Full Paper

Biosynthesis of glycolipid MPIase (membrane protein integrase) is independent of the genes for ECA (enterobacterial common antigen)

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MPIase (membrane protein integrase) is an essential glycolipid that drives protein integration into the inner membrane of *E. coli*, while glycolipid ECA (enterobacterial common antigen) is a major component at the surface of the outer membrane. Irrespective of the differences in molecular weight, sub-cellular localization and function in cells, the glycan chains of the two glycolipids are similar, since the repeating unit comprising the glycan chains is the same. A series of biosynthetic genes for ECA, including ones for the corresponding nucleotide sugars, have been identified and extensively characterized. In this study, we found that knockouts as to the respective genes for ECA biosynthesis can grow in the minimum medium with the normal expression level of MPIase, indicating that MPIase can be biosynthesized *de novo* without the utilization of any compounds generated through ECA biosynthesis. Conversely, ECA was expressed normally upon MPIase depletion. From these results, we conclude that the biosynthetic genes for MPIase and ECA are independent.

Key Words: biosynthesis; cell envelope; *cdsA*; ECA; MPIase; *wec* genes; *ynbB*

Abbreviations: MPIase, membrane protein integrase; DAG, diacylglycerol; Fuc4NAc, 4-acetamido-4-deoxyfucose; ManNAcA, 2-acetamido-2-deoxymannuronic acid; GlcNAc, N-acetylglucosamine; ECA, enterobacterial common antigen; TCA, trichloroacetic acid

Introduction

MPIase (membrane protein integrase) is a glycolipid that drives membrane protein integration into the inner membrane of *E. coli* (Endo and Nishiyama, 2015; Nishiyama and Shimamoto, 2014; Nishiyama et al., 2006, 2010, 2012). MPIase integrates a subset of membrane proteins of small size and, with a transmembrane domain at the C-terminus, leads us to propose the concept of ‘glycolipozyme’ (Nishiyama et al., 2012). For the membrane integration requiring signal recognition particle and SecYEG, MPIase is necessary (Nishiyama et al., 2006). Moreover, MPIase is also involved in protein translocation of presecretory proteins (Nishiyama et al., 2006; Sawasato et al., 2019a, b), by affecting the dimer structure of SecYEG (Moser et al., 2013).

The structure of MPIase has been determined to be as shown in Fig. 1 (Nishiyama et al., 2012). MPIase contains a glycan chain and diacylglycerol (DAG) linked through pyrophosphate. The glycan chain is composed of ~10 repeats of a unit of three N-acetylated amino sugars, 4-acetamido-4-deoxyfucose (Fuc4NAc), 2-acetamido-2-deoxymannuronic acid (ManNAcA), and N-acetylglucosamine (GlcNAc). This glycan is similar to that of glycolipid ECA (enterobacterial common antigen) (Fig. 1), and is exposed on the outer surface of the outer membrane (Kuhn et al., 1988; Rick and Silver, 1996; Rinno et al., 1980). Although the number of repeats in ECA is more heterogeneous and longer (18~55) than that in MPIase (9~11), the repeating unit of the two glycolipids is the same (Nishiyama et al., 2012). One more difference in these glycolipids is that the linker between the glycan and DAG is monophosphate in ECA (Fig. 1) (Nishiyama et al., 2012). In addition to the similarity of the glycan chains, the fact

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Fig. 1. Chemical structures of MPIase and ECA.

The repeating unit of the glycan chain consists of Fuc4NAc, ManNAcA and GlcNAc. The number of the repeat (n) is 9–11 for MPIase, and 18–55 for ECA, respectively. The linker between the glycan chain and diacylglycerol is pyrophosphate in MPIase and monophosphate in ECA, respectively. R^1 , acyl chains of C_{16} or C_{18} ; R^2 , Ac or H. The structures of compound I and DGP-disaccharide are also indicated.

Fig. 2. Biosynthetic pathways for MPIase and ECA.

Schematic representations for MPIase (A) and ECA (B) biosynthesis are shown. G, GlcNAc; M, ManNAcA; F, Fuc4NAc; Glc, glucose; R, rhamnose.

Table 1. Bacterial strains used in this study, and their doubling times in M9 medium supplemented with glycerol.

Strain	Genotype	Doubling time (h)*
BW25113	<i>rrmB3 ΔlacZ4787 hsdR514 Δ(araBAD)567 Δ(rhaBAD)568 rph-1</i>	2.4+/-0.2
JW3029-KC	BW25113 <i>ΔbacA::kan</i>	2.1+/-0.3
JW3758-KC	BW25113 <i>ΔwecA::kan</i>	1.9+/-0.1
JW5600-KC	BW25113 <i>ΔwecB::kan</i>	1.8+/-0.2
JW5599-KC	BW25113 <i>ΔwecC::kan</i>	2.1+/-0.4
JW5597-KC	BW25113 <i>ΔwecD::kan</i>	2.3+/-0.3
JW3765-KC	BW25113 <i>ΔwecE::kan</i>	2.1+/-0.2
JW5596-KC	BW25113 <i>ΔwecF::kan</i>	2.2+/-0.2
JW3770-KC	BW25113 <i>ΔwecG::kan</i>	2.1+/-0.1
JW5598-KC	BW25113 <i>ΔrffG::kan</i>	2.2+/-0.4
JW3763-KC	BW25113 <i>ΔrffH::kan</i>	2.2+/-0.1
JW2024-KC	BW25113 <i>ΔrmlA::kan</i>	2.1+/-0.2
JW2026-KC	BW25113 <i>ΔrmlB::kan</i>	2.1+/-0.3
JW3766-KC	BW25113 <i>ΔwzxE::kan</i>	1.9+/-0.6
JW5601-KC	BW25113 <i>ΔwzzE::kan</i>	2.2+/-0.3
NF07	BW25113 <i>ΔrffH ΔrmlA::kan</i>	1.7+/-0.1
NF08	BW25113 <i>ΔrffG ΔrmlB::kan</i>	1.9+/-0.2
KS23/pAra-CdsA	MC4100 <i>ara⁺ ΔcdsA::cat ΔynbB/pAra-CdsA (ParaB-cdsA spc^r)</i>	nd

*Doubling time is indicated as average +/- standard deviation calculated for four independent experiments. nd, not determined.

that ManNAcA and Fuc4NAc are not the major sugars suggests that the biosynthesis of these sugars is shared in biosynthesizing the two glycolipids.

ECA is biosynthesized by the series of gene products summarized in Fig. 2B (Erbel et al., 2003; Jorgenson et al., 2016; Mitchell et al., 2018). The repeating unit is synthesized on undecaprenol on the cytoplasmic surface of the inner membrane, followed by translocation to the periplasmic surface and then polymerization (Erbel et al., 2003; Mitchell et al., 2018). On the other hand, the unit for MPIase is synthesized on phosphatidic acid (Fig. 2A) (Sawasato et al., 2019a), reflecting the difference in sub-

cellular localization. The first step of MPIase biosynthesis is catalyzed by CdsA/YnbB, a CDP-DAG synthase, through incorporation of GlcNAc-phosphate into CDP-DAG (Sato et al., 2019; Sawasato et al., 2019a). Depletion of CdsA/YnbB results in MPIase depletion (Sato et al., 2019; Sawasato et al., 2019a). To determine whether or not the biosynthetic genes overlap in the biosynthesis of the two glycolipids, we examined the effects of gene disruption of the respective genes on expression of the other glycolipid. We found that the biosynthetic genes for glycolipids MPIase and ECA are independent.

Materials and Methods

Bacterial strains and media. The bacterial strains used in this study are listed in Table 1. The Keio collection (Baba et al., 2006) was obtained from NBRP (NIG, Japan): *E. coli*. MPIase was depleted by cultivating KS23/pAra-CdsA cells in the absence of arabinose, as described (Sawasato et al., 2019a). M9 minimum medium supplemented with 0.2% glycerol and LB broth (Maniatis et al., 1982) were used.

NF07 ($\Delta rffH \Delta rmlA::kan$) and NF08 ($\Delta rffG \Delta rmlB::kan$) were constructed as follows. The *kan* cassette in JW3763-KC (BW25113 $\Delta rffH::kan$) and JW5598-KC (BW25113 $\Delta rffG::kan$) was removed using plasmid pCP20 (Cherepanov and Wackernagel, 1995), followed by introducing the $\Delta rmlA::kan$ and $\Delta rmlB::kan$ alleles, respectively, by P1 transduction, as described (Datsenko and Wanner, 2000).

Immunoblot analysis. The cell culture was treated with 5% trichloroacetic acid (TCA) to precipitate cellular compounds (Sato et al., 2019). The precipitates were analyzed by SDS-PAGE, followed by transfer to PVDF membranes (Millipore), as described (Nishiyama et al., 1992). The membranes were dried, and then coated with 0.1% poly(isobutyl methacrylate) in cyclohexane for 75 s. After drying, the membranes were treated with 10% horse serum in TBS buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl), followed by incubation with anti-MPIase antiserum (Sawasato et al., 2019b). The bands that reacted with the anti-MPIase antibody (Nishiyama et al., 2012) were visualized by means of a phosphatase-labeled second antibody (Sugai et al., 2007). The bands were quantified using an ATTO CS analyzer. As a loading control, the level of RpmJ was visualized by means of the anti-RpmJ antibody (Ikegami et al., 2005).

TLC analysis and immunostaining of ECA. Cellular extracts, solubilized in 2.5% SDS, were spotted onto TLC sheets (Merck), followed by development with Solvent C (chloroform:ethanol:water = 3:7:4) (Nishiyama et al., 2010). ECA was then detected by immunostaining with an anti-MPIase antibody, which also reacts with ECA (Nishiyama et al., 2012). The TLC sheets were dried and then coated with poly(isobutyl methacrylate) as described above. After they had been dried, the TLC sheets were incubated in TBS buffer containing 10% horse serum, followed by incubation with anti-MPIase antiserum for 2 h at 37°C. Immunodecorated materials were then visualized as described (Sugai et al., 2007).

Results

Mutants as to ECA biosynthesis cultivated in the minimum medium express MPIase as much as the wild type strain

MPIase is essential for cell growth (Sato et al., 2019; Sawasato et al., 2019a), while ECA is not (Rick and Silver, 1996). If common gene products are utilized for the biosynthesis of precursors for Fuc4NAc and ManNAcA, characterized for MPIase and ECA, ECA mutants deficient in the biosynthesis of the respective nucleotide sugars would not be able to grow in the minimum medium

Fig. 3. The MPIase level is not affected in mutants as to ECA biosynthesis.

A. Overnight cultures of the indicated strains grown in LB medium were washed three times with M9 minimum medium, followed by inoculation into M9 minimum medium supplemented with 0.2% glycerol. The cell cultures were shaken at 37°C, the turbidity being monitored at 660 nm. B. The expression levels of MPIase in the mutants. When the growth reached the late log phase, an aliquot (0.5 mL) was withdrawn and treated with 5% TCA. The precipitate equivalent to 1.3×10^6 cells was then analyzed by SDS-PAGE and immunoblotting. The RpmJ (upper part of each panel) and MPIase (lower part) bands were visualized. The relative MPIase level of each mutant to that of WT was shown at the bottom of the MPIase blots. Standard deviations were calculated from three independent experiments. C. The expression levels of ECA in the mutants. The same samples as in B were subjected to TLC analysis, followed by immunostaining of ECA.

when such sugars had not been added to the medium. We cultivated the ECA mutants listed in Table 1 in M9 medium supplemented with glycerol as a carbon source (Fig. 3A). While the steady-state level differed, the doubling times at the log phase were similar among all the mutants examined (Table 1), indicating that MPIase biosynthesis, essential for growth, was not impaired in these mutant cells. When the expression level of MPIase in these mutants was determined, essentially the MPIase level remained the same in all the mutants (Fig. 3B), consistent with the results for cell growth (Fig. 3A). The normal expression of MPIase in knockouts as to genes involved in nucleotide sugar synthesis (*wecB-E*, *rffG-H* and *rmlA-B*) strongly suggest the presence of another set of genes dedicated to MPIase biosynthesis.

Next, we checked the ECA expression by immunostaining. All the *wec* mutants and the *rffH* mutant did not express a detectable amount of ECA (Fig. 3C). In the other mutants, the expression level of ECA was simi-

Fig. 4. The *rffH/rmlA* and *rffG/rmlB* double knockouts express MPIase. A. NF07 and NF08 cells were cultivated in M9/glycerol medium at 37°C, as described in Fig. 3A. The turbidity at 660 nm was monitored. B. The expression levels of MPIase in the mutants. The MPIase level was determined as described in Fig. 3B. C. The expression levels of ECA in the mutants. The ECA level was determined as described in Fig. 3C.

lar to that in the WT strain (Fig. 3C). The *rmlA* and *rmlB* genes (see Fig. 2B) are included in the *rml* (or *rfb*) operon that is involved in LPS biosynthesis. It is reported that dTDP-glucose is also utilized to biosynthesize dTDP-rhamnose, with which rhamnose is attached to LPS (Giraud and Naismith, 2000). Since the *rffH* mutant did not express a detectable amount of ECA, it is likely that the expression level of *rmlA* is very low. Since ribosome profiling analysis revealed that the protein synthesis rate for *rmlA* was faster than that of *rffH* (Li et al., 2014), the RmlA protein might be unstable in the cells. We next constructed an *rffH/rmlA* double knockout (NF07) and an *rffG/rmlB* double knockout (NF08), since these gene pairs catalyzed the same steps to biosynthesize dTDP- α -D-glucose and dTDP-4-dehydro-6-deoxy- α -D-glucose, respectively (Sivaraman et al., 2002). These double knockouts grew as well as other mutants in the M9/glycerol medium (Fig. 4A), with a doubling time comparable to those of other mutants (Table 1). MPIase was still expressed in these mutants as in other mutants (Fig. 4B), confirming the presence of genes for Fuc4NAc biosynthesis dedicated for MPIase biosynthesis. In NF07, no ECA expression was observed (Fig. 4C). On the other hand, ECA was normally expressed in NF08 (Fig. 4C), suggesting that unknown gene product(s) including that for MPIase biosynthesis can catalyze this reaction by RffG/RmlB, which is shared by

Fig. 5. ECA expression in MPIase-depleted strain is not impaired.

A. MPIase-depletion upon *cdsA* and *ynbB* depletion. KS23 cells harboring pAra-CdsA, grown overnight in the presence of 0.002% arabinose, were washed three times with fresh LB medium, followed by inoculation into the LB medium supplemented (+), or not supplemented (-), with 0.002% arabinose. After the growth of KS23 ceased in the absence of arabinose, an aliquot (0.5 mL) of the culture was treated with 5% TCA. The precipitate equivalent to 1.3×10^6 cells was subjected to immunodetection of MPIase. B. ECA levels on *cdsA* and *ynbB* depletion. The same samples as used in A were subjected to immunostaining of ECA. As controls, samples prepared from strains BW25113 (WT) and JW3758-KC ($\Delta wecA$) were also analyzed.

ECA and LPS biosynthesis.

In the $\Delta wzxE$ and $\Delta wzxE$ mutants, no effect on either MPIase or ECA biosynthesis was observed (Figs. 3B and C). These gene products are considered to catalyze Lipid III translocation and polymerization of the glycan unit, respectively (see Fig. 2B). Since MPIase should function on the cytoplasmic surface of the inner membrane, these genes are not likely to be involved in MPIase biosynthesis. For ECA biosynthesis, another set of genes might be involved in these processes. Alternatively, lipid III might migrate on TLC like mature ECA, giving a similar spot to mature ECA (Fig. 3C).

The expression level of ECA does not decrease upon MPIase depletion

We next examined the effect of MPIase depletion on ECA biosynthesis (Fig. 5). The *cdsA* and *ynbB* gene products, CDP-DAG synthases, catalyze MPIase biosynthesis (Sato et al., 2019; Sawasato et al., 2019a) (Fig. 2A). When KS23 ($\Delta cdsA \Delta ynbB$)/pAra-CdsA was cultivated in the absence of arabinose, MPIase was depleted (Fig. 5A), as reported (Sawasato et al., 2019a). Under the MPIase-depleted conditions, ECA expression was not impaired but increased several fold (Fig. 5B). The reason for the increase is not clear, however it is possible that perturbation of phospholipid biosynthesis by the *cdsA* depletion had a pleiotropic effect. Nonetheless, upon MPIase depletion, ECA biosynthesis was not impaired, indicating that MPIase biosynthesis does not overlap with ECA biosynthesis.

Discussion

In this study, we analyzed the MPIase level in mutants as to ECA biosynthesis, and, conversely, the ECA level in mutants in MPIase biosynthesis. The MPIase level in all

the ECA mutants did not change, and, conversely, the ECA level in the MPIase mutants was not impaired. These results clearly indicate that the biosynthetic genes for glycolipids MPIase and ECA are independent. While the possibility cannot be completely excluded that the strains in the Keio collection used in this study have conferred the unexpected mutations in the process of gene disruption through the lambda Red recombination (Baba et al., 2006; Datsenko and Wanner, 2000), it is highly likely that the system dedicated to MPIase biosynthesis is present.

While the glycan chains of the two glycolipids are quite similar, the biosynthesis mechanisms are not the same at all. A repeating unit of three sugars is biosynthesized on undecaprenol in ECA (Erbel et al., 2003; Jorgenson et al., 2016; Mitchell et al., 2018), while it is biosynthesized on phosphatidic acid in MPIase (Nishiyama et al., 2012). In the *wzxE* mutant, the repeating unit on undecaprenol (Lipid III) is accumulated on the cytoplasmic surface of the inner membrane (Rick et al., 2003), where MPIase should be biosynthesized. Since the MPIase level in this mutant was not increased, it is strongly suggested that Lipid III is not utilized for MPIase biosynthesis.

It is reported that ManNAcA-GlcNAc-PP-undecaprenol (Lipid II) is accumulated in an *rmlA* mutant of an *S. typhimurium* strain due to a lack of Fuc4NAc (Rick et al., 1998). In this mutant, ManNAcA-GlcNAc-PP-DAG (DGP-disaccharide), a putative precursor for MPIase, is also accumulated (Rick et al., 1998), indicating that the repeating unit for MPIase is biosynthesized on phosphatidic acid (Sawasato et al., 2019a). In *E. coli*, the *rmlA* mutant expressed a normal level of ECA, but the *rffH* mutant, defective in the same reaction, did not. Therefore, the *E. coli rffH* gene is equivalent to the *S. typhimurium rmlA* one. Nonetheless, both mutants expressed a normal level of MPIase, indicating that there is another system for Fuc4NAc biosynthesis dedicated to MPIase biosynthesis. Since it is also reported that this mutant expresses a trace amount of ECA (Rick et al., 1998), it is plausible that Fuc4NAc, biosynthesized by the system for MPIase biosynthesis, was utilized for ECA biosynthesis. Therefore, a trace amount of ECA was expressed, and, in exchange, ManNAcA-GlcNAc-PP-DAG was accumulated. It is also suggested that the genes for MPIase biosynthesis committed to Fuc4NAc biosynthesis are upregulated to warrant the normal level of MPIase expression.

ManNAcA and Fuc4NAc in the repeating unit are characteristic sugars for the two glycolipids. The presence of a biosynthesis system for Fuc4NAc in MPIase biosynthesis has been suggested, as discussed above. In the case of ManNAcA, the *wecB* and *wecC* genes are responsible for ManNAcA biosynthesis in ECA biosynthesis (Erbel et al., 2003; Jorgenson et al., 2016; Mitchell et al., 2018). These gene products convert UDP-GlcNAc into UDP-ManNAcA. Since MPIase is expressed normally in the *wecB* and *wecC* mutants as well, there should be another system for ManNAcA biosynthesis dedicated to MPIase biosynthesis. In this case, it is possible that another nucleotide sugar than UDP-ManNAcA is utilized for MPIase biosynthesis. The identification of biosynthetic genes for MPIase is in progress in our laboratory.

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