**Introduction**

MPIase (membrane protein integrase) is a glycolipid that drives membrane 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, subcellular 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

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**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, subcellular 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.
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 subcellular 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 (ΔrffH ∆rmLA::kan) and NF08 (ΔrffG ∆rmLB::kan) were constructed as follows. The kan cassette in JW3763-KC (BW25113 ΔrffH::kan) and JW5598-KC (BW25113 ΔrffG::kan) was removed using plasmid pCP20 (Cherepanov and Wackernagel, 1995), followed by introducing the ΔrmLA::kan and ΔrmLB::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., 2007) were visualized 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 (Sugai et al., 2007). The bands were quantified using an ATTO CS analyzer. 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.

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 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-
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 ECA and LPS biosynthesis.

In the ΔwzxE and ΔwzzE 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 (∆cdsA ∆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 glycolchains 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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References

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y. et al. (2006) Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol. Syst. Biol., 2, 2006 0008.

Cherepanov, P. P. and Wackernagel, W. (1995) Gene disruption in Escherichia coli: TeK and Km6 cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene, 158, 9–14.

Datsenko, K. A. and Wanner, B. L. (2000) One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc. Natl. Acad. Sci. USA, 97, 6640–6645.

Endo, Y. and Nishiyama, K. (2015) Relationship between glycolipolzyme MPIase and components comprising the protein transport machinery. Med. Res. Archives, 2, 11.

Erbel, P. J., Barr, K., Gao, N., Gerwig, G. J., Rick, P. D. et al. (2003) Identification and biosynthesis of cyclic enterobacterial common antigen in Escherichia coli. J. Bacteriol., 185, 1995–2004.

Giraud, M. F. and Naismith, J. H. (2000) The rhannose pathway. Curr. Opin. Struct. Biol., 10, 687–696.

Ikegami, A., Nishiyama, K., Matsuyama, S., and Tokuda, H. (2005) Disruption of rmpl encoding ribosomal protein L36 decreases the expression of secD upstream of the sec operon and inhibits protein translocation in Escherichia coli. Bionc. Biotechnol. Biochem., 69, 1595–1602.

Jorgenson, M. A., Kannan, S., Laubacher, M. E., and Young, K. D. (2016) Dead-end intermediates in the enterobacterial common antigen pathway induce morphological defects in Escherichia coli by competing for undecaprenyl phosphate. Mol. Microbiol., 100, 1–14.

Kuhn, H. M., Meier-Dieter, U., and Mayer, H. (1988) ECA, the enterobacterial common antigen. FEMS Microbiol. Rev., 4, 195–222.

Li, G.-W., Burkhardt, D., Gross, C., and Weissman, J. S. (2014) Quantifying absolute protein synthesis rates reveals principles underlying allocation of cellular resources. Cell, 157, 624–635.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, CSHL Press, New York.

Mitchell, A. M., Srikumar, T., and Silhavy, T. J. (2018) Cyclic enterobacterial common antigen maintains the outer membrane permeability barrier of Escherichia coli in a manner controlled by YhdP. mBio, 9, e0132118.

Moser, M., Nagamori, S., Huber, M., Tokuda, H., and Nishiyama, K. (2013) Glycolipolzyme MPIase is essential for topology inversion of SecG during preprotein translocation. Proc. Natl. Acad. Sci. USA, 110, 9734–9739.

Nishiyama, K. and Shimamoto, K. (2014) Glycolipolzyme membrane protein integrase (MPIase): recent data. Biomol. Concepts, 5, 429–438.

Nishiyama, K., Mizushima, S., and Tokuda, H. (1992) The carboxy-terminal region of SecE interacts with SecY and is functional in the reconstitution of membrane protein translocation activity in Escherichia coli. J. Biol. Chem., 267, 7170–7176.

Nishiyama, K., Ikegami, A., Moser, M., Schiltz, E., Tokuda, H. et al. (2006) A derivative of lipid A is involved in signal recognition particle/SecYEG-dependent and -independent membrane integrations. J. Biol. Chem., 281, 35667–35676.

Nishiyama, K., Maeda, M., Abe, M., Kanamori, T., Shimamoto, K. et al. (2010) A novel complete reconstitution system for membrane integration of the simplest membrane protein. Biochem. Biophys. Res. Commun., 394, 733–736.

Nishiyama, K., Maeda, M., Yanagisawa, K., Nagase, R., Komura, H. et al. (2012) MPIase is a glycolipolzyme essential for membrane protein integration. Nat. Commun., 3, 1260.

Rick, P. D. and Silver, R. P. (1996) Enterobacterial common antigen and capsular polysaccharides. In Escherichia coli and Salmonella, Cellular and Molecular Biology, 2nd ed., ed. by Neidhardt, F. C.,...
ASM Press, Washington, D.C., pp. 104–122.

Rick, P. D., Hubbard, G. L., Kitaoka, M., Nagaki, H., Kinoshita, T. et al. (1998) Characterization of the lipid-carrier involved in the synthesis of enterobacterial common antigen (ECA) and identification of a novel phosphoglyceride in a mutant of Salmonella typhimurium defective in ECA synthesis. *Glycobiology*, 8, 557–567.

Rick, P. D., Barr, K., Sankaran, K., Kajimura, J., Rush, J. S. et al. (2003) Evidence that the *wzxE* gene of *Escherichia coli* K-12 encodes a protein involved in the transbilayer movement of a trisaccharide-lipid intermediate in the assembly of enterobacterial common antigen. *J. Biol. Chem.*, 278, 16534–16542.

Rinno, J., Goleccki, J. R., and Mayer, H. (1980) Localization of enterobacterial common antigen: immunogenic and nonimmunogenic enterobacterial common antigen-containing *Escherichia coli*. *J. Bacteriol.*, 141, 814–821.

Sato, R., Sawasato, K., and Nishiyama, K. I. (2019) YnbB is a CdsA paralogue dedicated to biosynthesis of glycolipid MPIase involved in membrane protein integration. *Biochem. Biophys. Res. Commun.*, 510, 636–642.

Sawasato, K., Sato, R., Nishikawa, H., Iimura, N., Kamemoto, Y. et al. (2019a) CdsA is involved in biosynthesis of glycolipid MPIase essential for membrane protein integration in vivo. *Sci. Rep.*, 9, 1372.

Sawasato, K., Suzuki, S., and Nishiyama, K. (2019b) Increased expression of the bacterial glycolipid MPIase is required for efficient protein translocation across membranes in cold conditions. *J. Biol. Chem.*, 294, 8403–8411.

Sivaraman, J., Sauve, V., Matte, A., and Cygler, M. (2002) Crystal structure of *Escherichia coli* glucose-1-phosphate thymidylyltransferase (RifH) complexed with dTTP and Mg$^{2+}$. *J. Biol. Chem.*, 277, 44214–44219.

Sugai, R., Takemae, K., Tokuda, H., and Nishiyama, K. (2007) Topology inversion of SecG is essential for cytosolic SecA-dependent stimulation of protein translocation. *J. Biol. Chem.*, 282, 29540–29548.