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Modification of *Escherichia coli* Lipid A by the Introduction of Myristoyltransferase Gene Cloned from *Klebsiella pneumoniae*

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Abstract

Late stages of lipid A biosynthesis of *Escherichia coli* are transfer reactions of lauric acid (C12:0) and myristic acid (C14:0) to the hydroxyl group of 3-hydroxy-myristic acid (3-OH-C14:0). In the previous study we constructed the mutant strains with disrupted C12:0-transferase and C14:0-transferase genes and used those mutant strains for the modification of lipid A by the introduction of foreign acyltransferase genes. In the study reviewed here, the C14:0-transferase gene (*lpxL2*) of *Klebsiella pneumoniae* was cloned and introduced to the mutant strains by transformation to modify the lipid A structure. Lipopolysaccharide (LPS) preparations of the transformants were analyzed through chemical modification and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and were proved to have the lipid A with one C14:0, or two C14:0, one of which replaced C12:0 bound to 3-OH-C14:0 at the C2-position of the non-reducing end glucosamine. The IL-6-inducing activity of the LPS with C14:0 was measured, and compared with that of the original LPS with C12:0. The activity of LPS with C14:0 was found to be comparable with that of LPS with C12:0, suggesting that C14:0 can replace C12:0 without changing the immune-stimulating activity of lipid A.

Keywords

Lipid A
Fatty acid transferase
Mass spectrometry
IL-6
E. coli

1. Introduction

The importance of the structure of lipopolysaccharide (LPS), particularly the carbon chain of fatty acids and the position of their linkages, for the immune activity exhibited was demonstrated in the chemical synthesis of lipid A by Imoto *et al.* in the 1980s [1]. On the other hand, if *Escherichia coli* can synthesize lipid A with various structures, it would be a useful method for the production of immunostimulants which is different from chemical synthesis. From the point of view of modifying the structures involved in activity, the easiest way is to replace lauric acid ($C_{12:0}$) and myristic acid

($C_{14:0}$), which are added in the later part of *E. coli* lipid A biosynthesis to form branched-chain structures, with other fatty acids or to change the bonding position. To modify the structure, we generated disruption mutants of the $C_{12:0}$ transferase gene or the $C_{14:0}$ transferase gene with reference to other studies and introduced the Salmonella $C_{16:0}$ transferase gene into these strains [2], which successfully synthesized lipid A with a structure not normally found in *E. coli* [3,4]. Using a similar approach, it is thought that lipid A with a variety of fatty acid branched-chain structures could be produced by introducing fatty acid transferase genes from various gram-negative bacteria into the aforementioned mutant strains. In the course of investigating fatty acid transferases, it was recently reported that *Klebsiella pneumoniae* possesses an enzyme gene that transfers $C_{14:0}$ to the $C_{12:0}$ position of *E. coli* lipid A [5]. We, therefore, cloned the gene, introduced it into a mutant strain, and investigated changes in the structure and immune activity of lipid A as part of the aforementioned research [6].

2. Changes in the lipid A fatty acid composition by the introduction of the myristoyltransferase gene.

In our previous studies, a wild-type strain (strain KGU0107), a $C_{14:0}$ transferase gene disruption strain (strain KGU0221), and a double mutant strain (strain KGU0377) in which the $C_{12:0}$ and $C_{16:0}$ transferase genes were disrupted were generated from *E. coli* BL21 [3]. The KGU0377 strain lacks $C_{12:0}$ and therefore also has little $C_{14:0}$.

K. pneumoniae was reported to possess a $C_{14:0}$ transferase gene (*lpxL2*), which is separate from the $C_{12:0}$ transferase, to transfer $C_{12:0}$ or $C_{14:0}$ at the $C_{12:0}$ position of *E. coli* lipid A [5]. Therefore, following this paper, the $C_{14:0}$ transferase gene was amplified by polymerase chain reaction (PCR) and cloned using the vector plasmid pUC119. The resulting plasmid, pUC119-lpxL2, was introduced into the aforementioned mutant strains KGU0377 or KGU0221 by transformation and the fatty acid composition of the LPS was examined using gas chromatography. The results showed that the amount of $C_{14:0}$ in one molecule of lipid A of the transformant strain KGU0485, obtained from strain KGU0377, increased from about 0.2 molecules to more than two molecules. In the KGU0221 strain, the $C_{12:0}$ of lipid A was almost completely eliminated by plasmid transfection, and the $C_{14:0}$ of lipid A was reduced to about 0.2 molecules in the KGU0485 strain, suggesting that this extreme increase was not only due to the action of the transfected *lpxL2* but also to the action of the $C_{14:0}$ transferase native to *E. coli*, which transferred $C_{14:0}$ at the $C_{12:0}$ position. In the KGU0221 strain, the $C_{12:0}$ of lipid A was almost completely eliminated by the introduction of the plasmid, and instead, $C_{14:0}$ was increased to about one molecule per lipid A. The reason why $C_{12:0}$ was almost completely replaced by $C_{14:0}$ in strain KGU0496 was considered to be the copy number of the vector plasmid (pUC-based plasmids have high copy number), i.e. the quantitative effect of the gene, which preferentially caused the $C_{14:0}$ transfer.

3. Analysis of lipid A structure by mass spectrometry

Mass spectrometry is suitable for the accurate analysis of the number of fatty acids in the lipid A molecule. In our laboratory, LPS has been hydrolyzed with a weak acid (0.1 M HCl, 100°C, 30 min) and the resulting C1-position dephosphorylated lipid A analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), and analytical data have been accumulated. Lipid A from KGU0496 and KGU0485 was analyzed using this method, and the peak with the highest mass number, *m/z* 1745, was detected in KGU0485, and *m/z* 1534 in KGU0496. These peaks indicate the presence of two or one molecule of $C_{14:0}$ in lipid A, respectively.

As for the binding position of $C_{14:0}$, it is reasonable to assume that $C_{14:0}$ is transferred to the usual $C_{12:0}$ position, but to confirm this, the dephosphorylated lipid A used in the above analysis was further hydrolyzed

under weak alkaline conditions (150-fold diluted triethylamine, 100°C, 2 h) and analyzed by MALDI -TOF MS. As shown in **Figure 1**, two peaks with two molecules of $C_{14:0}$ and one peak with one molecule of $C_{14:0}$ were detected for KGU0485 lipid A. The peaks with the highest intensities were 2 molecules of 3-hydroxymyristic acid (3-OH-$C_{14:0}$) and one molecule of $C_{14:0}$. One molecule of $C_{14:0}$ was bound to 3-OH-$C_{14:0}$ attached to the hydroxyl group at the C3 position of glucosamine and was easily released by weak alkaline treatment, while the other molecule was bound to 3-OH-$C_{14:0}$ attached to the amino group at the C2 position, indicating that the other molecule is relatively stable. On the other hand, for KGU0496 strain lipid A, as shown in **Figure 2**, all peaks contained one molecule of $C_{14:0}$, indicating that $C_{14:0}$ was not released by this weak alkaline degradation. Therefore, this $C_{14:0}$ was shown to be bound to the 3-OH-$C_{14:0}$ bonded to the amino group. It should be noted that the fact that these $C_{14:0}$ that are not bound to 3-OH-$C_{14:0}$ bonded to glucosamine on the reducing end was also confirmed by the detection of oxonium ions from non-reducing terminal glucosamine and by a combination of other chemical degradations and mass spectrometry, which the details are omitted.

Based on the above information, lipid A of strains KGU0485 and KGU0496 was deduced as shown in **Figures 3A** and **3B**. Both structures were considered to have a $C_{14:0}$ substitution at the original $C_{12:0}$ position.

4. Immunological activity of LPS with modified lipid A

The IL-6 production-inducing activity of LPS from KGU0485 and KGU0496 strains obtained by transfection with the $C_{14:0}$ transferase gene was examined as an indicator of immune activity and

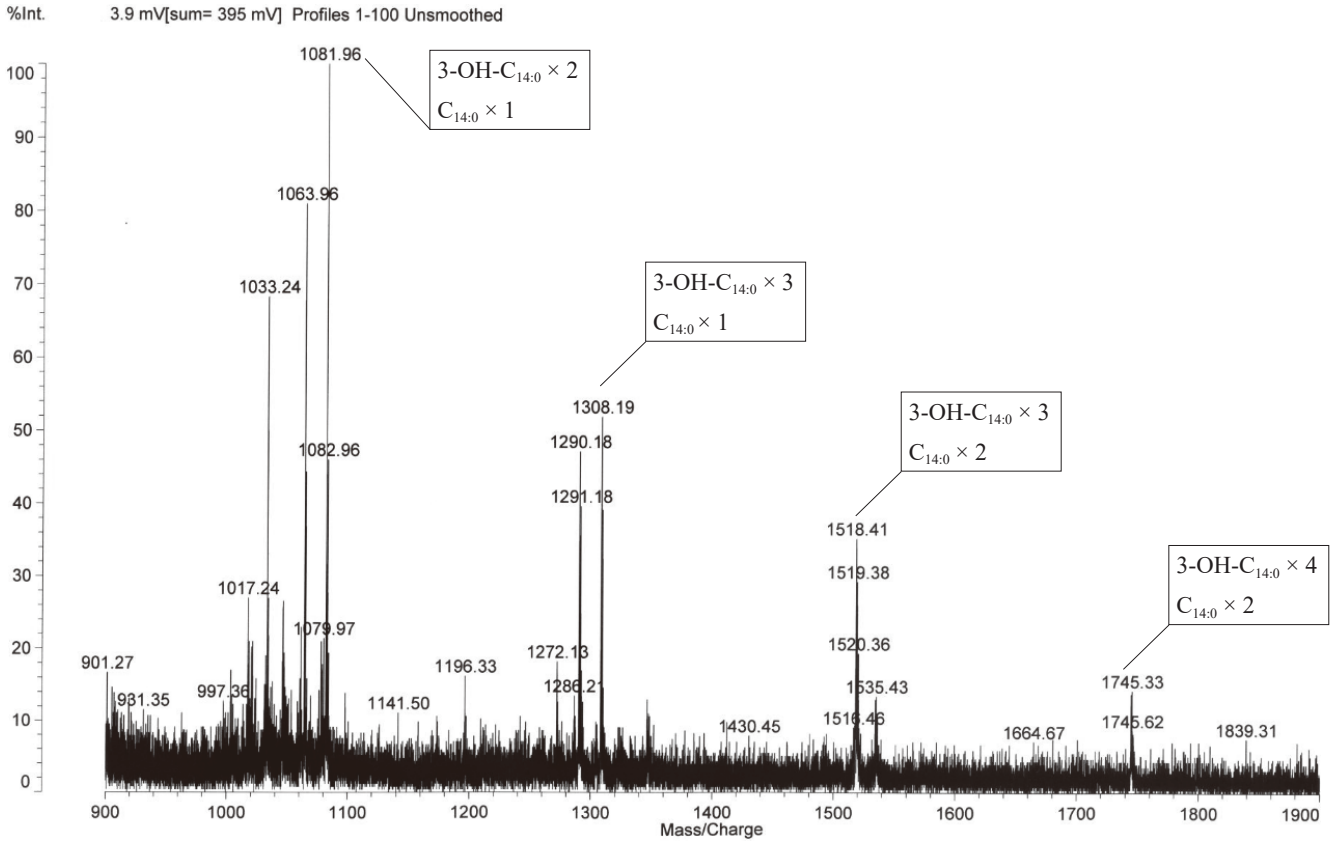

Figure 1. MALDI-TOF mass spectra of lipid A degradation products obtained from KGU0485 strain LPS by weak acid hydrolysis and weak alkaline hydrolysis. Anion $[M-H]^-$ detected in negative mode.

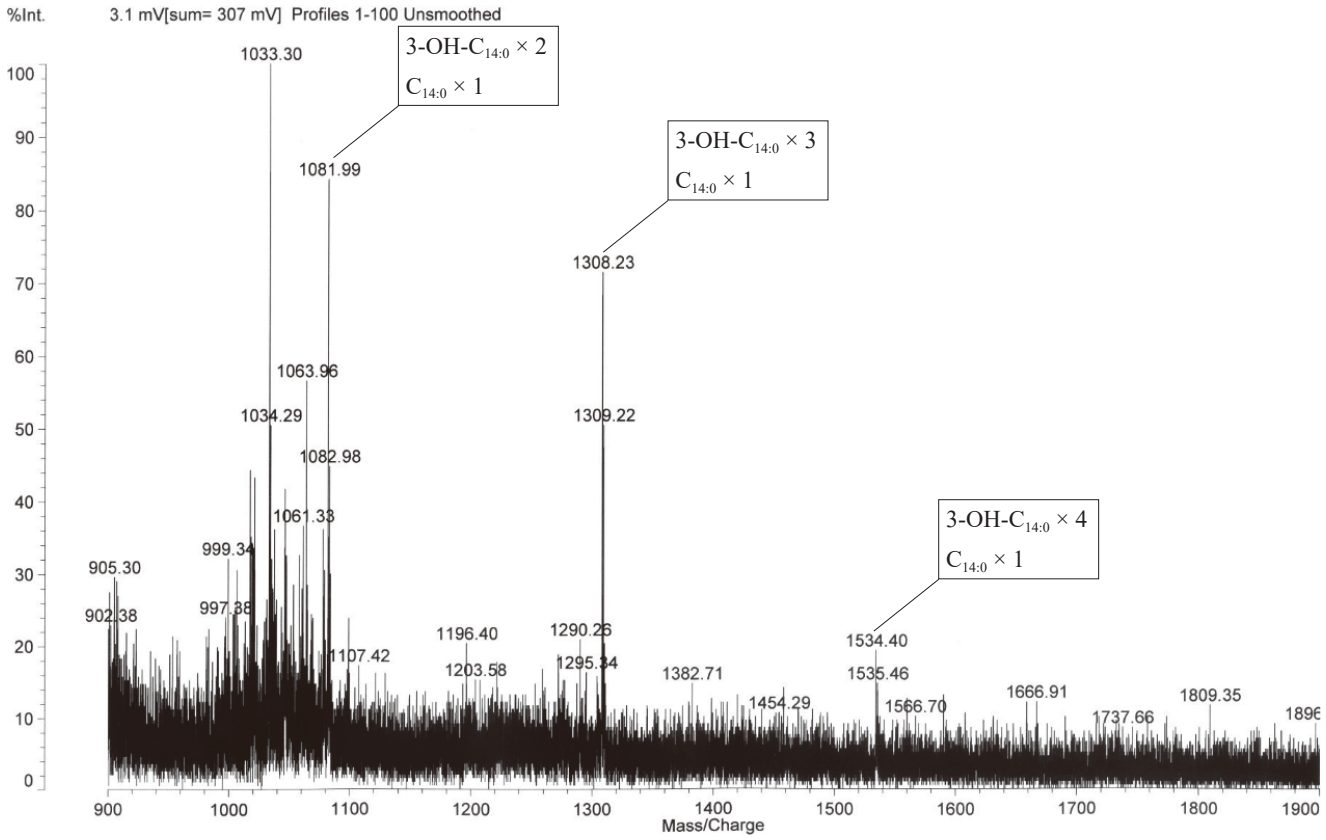

Figure 2. MALDI-TOF mass spectra of lipid A degradation products obtained from KGU0496 strain LPS by weak acid hydrolysis and weak alkaline hydrolysis. Anion $[M-H]^-$ detected in negative mode

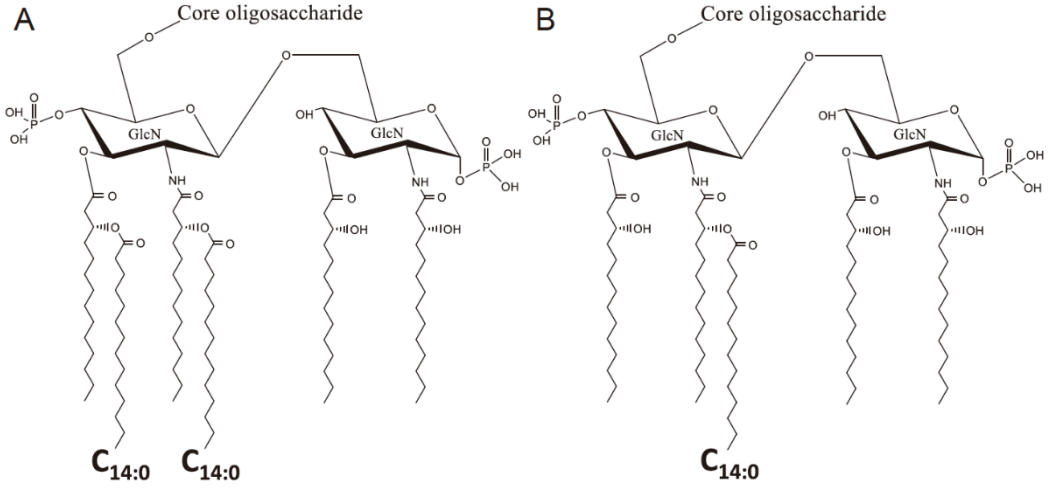

Figure 3. Predicted chemical structure of myristate transferase transgene strain lipid A. (A) Chemical structure of KGU0485 strain lipid A; (B) Chemical structure of KGU0496 strain lipid A.

compared with that of the original strains. The human-derived monoblast-like cell line U937 cells were used for the assay and incubated with phorbol myristate acetate (PMA) at 100 nM for 3 days, then stimulated with LPS of each strain and incubated for 6 hours, after which IL-6 produced in the culture medium was quantified using sandwich enzyme-linked immunosorbent assay (ELISA).

Figure 4A compares the LPS activity of the wild-type strain (KGU0107) and two gene disruption mutants, KGU0377 and KGU0221 extracted from a previous paper [3], showing that the KGU0377 LPS lacks both $C_{12:0}$ and $C_{14:0}$ and is therefore extremely inactive compared with the wild-type LPS. In contrast, the activity of the LPS of strain KGU0221 was stronger than that of strain KGU0377 and was comparable to that of strain KGU0107 at high concentrations. Based on these data, the results of the KGU0485 and KGU0496 LPS assays showed interesting results: the LPS of the KGU0485 strain was as active as that of the wild-type KGU0107 strain and the LPS of the KGU0496 strain was as active as the LPS of KGU0221 strain (**Figure 4B**). As mentioned above, lipid A in the KGU0485 strain LPS has $C_{12:0}$ replaced by $C_{14:0}$ in the wild strain lipid A, and lipid A in the KGU0496 strain LPS also has the $C_{12:0}$ replaced by $C_{14:0}$ structure in the KGU0221 strain lipid A. Therefore, these results indicate that $C_{14:0}$ can replace $C_{12:0}$ in terms of immune activity. Considering this from the receptor point of view, it is assumed that the LPS receptor, toll-like receptor 4 (TLR4), does not discriminate between 3-OH-$C_{14:0}$ bound to the C2-position of the non-reducing terminal glucosamine and the fatty acid forming the branched chain, which differs by two carbon chains.

5. Conclusion

With regard to the modification of the fatty acid branched chain structure of lipid A, it is relatively easy to change the carbon chain of the fatty acid in the case of chemical synthesis, and it seems possible to change the bonding position as well. In contrast, the method of modifying the lipid A structure by introducing a fatty acid transferase has the disadvantage that the structure that can be produced is defined by the type and properties of the enzyme. However, it may be interesting in terms of the unexpectedness of the structures that can be produced. Furthermore, as large quantities of LPS can be easily obtained by cultivating

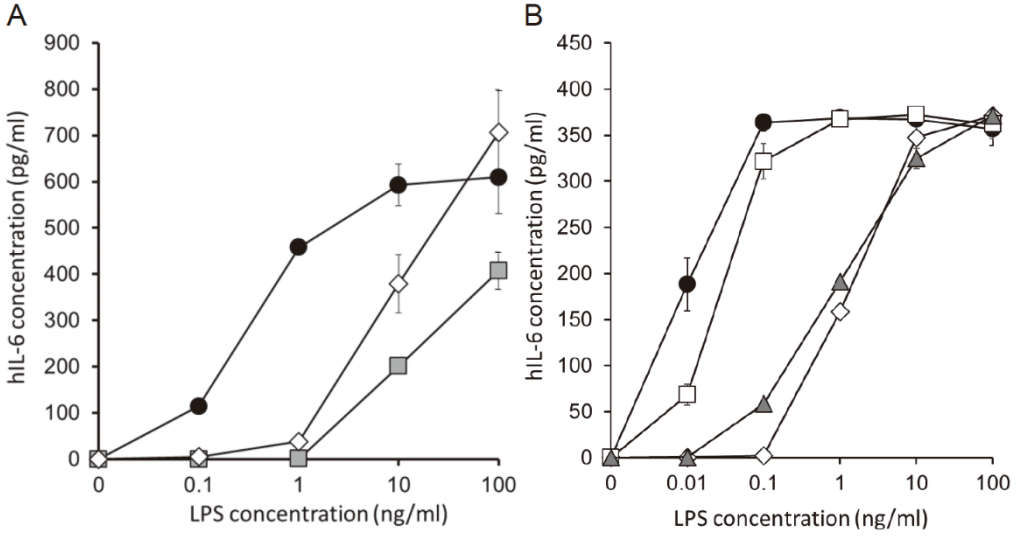

Figure 4. The activity of LPS from wild-type, fatty acid transferase gene disruption, and myristoyltransferase transgenic strains in inducing IL-6 production in U937 cells. (A) ●, KGU0107 strain (wild strain); ◇, KGU0221 strain; ■, KGU0377 strain; (B) ●, KGU0107 (wild strain); ◇, KGU0221; □, KGU0485; ▲, KGU0496.

the bacteria, this aspect is expected to have a wider range of applications. Further modifications of *E. coli* lipid A using fatty acid transferases from other bacterial species will be carried out in the future.

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Disclosure statement

The authors declare no conflict of interest.

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