, which was used for specific detection of the deletion in the *waaL* gene of Clm sensitive bacteria (Clm^S). Further verification of deletion was performed by PCR.

Presence of virulence plasmid and expressing of YadA protein was checked to be immunoblotting with mAb anti-Yad A (Fig. 1).

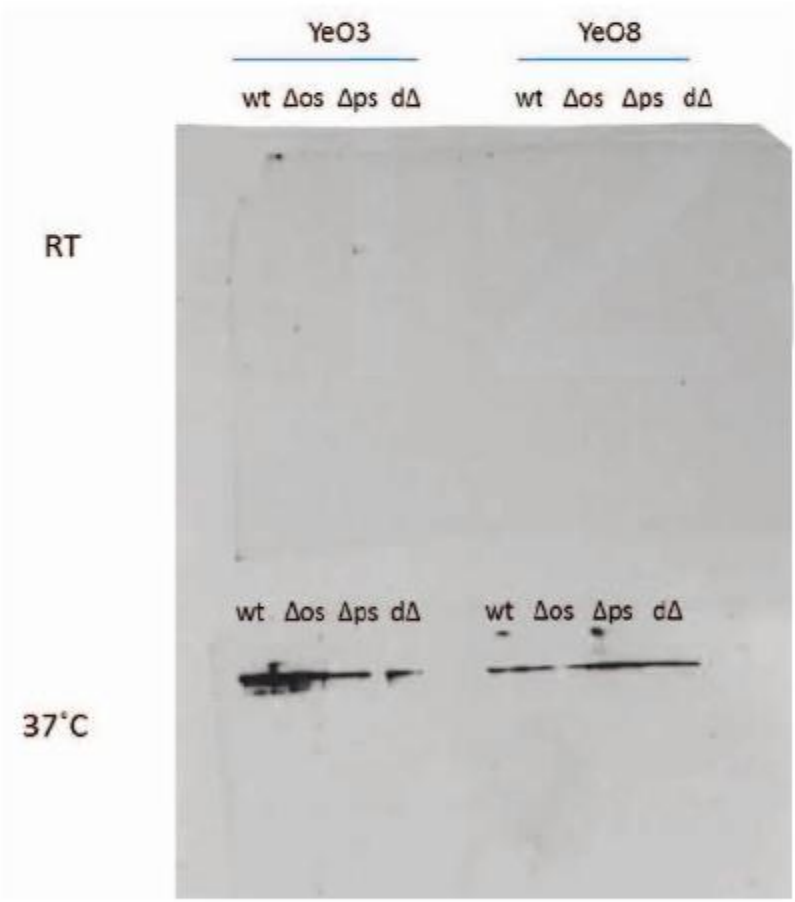

Fig. 1. Immunoblot analysis with mAb specific for YadA (wt-wild type, Δos & Δps – $waaL_{OS}$ & $waaL_{PS}$ single mutants, dΔ – double mutants)

CP/AP- and AP-mediated killing. According to the obtained results, we can see a clear difference between surviving of wild type bacteria and *waaL*-kock-out mutants of YeO3 and YeO8 in 30 min exposition with human serum. The single deletion of $waaL_{OS}$ and $waaL_{PS}$ genes reduced resistance of bacteria to NHS 2.5–5 times, respectively, compared to wild type YeO3. Double ligase mutants of YeO3 under NHS treatment didn't survive at all (Fig. 2A). AP-mediated killing with the same samples was in 2.4 (YeO3_os) and 1.3 (YeO3_ps & YeO3_os_ps) times more efficient than wild type ones (Fig. 2B).

Furthermore, single and double ligase mutants of YeO8 showed similar decreasing of resistance to NHS as YeO3 ones (Fig. 2C). In AP-mediated killing experiment with YeO8 mutants, however, we didn't follow the same trend. The single ligase mutants survived as well as wild type bacteria and double mutants in two times better then others (Fig. 2D).

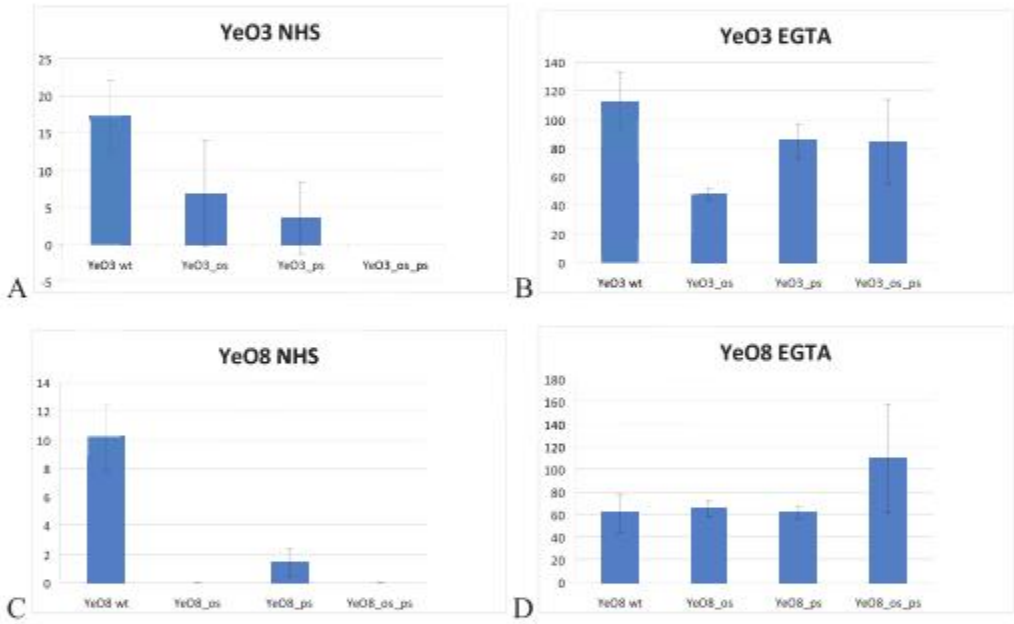

Fig. 2. Survival percentage of YeO3 ligase mutants in NHS (A) & EGTA-treated serum (B) and YeO8 (C, D)

These findings show that LPS' ligase deletion contributes to human serum resistance of *Yersinia enterocolitica* O:3 and O:8 bacteria. In general, bacterial exposition with normal serum caused total killing of double ligase mutants and provided much less surviving of single ones compared to wild type. It was some difference between serotypes but common tendency was the same.

Comparing two virulent serotypes, we have noticed some difference in surviving. It can be explained by LPS structure distinctions between YeO3 & YeO8 and the complement proteins deposition potential. It was observed that O-Ag, as a single

factor, could prevent deposition of covalently bound C3b on YeO3 bacteria after exposing bacteria to EGTA-Mg-treated serum in the beginning of incubation [2]. Indeed, we can follow sharp decreasing of surviving among $waaL_{OS}$ ligase mutants of YeO3 after exposing bacteria in EGTA-Mg-treated serum. On the other hand, we didn't observe the same tendency with YeO8 $waaL_{OS}$ ligase mutants and with double mutants of both serotypes. In the same way, survival rate of *waaL*-knock-out mutants of *Edwardsiella tarda* declined dramatically compared to parent strain [3].

It was considered that O-Ag played an important role in inhibition of the early phase of alternative pathway activation [4]. As for OC, previous studies do not indicate any direct involvement of OC in serum resistance [5]. It was shown that OC-positive, YadA- and Ail-negative strains were efficiently killed by complement. In the absence of YadA, however, OC seemed to potentiate Ail-mediated resistance [2].

Conclusions

In this work we have characterized the biological role of WaaL ligases in serum killing system. The results of normal serum killing showed clear reduction of serum resistance among single and double ligase mutants for both serotypes. YeO8 ligase mutants, however, showed full resistance to AP-mediated killing. Further work will be needed to clarify the role of WaaL LPS' ligases of *Y. enterocolitica* for virulence *in vivo*.

Table 1.

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, KmR	This work
	YeO3_ Δos_ Δps	$waaL_{os}$::pSW23T-lig1727su $waaL_{ps}$::pSW29-lig-532del, KmR	This work
	8081	YeO8 wild type strain, patient isolate	[7]
	8081-L2	R^-M^+ derivative of wild-type strain 8081; serotype O:8; pYV+	[8]
	YeO8_Δos	$waaL_{os}$::pSW23T-lig1727su, pYV+, ClmR	This work
	YeO8_Δps	$waaL_{ps}$::pSW29-lig532del, KmR	This work
	YeO8_Δos_ Δps	$waaL_{os}$::pSW23T-lig1727su $waaL_{ps}$, ClmR::pSW29-lig532del, KmR	This work
Escherichia coli	ω7249	B2163Λnic35, *E. coli* strain for suiside vector delivery, requirement for diaminopimelic acid 0.3mM, KmR	[9]
	S17-1λ pir	A-pir lysogen of S17-1, *E. coli* strain for suiside vector delivery	[10]
	DH10B	F- mcrA Δ(mrr-hsdRMS-mcrBC), Φ80lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ- rpsL nupG tonA	Life Technologies

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УЧАСТЬ WAAL ЛІГАЗ БАКТЕРІЙ *YERSINIA ENTEROCOLITICA* O:3 ТА O:8 В ФОРМУВАННІ РЕЗИСТЕНТНОСТІ ДО СИРОВАТКИ КРОВІ

Реферат

Метою даної роботи було дослідження участі WaaL лігаз в біосинтезі ліпополісахариду (ЛПС) бактерій *Yersinia enterocolitica* серотипів O:3 та O:8; їх ролі в формуванні фенотипу ЛПС та резистентності *waaL*-мутантів до сироватки крові. WaaL лігази каталізують приєднання О-антигену (O-Ag) та зовнішнього полісахаридного кору (ОС) до ліпіду А в процесі біосинтезу ліпополісахариду грамнегативних бактерій. В геномі бактерій *Y. enterocolitica* було ідентифіковано три гени лігаз, які були названі $waaL_{OS}$, $waaL_{PS}$ та $waaL_{NS}$. **Методи**. Нокаутні мутанти по генам лігаз *waaL* були створені шляхом обміну алелями. Візуалізація фенотипів ЛПС створених мутантів відбувалась шляхом забарвлення сріблом гелю DOC-PAGE та імуноблоту з специфічними моноклональними антитілами до кору та О-полісахариду. Оцінювали виживання бактерій в нормальній сироватці крові (присутні класичний, лектиновий та альтернативний шлях активації комплементу) та обробленій EGTA (тільки альтернативний шлях). **Результати**. Отримані результати підтверджують участь WaaL лігаз в синтезі належної молекули ЛПС та відіграють важливу роль в формуванні резистентності до бактерицидної дії сироватки крові. **Висновки**. Лігази ЛПС бактерій демонструють низьку субстрату специфічність. За даних умов участь $WaaL_{OS}$ лігази в лігуванні ОС та ОПС на ліпід А є істотнішою ніж $WaaL_{PS}$.

Ключові слова: WaaL лігаза, ліпополісахарид, *Yersinia enterocolitica*, резистентність сироватки.

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УЧАСТИЕ WAAL ЛИГАЗ БАКТЕРИЙ *YERSINIA ENTEROCOLITICA* O:3 И O:8 В ФОРМИРОВАНИИ РЕЗИСТЕНТНОСТИ К СЫВОРОТКЕ КРОВИ

Реферат

Целью работы было исследование участия WaaL лигаз в биосинтезе липополисахарида (ЛПС) бактерий *Yersinia enterocolitica* серотипов O:3 и O:8; их роли в формировании фенотипа ЛПС и устойчивости *waaL*-мутантов к сыворотке крови. WaaL лигази катализируют присоединение О-антигена (O-Ag) и внешнего полисахаридного кора (ОС) к липиду А в процессе биосинтеза ЛПС грамнегативных бактерий. В геноме бактерий *Y. enterocolitica* было идентифицировано три гена лигаз, которые были названы $waaL_{OS}$, $waaL_{PS}$ и $waaL_{XS}$. **Методы.** Нокаутные мутанты по генам лигаз *waaL* были созданы путем обмена аллелями. Визуализация фенотипов ЛПС созданных мутантов происходила путем окраски серебром гелей DOC-PAGE и иммуноблота со специфическими моноклональными антителами к кору и О-полисахариду. Оценивали выживание бактерий в нормальной сыворотке крови (присутствуют классический, лектиновый и альтернативный путь активации комплемента) и обработанной EGTA (только альтернативный путь). **Результаты.** Полученные результаты подтверждают участие WaaL лигаз в синтезе надлежащей молекулы ЛПС и играют важную роль в формировании резистентности к бактерицидному действию сыворотки крови. **Выводы.** Лигазы ЛПС бактерий демонстрируют низкую субстратную специфичность. При данных условиях участие $WaaL_{OS}$ лигазы в лигировании ОС и ОПС на липид А является более значимым чем $WaaL_{PS}$.

Ключові слова: WaaL лигаза, липополисахарид, *Yersinia enterocolitica,* резистентность сыворотки.

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