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**Secretion Systems and Applications of Microbial
Lactate-Based Oligomers**

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Chapter 1

General Introduction

1.1.Petroleum-based plastics

Plastics are a class of organic polymers composed of long, chain-like molecules made of thousands of building blocks (monomers) and with high average molecular weight (1). Synthetic plastics are produced in a chemical process and derived from fossil fuel feedstocks. These materials possess highly versatile and desirable properties, such as strength, durability, and light weight (1). These properties can be tailored by varying monomer type and sequence, polymerization process, polymer superstructures, and processing technologies (2). Thus, plastics have been extensively used in packing materials, households, automobiles, and in a variety of other applications (3). Due to the increased demand of plastics materials in the modern life, the global production of plastics has increased exponentially since 1950, with approximately 322 million metric tons produced in 2015 (4).

Nevertheless, in the economic point of view, due to the finite supply of petroleum, the behavior of the oil price in future is uncertain and it would affect the global production and distribution of synthetic plastics. Furthermore, the emission of greenhouse gases in the industrial production of petroleum-based plastics aggravates the atmospheric pollution. Thus, there is a recent demand for greener materials with lower carbon footprint, which is supported by the creation of new environmental legislations and regulations (2).

1.2.Biobased plastics

The overdependence on petroleum resources can be alleviated by the development and utilization of plastics entirely or partially produced from renewable resources, known as biobased plastics (5). Renewable carbon sources can be obtained from plant and wood biomass, such as starch, cellulose, hemicellulose, lignin, or plant oil, which are produced by photosynthesis from atmospheric carbon dioxide (6). After use, biobased plastics can be burned, biodegraded or composted and the generated carbon dioxide converted again into biomass by

photosynthesis (Figure 1.1). Thus, biobased polymers entirely produced from renewable carbon sources are carbon neutral materials (6). Among biobased polymers, polylactide (PLA) and polyhydroxyalkanoates (PHAs) are the most representative examples of polymers 100% derived from renewable resources (5).

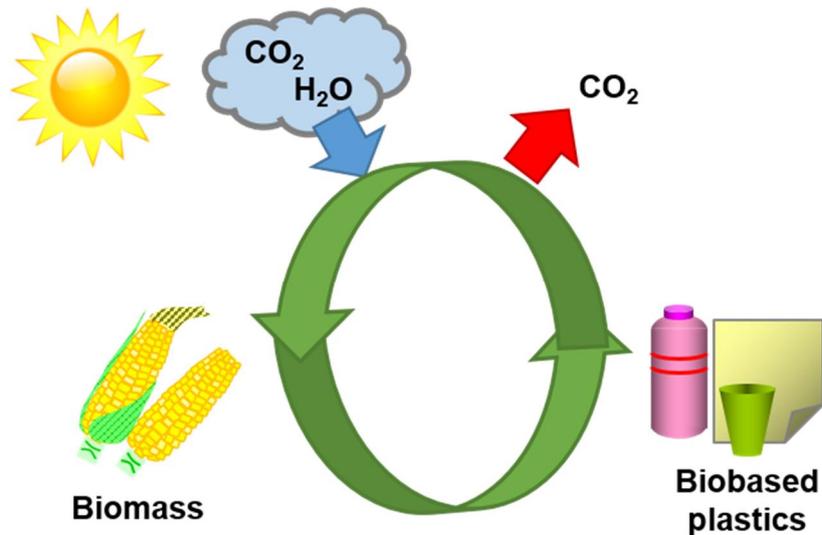


Figure 1.1. The carbon neutrality of biobased plastics.

1.2.1. Polylactide (PLA)

PLA is one of the major commercially available thermoplastics derived from renewable carbohydrate rich-products, such as corn, sugarcane, potato starch, and tapioca starch (7-9). The production capacity of PLAs reached 195,000 tons in 2013, representing about 3.8% of the total production of biobased plastics (partially or 100% derived from renewable resources) (10), and by 2020 the production is expected to reach about 450,000 tons (11). PLAs are compostable and biocompatible materials that have been extensively applied in the biomedical field (12, 13) and which are being increasingly used in commodity applications, such as packaging (14, 15) and textiles (16, 17).

PLA is mainly synthesized in the industry by two routes. High-molecular weight PLA can be produced via direct polycondensation in an azeotropic solution, a method released and utilized by Mitsui Toatsu Chemicals Inc. PLA is synthesized by a direct condensation of lactic acid while the condensation water is continuously removed by the azeotropic distillation (18). The main drawbacks of this method are the necessity of organic solvents for the azeotropic distillation and the presence of considerable catalyst impurities in the final polymer due to the high levels needed for acceptable reaction rates (19).

In contrast, NatureWorks LLC, the major global producer of PLA (18), synthesizes high-molecular weight PLA via ring-opening polymerization (ROP) through the formation of the cyclic dimer (lactide) of lactic acid (Figure 1.2). This method is based on the original Cargill-Dow patented process (20) which combines a solvent-free and a distillation process for producing PLA with controlled molecular weights (18). In this process, lactic acid is mainly produced by carbohydrate fermentation, and the purification of lactic acid from culture medium is typically performed by calcium salt precipitation and subsequent acidification, which generates salt as a byproduct (21). To produce lactate (LA) oligomers, the purified lactic acid is conventionally condensed at increased temperature, and water is removed under vacuum for several hours (22). Subsequently, LA oligomers are depolymerized to generate lactides, which are finally polymerized into high-molecular weight PLA via metal-catalyzed ROP (23). Although this is the main industrial route, the multistep chemo-bio process is considered complex and expensive relative to petroleum-based polymers (12). Among the steps, the production of lactide from LA, which comprises the oligomerization of LA and lactide synthesis from LA oligomers, contributes to 30% of the PLA cost (22).

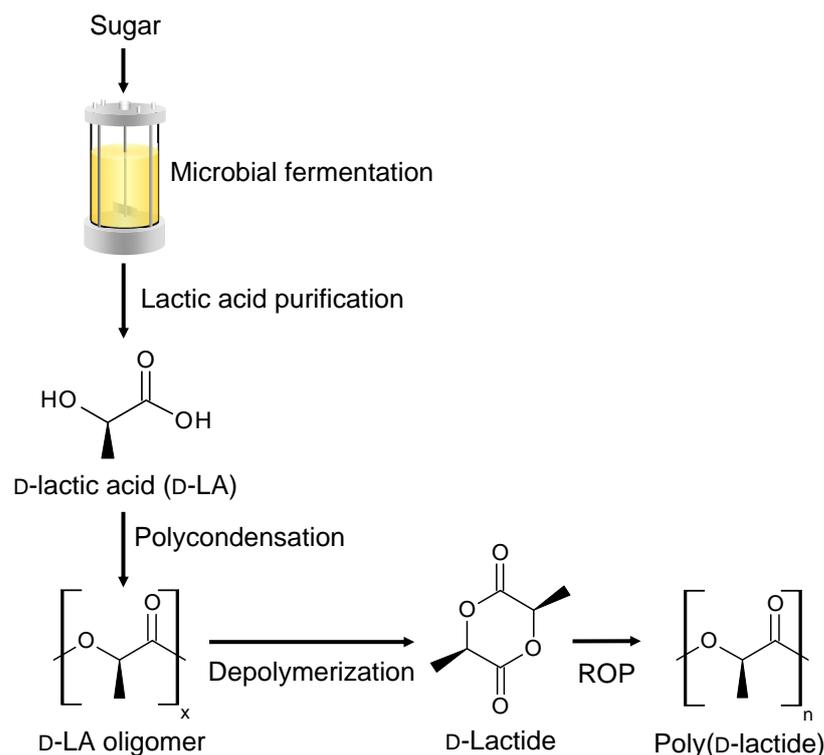


Figure 1.2. Scheme of the conventional route for PLA production via ring-opening polymerization (ROP).

1.2.2. Polyhydroxyalkanoates (PHA)

Polyhydroxyalkanoates (PHAs) are a family of microbial polyesters. Both native and recombinant microorganisms have been used for producing PHAs via fermentation process (24) from carbon sources, such as sugars and plant oils (25). In general, a continuous transesterification using hydroxyalkanoic acids as monomer units is catalyzed by a PHA synthase (PhaC) yielding high-molecular weight polyesters (26). To date, about 150 hydroxyalkanoic acids are accounted as possible monomers (27), enabling the generation of diverse polyesters as homo-, random-, block copolymers and thiopolyesters. PHAs exhibit a wide range of material properties and functionalities and are mostly biocompatible and biodegradable, thus suitable for numerous biomedical and industrial applications (28, 29). PHAs are synthesized intracellularly and accumulated as hydrophobic inclusions. For

recovering PHA from cell biomass, extraction using organic solvents is the most used method (27).

1.3. The first microbial factory of LA-based polymers

The engineering of PHA biosynthetic system for incorporating a wide range of monomers including unusual monomer constituents into PHAs is an attractive feature of this microbial system (25). In 2008, with the motivation to create an environmentally friendly alternative to the conventional bio-chemo process of PLA production, the first microbial platform of LA-based polyester poly(lactate-co-3-hydroxybutyrate) [P(LA-co-3HB)] was established (30). Lactic acid is an unusual monomer unit to PHA synthases, and microbially synthesized LA-based polymers had never been reported before. Thus, this achievement was only possible with the discovery of a Ser325Thr/Glu481Lys mutant of PHA synthase from *Pseudomonas* sp. 61-3 [PhaC_{1PS}(ST/FS/QK)] with acquired LA-polymerizing activity, named D-specific LA-polymerizing enzyme (LPE).

P(LA-co-3HB) is biosynthesized by successive enzymatic reaction steps, as follows (Figure 1.3): (i) generation of lactyl-coenzyme A (LA-CoA) by propionyl-CoA transferase (PCT), (ii) supply of 3-hydroxybutyryl-CoA(3HB-CoA) from acetyl-CoA (PhaA and PhaB), and (iii) copolymerization of LA-CoA and 3HB-CoA by the LPE. These enantiomerically pure polyesters could be produced from renewable feedstock and were intracellularly accumulated as hydrophobic inclusions within recombinant cells, such as *Escherichia coli* (30) and *Corynebacterium glutamicum* (31). In addition, as an effort to increase the LA fraction in P(LA-co-3HB), an evolved LPE, PhaC_{1PS}(ST/FS/QK), was obtained by the directed evolution of the original LPE (32).

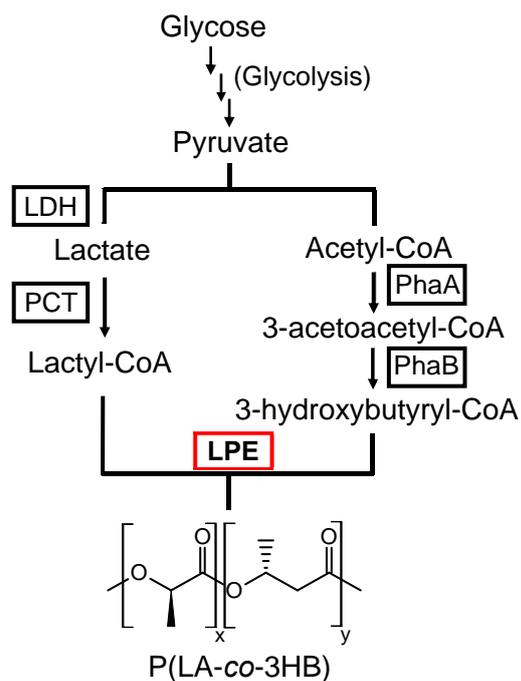


Figure 1.3. The biosynthetic pathway for the lactate (LA)-based polyester in recombinant *E. coli*. LDH, lactate dehydrogenase; PCR, propionyl-CoA transferase; PhaA, β -kethiolase, PhaB, NADPH-dependent acetoacetyl-CoA reductase; LPE, LA-polymerizing enzyme.

By varying the culture conditions, bacterial strains, and PhaC mutants, P(LA-co-3HB) with different LA fractions, ranging from 6 to 99.3 mol% LA (30, 31, 33-35), can be produced. In particular, the biosynthesized poly(D-lactide) (PDLA)-like polymers tended to have relatively low molecular weights ($< 10^4$ g/mol) (31, 35), revealing an inverse correlation between LA fraction and polymer molecular weight. Possibly, D-lactyl-CoA is still not the preferred substrate for LPE thus causing chain termination before high-molecular weight polymers are polymerized.

1.4. The aim of this thesis

For the industrial production of PLA, although ROP is the main route, it is a multi-step chemo-bio process which requires additional purification steps, so it is considered relatively complex and expensive (36). Therefore, there is room for improvement in the bioprocessing and manufacture of PLA to improve its price competitiveness with conventional polyesters.

Since in the microbial system of LA-based polyesters PDLA-like polymers are synthesized with relatively low molecular weights, the development of a bacteria-based system for PDLA-like oligomer production came to light. In addition, based on the knowledge that compounds such as lipids (37), polysaccharides (e. g. xanthan and gellan) (38), and amino acids (39), are bacterially secreted, the most interesting aspect of this research was the possibility of LA oligomers secretion by *E. coli*. The establishment of a secretion system of D-LA oligomers would greatly contribute to the industrial production of PLA as a *ö*biosynthetic shortcutö to provide LA oligomers in the chemo-bio process. In addition, the production of optically pure D-LA oligomers can be beneficial due to the superior thermal properties of PLLA/PDLA stereocomplex, as well as advantageous in the commercial price ratio of D-LA (high price) to L-LA (low price). Therefore, the primary aim of this study was to produce D-LA oligomers in recombinant *E. coli* expressing LPE and explore the possibility of D-LA oligomers secretion.

The first chapter of this thesis is the general introduction. In Chapter 2, the possibility of D-LA oligomers production and secretion by *E. coli* during the microbial production of P(LA-co-3HB) was evaluated. First, the detection of D-LA oligomers in the culture supernatant of recombinant *E. coli* grown on glucose was attempted. Once the D-LA-based oligomers (D-LAOs), which are co-oligomers of LA and 3HB, were surprisingly found to be secreted, but with low production, improving the secretion system was the next target. To increase the secretory production, increasing the frequency of chain transfer (CT) reaction by adding alcoholic compounds acting as CT agents into the culture medium was performed. The

monomer composition, terminal structure, and molecular weight of the biosynthesized oligomers were well characterized. In addition, the features of this new established microbial system were discussed. The goal in Chapter 3 was to utilize the microbial secreted D-LAOs as a substrate for synthesizing lactide, which is the monomeric unit used in the ROP for PLA production. Initially, the conversion of D-LAOs into lactide via thermal depolymerization using metal catalyst was attempted. Next, the strategy adopted to further improve the conversion of D-LAOs into lactide was to increase the LA fraction in D-LAOs. In Chapter 4, the D-LAOs secretion phenomenon was explored and the existence of membrane protein transporters mediating the secretion of D-LAOs in *E. coli* was investigated. The effect of the individual deletion of genes encoding membrane proteins related to the transport of organic compounds on the ability to secrete D-LAOs by *E. coli* was evaluated. The effect of the overexpression of membrane proteins selected as candidates of D-LAOs transporters was also assessed. As a result, a model of D-LAOs secretion routes was proposed. Finally, the thesis conclusion is presented in Chapter 5.

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Chapter 2

**Finding and improvement of D-lactate-based oligomers secretory
production by engineered *Escherichia coli***

2.1. Introduction

In 2008, our research group established the first microbial platform for the production of D-LA-based polyesters using an engineered PHA synthase (PhaC) named D-specific lactate-polymerizing enzyme (LPE) (1). These enantiomerically pure polyesters (2) could be synthesized using *Escherichia coli* (1) and *Corynebacterium glutamicum* (3). During the course of our research, we found that the poly (D-lactide) (PDLA)-like polymers synthesized by these bacteria tended to have relatively low molecular weights ($< 10^4$) (3, 4). This result inspired us to develop a bacteria-based system for PDLA-like oligomer production. Thus, in the present study, we initially explored the existence of an oligomeric fraction in the system of LA-based polymers in recombinant *E. coli* expressing LPE. However, besides the detection of D-lactate-based oligomers (D-LAOs), which are co-oligomers of LA and 3HB, inside the cells, surprisingly, a small amount of D-LAOs was also detected in the medium. This finding indicated that (i) D-LAOs can be synthesized during the LA-based polymer production, and (ii) the synthesized D-LAOs can be spontaneously secreted from the cells without the introduction of exogenous exporters (Figure 2.1). In practical applications, the D-LAO-secretion system has the potential to contribute to the industrial production of PDLA and its copolymers as a biotechnological shortcut route in the production process, which eliminates the necessity of LA purification and oligomerization, thus reducing the energy, time and costs of PLA production.

To improve the secretory production of D-LAOs by *E. coli*, we hypothesized that the synthetic capacity of D-LAOs would be determined by the frequency of chain transfer (CT) reaction during the polymerization. Compounds bearing hydroxyl groups, such as diethylene glycol (DEG), are known to act as CT agents and reduce the molecular weight of microbial polyesters (5, 6). Therefore, in this study, we attempted to use CT agents to enhance the D-LAO production and/or secretion by *E. coli*. In fact, CT agents were found to be a critical factor for the efficient secretory production of D-LAOs. Furthermore, because the CT agent was

bound to the oligomer at the carboxyl terminal (Figure 2.1), the D-LAOs with diverse monomer composition and terminal structures have the potential to be derivatized into a variety of LA-based polymers.

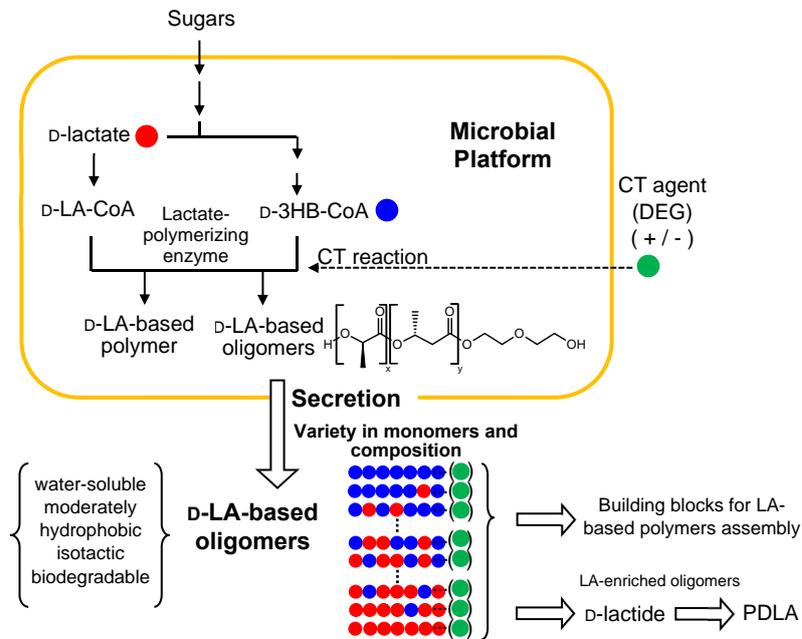


Figure 2.1. Microbial platform for secretory production of D-LAOs and their potential.

2.2. Materials and methods

2.2.1. Bacterial strain and plasmids

Escherichia coli BW25113 was used as the host strain. Expression vector pTV118NpctphaC1_{Ps}(ST/FS/QK)AB harboring the genes encoding propionyl-CoA transferase (PCT) from *Megasphaera elsdenii*, 3-hydroxybutyryl-CoA (3HB-CoA)-supplying enzymes - ketothiolase and acetoacetyl-CoA reductase (PhaA, PhaB) from *Ralstonia eutropha* and Ser325Thr/Phe392Ser/Gln481Lys mutated PhaC [PhaC1_{Ps}(ST/FS/QK)] from *Pseudomonas* sp. 6163 (7) was used for D-LAO production.

2.2.2. Culture conditions

Recombinant *E. coli* cells harboring pTV118NpctphaC1_{Ps}(ST/FS/QK)AB were cultivated in 10 mL glass test tubes containing Luria Bertani (LB) medium (1.7 mL) with 20 g L⁻¹ glucose and 100 mg L⁻¹ ampicillin at 30 °C for 48 h with reciprocal shaking at 180 rpm. The cultivations were supplemented with the following CT agents: polyethylene glycol 200 (PEG200, average molecular weight = 200), diethylene glycol (DEG), ethylene glycol (EG), and ethanol. PEG200 (Kishida Chemical, Japan), DEG, and EG (Junsei, Japan) were added at 1, 3, and 5% (v/v) concentrations, whereas ethanol was supplemented at concentrations of 5, 10, and 20 g L⁻¹ (w/v). Cultures without any CT agent supplementation were also performed.

2.2.3. Cell viability measurements

Cells cultured in medium without CT agent and with 5% DEG were assayed for cell viability at 48 h using 4',6-diamidino-2-phenylindole (DAPI)/propidium iodide (PI) staining method. 1 mL cell culture was harvested at 2300g for 5 min, and washed twice with 1 × phosphate buffered saline (PBS) (134 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4). The cells were resuspended in 1× PBS and the cell density was adjusted to

10^9 cells/mL. One microliter DAPI (1 mg/mL) and 5 μ L PI (300 μ M) were added into 954 μ L microbial suspension and gently mixed. The cells were incubated at room temperature (RT) for 5 min in the dark. Fluorescence microscopy was performed with filters of excitation (EX, 360/40), dichromatic mirror (DM, 400), and barrier (BA, 460/50) for DAPI, and EX 545/25, DM 565 and BA 605/70 for PI using BZX700 (Keyence, Japan). Images were generated after haze reduction treatment (BZ-X S14 Analyzer, Keyence, Japan). The dead cells (negative control) were prepared with the heat treatment at 65 °C for 15 min using the cells harvested at 36 h. Colony forming units (CFU) were determined using cells grown for 48 h without CT agent and with 5% DEG. The cells were spread onto LB agar plates containing 20 g L⁻¹ glucose and 100 mg L⁻¹ ampicillin with proper dilution. Individual colonies were counted after overnight incubation at 30 °C. The values of CFU/mL are presented as the mean of biological triplicates.

2.2.4. Extracellular and intracellular LA-based products extraction

After cultivation, the bacterial cultures were centrifuged at 13,000 g for 10 min to prepare cell-free culture supernatants. The culture supernatant was passed through a hydrophilic nylon membrane filter with a pore size of 0.22 μ m. Extracellular D-LAOs were extracted from the cell-free culture supernatant by two-phase extraction using chloroform. The extraction was performed by adding 1 volume of organic solvent to 1 volume of supernatant and mixing vigorously. After centrifugation at 2,300 g for 5 min, the chloroform phase was added to a new test tube, and 1 volume of H₂O was added and mixed vigorously to remove excess CT agent and LA/3HB monomers. The H₂O-washing step was repeated twice. To extract intracellular D-LAOs and polymer, lyophilized cells were soaked in chloroform for 2 days at RT. Cell debris was removed by passing through a polytetrafluoroethylene (PTFE) filter, and a 10-fold volume of methanol was added and incubated at 4 °C for 1 day to precipitate

the polymer. Intracellular D-LAOs were considered to be the methanol-soluble polymerized products and were separated from the precipitated polymer by centrifugation at 2,700 g and 4 °C for 30 min and then filtrated using a PTFE filter.

2.2.5. Measurement of the extracellular D-LAO concentration

To estimate the concentration of the secreted D-LAOs in the culture supernatant, cell-free culture supernatant was analyzed before and after treatment with HCl. HCl was added to the supernatant at a final concentration of 2 N and incubated at 100 °C overnight to hydrolyze the D-LAOs. Subsequently, the mixture was neutralized with 2 N NaOH. The samples were analyzed by HPLC (Jasco, Japan) equipped with an Aminex HPX-87H column (Bio-Rad, USA) and a refractive index detector. The amount of oligomeric LAs was determined based on the difference in the LA concentrations of the samples after and before HCl treatment. The monomer composition of the oligomers produced with DEG supplementation was determined using liquid chromatography-mass spectrometry (LC-MS) (LCMS-8030, Shimadzu, Japan) equipped with a Mastro C18 column (150 mm), electrospray ionization (ESI) and a triple quadrupole mass analyzer. Carrier A: 5 mM ammonium acetate (pH 5.6) containing 10 mM dimethylbutylamine and 10% methanol; carrier B: methanol at a flow rate of 0.2 mL min⁻¹ in gradient mode. The ESI voltage was 3.5 kV in negative mode. Nitrogen was used as the nebulizer (3 mL min⁻¹) and drying gas (15.0 mL min⁻¹). [M-H]⁻ ions from LA ($m/z = 89$, retention time: 2.3 min) and 3HB ($m/z = 103$, retention time: 2.6 min) were monitored in selected ion monitoring mode.

2.2.6. Nuclear magnetic resonance (NMR) analyses of oligomers

NMR was performed in CDCl₃ with tetramethylsilane as the internal reference. ¹H NMR, ¹H-¹H correlation spectroscopy (COSY)-NMR and ¹H-¹³C heteronuclear multiple quantum coherence (HMQC) obtained at 400 MHz (for ¹H NMR) were recorded using a JEOL

JNM-ECS400 spectrometer (JEOL, Japan). ^1H - ^1H diffusion-ordered spectroscopy (DOSY)-NMR (500 MHz) was recorded using a Bruker AMX500 spectrometer (Bruker Daltonics). Benzoic acid (Wako, Japan) was used as an internal standard in ^1H NMR to quantify the extracted D-LAOs; the relaxation delay was set to 10 s, and the number of scans was 8.

2.2.7. ESI-time-of-flight (TOF)-MS analysis of oligomers

Extracted D-LAOs were subjected to ESI-TOF-MS analysis using a MicroTOF (Bruker Daltonics). The sample was injected directly by a syringe pump at RT at a flow rate of 180 $\mu\text{L h}^{-1}$. The ESI voltage was 4.5 kV in negative mode, and the drying temperature was 200 $^{\circ}\text{C}$. Nitrogen was used as the nebulizer (1.6 bar) and drying gas (9.0 mL min^{-1}). All spectra were recorded in the range of 5063000 m/z .

2.2.8. Intracellular D-LAOs and polymer analyses

Polymer and intracellular oligomers were ethanolyzed, and their concentrations and monomeric compositions were measured by gas chromatography (GC)-MS (GCMS-QP210, Shimadzu, Japan) equipped with an InertCap 1MS capillary column (GL Science, Japan). The molecular weight of high-molecular weight polymer was estimated by gel permeation chromatography (GPC, Jasco, Japan) equipped with a Shodex GPC KF-805 column (Showa Denko K. K., Japan). Polystyrene standards (Showa Denko K. K., Japan) were used for calibration (1).

2.3. Results and discussion

2.3.1. Detection of D-LAOs in the Culture Supernatant of Engineered *E. coli*

We investigated whether the P(LA-co-3HB)-producing *E. coli* could secrete oligomers including LA into the medium. The experimental design used in this work is shown in Figure 2.2. First, as a control, the water-soluble fraction of synthetic L-LA oligomers, which was in the range of approximately dimer to 14-mers, was extracted by two-phase extraction, whereby longer-size species in the oligomers migrated to the organic phase (extraction efficiency of 10%). Therefore, we attempted to concentrate oligomers from the culture medium of P(LA-co-3HB) producing *E. coli* using this method. The cells expressed four genes: *pct* to supply LA-CoA, *phaA* and *phaB* to supply 3HB-CoA, and the ST/FS/QK mutant of PHA synthase gene from *Pseudomonas* sp. 613 (*phaC1_{PS}*) as the evolved LPE (7). Recombinant *E. coli* grown on glucose has been reported to intracellularly accumulate the P(LA-co-3HB) copolymer (1). The supply of 3HB-CoA, which is a natural substrate of PHA synthase, is known to facilitate the incorporation of LA-CoA catalyzed by LPE (1). In fact, cells harboring no 3HB-CoA synthesis-related *phaAB* genes did not produce D-LAOs in *E. coli* (data not shown).

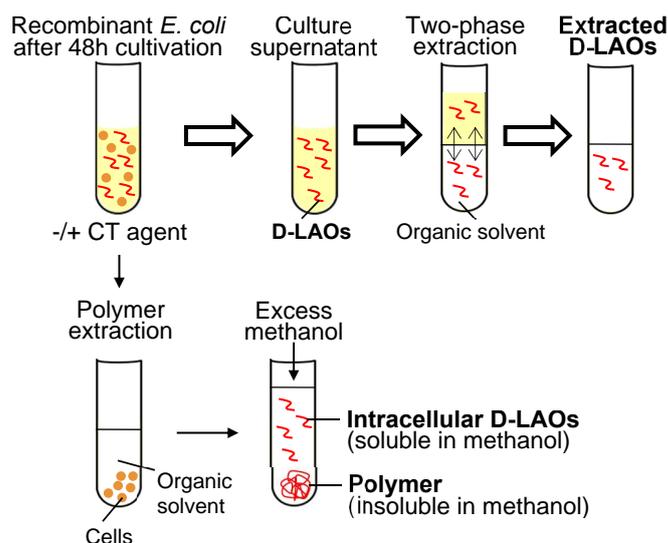


Figure 2.2. Experimental design for acquiring the samples analyzed here. The cell-free culture supernatant was extracted by two-phase extraction. The D-LAOs in organic solvent are referred to as extracted D-LAOs. The intracellular polymer was extracted with organic solvent from lyophilized cells, and the intracellular D-LAOs were obtained as a methanol-soluble fraction. The efficiency of CT agents was evaluated based on the concentration of oligomeric LA in the culture supernatant, before the two-phase extraction (see the Methods and Figure 2.5).

To determine the structures of the secreted compounds, the extracted fraction was subjected to ^1H NMR (Figure 2.3A) and ^1H ^1H COSY-NMR (Figure 2.4A). The components of the ^1H NMR resonances (in ppm) of the extracted fraction 4.9–5.2 ppm [1H, m, LA(1)] and 1.4–1.6 ppm [3H, m, LA(2)] were nearly identical to those of P(LA-co-3HB) (1, 2). The small variation in the chemical shifts might be attributable to the molecular weight difference between the polymers and oligomers. In addition, according to COSY-NMR (Figure 2.4A and B), the secreted compounds exhibited eight cross signals in the region of 3.9–4.4 ppm. Among them, resonances at 1.48/4.34 and 1.48/4.37 ppm were ascribed to the methine proton of the hydroxyl terminal LA unit in HO-LA*-3HB and HO-LA*-LA dyads, respectively, and the resonances at 1.26/4.19 and 1.26/4.22 ppm were ascribed to the methine proton of the hydroxyl terminal 3HB unit in HO-3HB*-3HB and HO-3HB*-LA dyads, respectively (8). The other four cross signals remain unidentified. On the basis of the ^1H NMR result, the extracted fraction was determined to contain oligomers consisting of 63 mol % LA, designated here as D-LAOs.

Furthermore, ESI-TOF-MS analysis of the sample detected a bimodal distribution of oligomers with periodic m/z values in the range of approximately 400–1400 (Figure 2.3B), corresponding to ~4- to 19-mer oligomers consisting of LA and 3HB units. The peak tops of the bimodal distribution were 7 and 12 mer, respectively.

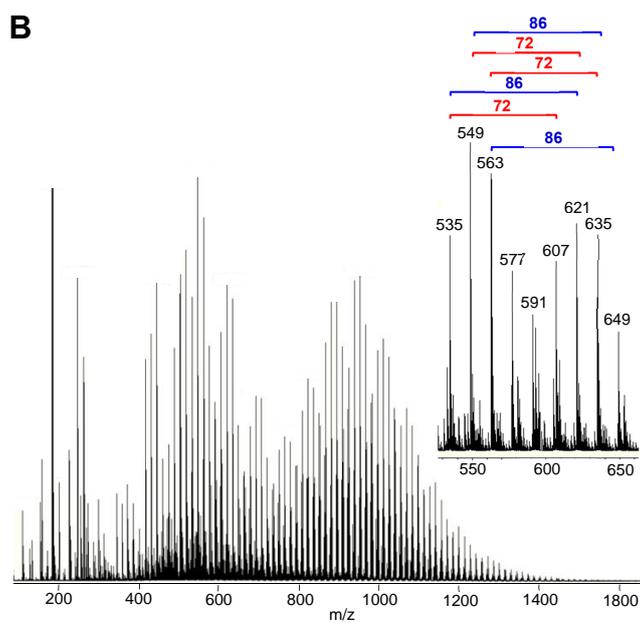
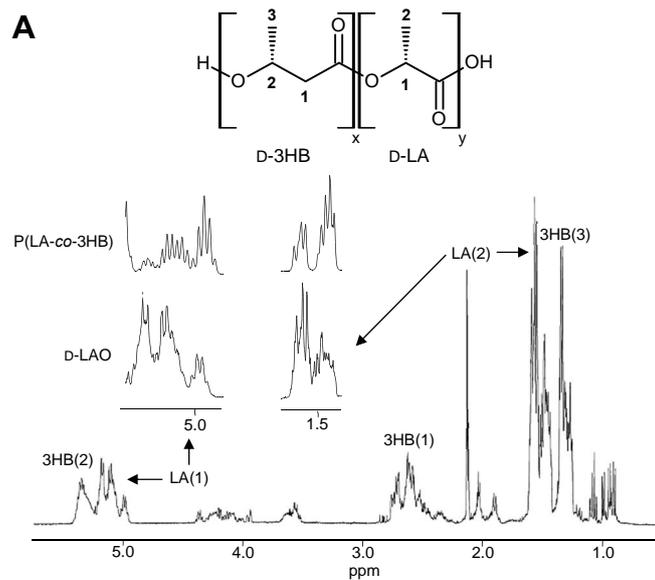


Figure 2.3. Structural analysis of extracted D-LAOs synthesized without a CT agent. (A) 1H NMR spectrum; (B) ESI-TOF-MS spectrum. The intervals between LA (72) and 3HB (86) units are represented in the magnified region from 550 to 650 m/z .

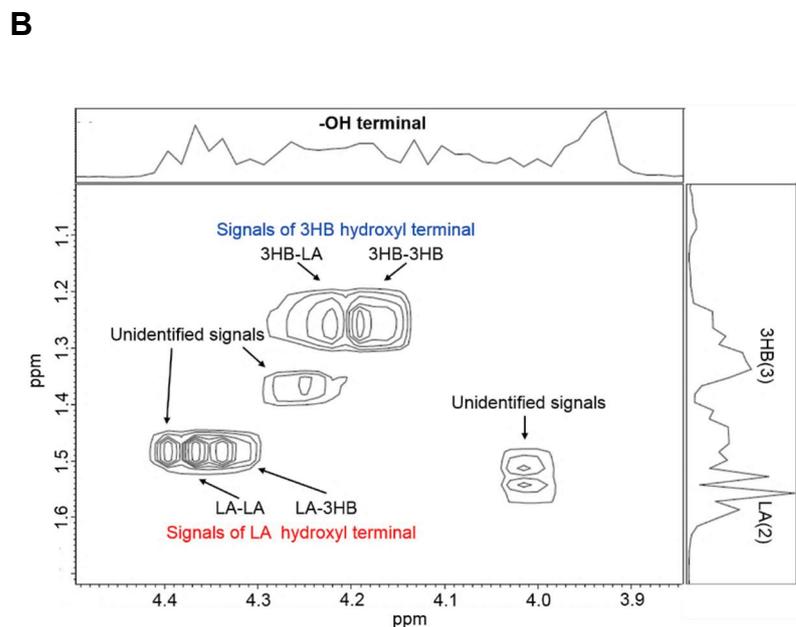
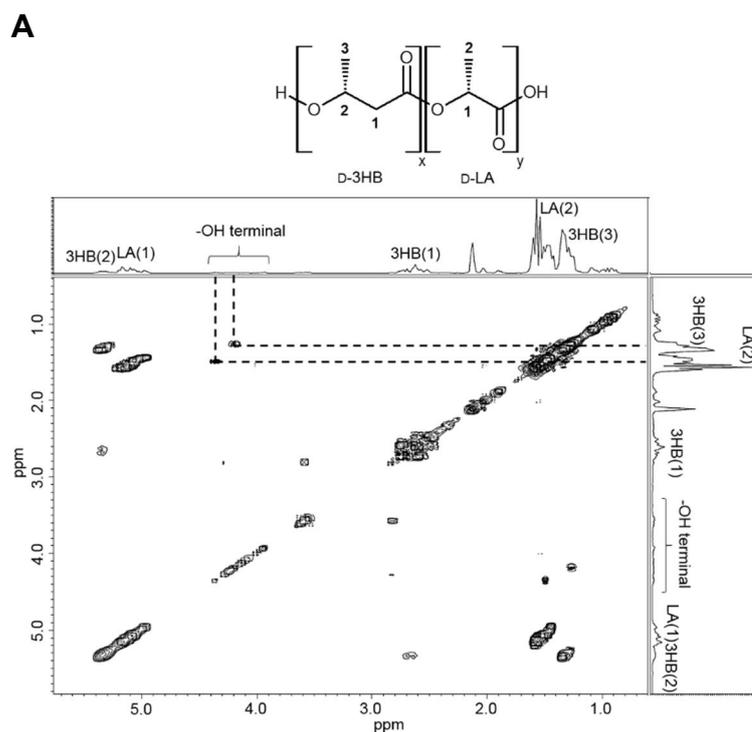


Figure 2.4. Evaluation of the hydroxyl terminal of extracted D-LAOs synthesized without a CT agent. (A) ^1H - ^1H COSY NMR (δ in ppm). The cross signals of the δOH terminal at 1.5 ppm/4.3 ppm and 1.4 ppm/4.2 ppm indicated that the extracted D-LAOs are oligomers. (B) Expansion of the region from 3.9 to 4.4 ppm on ^1H - ^1H COSY NMR. Cross signals of LA and 3HB -OH terminals were identified by comparison with synthetic trimer methyl esters of LA and 3HB.

2.3.2. Enhanced Secretion of D-LAOs by the Addition of CT Agents

The discovery of D-LAOs secretion prompted us to engineer the polymer-producing *E. coli* into an efficient oligomer-production system. For this purpose, we hypothesized that increasing the frequency of the CT reaction would facilitate the production of oligomers. In the termination step of polymer synthesis, PHA synthase has been suggested to lose its polymerization activity or transfer the polymer chain to a hydroxyl group-containing CT agent (5). Increasing the frequency of the CT reaction results in the production of polymers with lower molecular weights (9). Previous studies have demonstrated that low molecular weight PEGs (10-13) and short chain alcohols (9, 14) which were added to the medium, are effective CT agents capable of substantially reducing the molecular weight of the PHA polymer. Therefore, the abilities of four CT agents, PEG200, DEG, EG, and ethanol, to shift the polymer synthesis toward oligomer synthesis were evaluated.

The efficiency of the CT agents was evaluated based on the amount of oligomeric LA secreted into the medium. The amount of oligomeric LA was estimated by subtracting the LA (monomer) concentration in the culture supernatant from the total LA concentration, which was determined by hydrolyzing the oligomers with HCl (see Methods). As shown in Figure 2.5, DEG was the most effective CT agent for D-LAO production. The effectiveness of DEG as a CT agent was also supported by the drastic reduction in the molecular weight of the intracellularly accumulated polymers (Table 2.1). The number-average molecular weight (M_n) of the polymer produced without the addition of CT agent was 2.9×10^4 g/mol, while with the supplementation of 1, 3, and 5% DEG, polymers with M_n of 0.86×10^4 , 0.79×10^4 , and 0.61×10^4 g/mol, respectively, were produced. The maximum M_n reduction of 79% was achieved with the addition of 5% DEG. Our results agree with previous studies regarding the ability of PEGs to decrease the molecular weight of PHAs, whereby it was observed that the production of PHA leading to molecular weight reduction is enhanced for lower molecular weight PEGs. Shi

et al. (11) reported that among PEGs with M_n varying from 106 (DEG) to 10 000 g/mol, DEG was the most effective in causing the largest reduction in P(3HB) molecular weight by *Ralstonia eutropha* (currently known as *Cupriavidus necator*). Similarly, DEG was the most effective in regulating the P(3HB) molecular weight produced by *Alcaligenes latus* DSM 1122 (12).

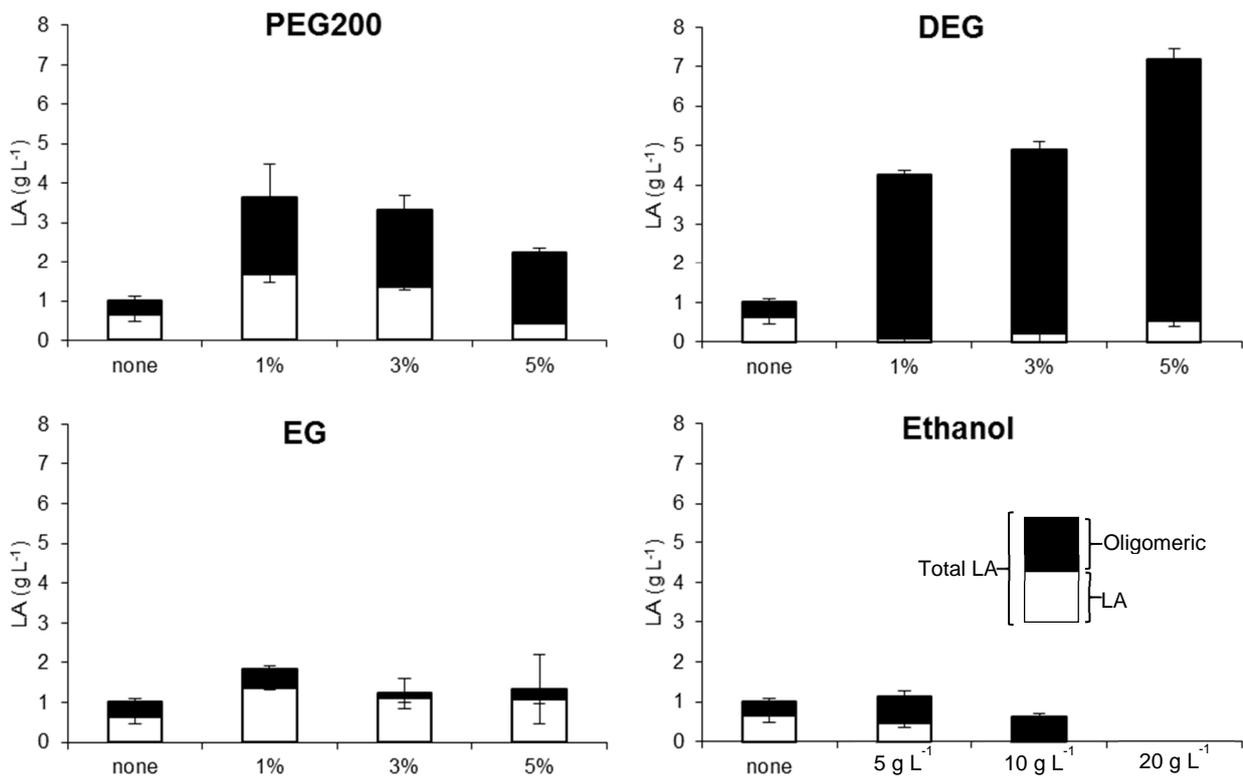


Figure 2.5. Effect of CT agent supplementation on D-LAOs production. Measurements of the LA concentration in the culture supernatant of recombinant *E. coli* before (white) and after (white + black) HCl treatment. The difference between the LA concentrations after and before treatment.

Table 2.1. Effect of CT agent supplementation on P(LA-co-3HB) biosynthesis in recombinant *E. coli*

CT agent	Concentration	CDW ^a (g L ⁻¹)	Polymer production ^a (g L ⁻¹)	molecular weight		<i>M_n</i> reduction (%)
				<i>M_n</i> (×10 ⁴)	<i>M_w</i> (×10 ⁴)	
None	0	5.9±0.16	3.8±0.12	2.9	14	0
PEG200 (% v/v)	1	5.8±0.18	1.9±0.07	1.7	4.2	43
	3	2.4±0.13	0.3±0.02	1.6	2.4	43
	5	1.0±0.06	nd	nm	nm	nm
DEG (% v/v)	1	6.4±0.24	2.2±0.08	0.86	2.2	70
	3	5.8±0.14	1.1±0.07	0.79	1.5	72
	5	3.6±0.03	0.8±0.07	0.61	0.80	79
EG (% v/v)	1	6.1±0.09	3.0±0.04	1.7	2.5	41
	3	6.8±0.42	3.0±0.67	1.8	4.5	36
	5	3.7±0.58	1.7±0.26	1.5	3.5	47
Ethanol (g L ⁻¹ , w/v)	5	8.4±0.17	3.7±0.09	1.7	6.2	41
	10	9.3±0.07	4.6±0.04	1.2	4.3	55
	20	1.1±0.08	0.1±0.01	nm	nm	nm

^a Data are presented as the mean ± standard deviation of three independent biological trials.

CDW: Cell dry weight.

nd: not detected.

nm: not measured.

The enhanced production of extracellular D-LAOs was not a result of cell lysis; indeed, microscopic observations of the cells revealed that they remained intact upon DEG addition at various concentrations (Figure 2.6). Moreover, DAPI/PI dual staining assay indicated that the viable cell ratio was as high as 90% (n = 120 cells), and there was no significant difference from no CT control (92%) (n = 83 cells) (Figure 2.7). In addition, the viable cell counts based on CFU/mL did not exhibit statistically significant difference ($P < 0.05$) between the conditions with 5% DEG ($9.77 \pm 4.97 \times 10^6$ CFU/mL) and no CT supplement ($2.23 \pm 1.82 \times 10^6$ CFU/mL). These results indicate that the production of D-LAOs in the medium was secreted by *E. coli* but was not caused by cell lysis.

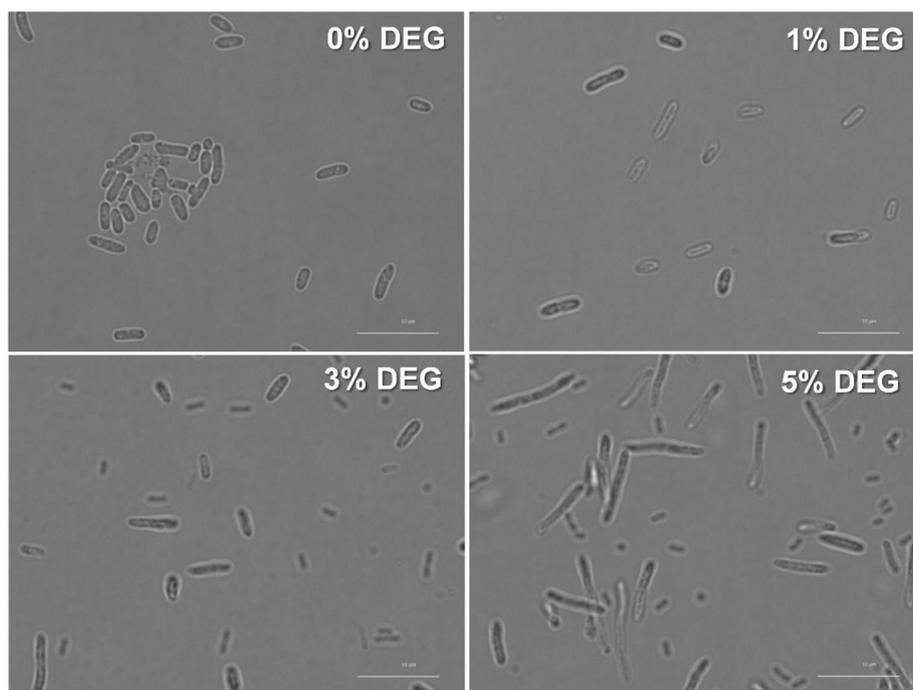


Figure 2.6. Microscopic observation of recombinant *E. coli* cells grown with and without DEG supplementation. The cells were cultivated for 48 h. No cell lysis was observed in all conditions tested. The bar represents 10 μm .

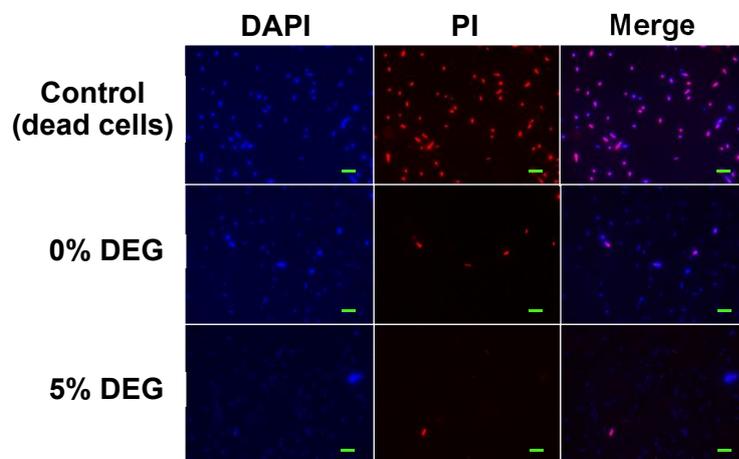


Figure 2.7. DAPI/PI dual staining assay after 48 h cultivation of recombinant *E. coli* cells grown with 0 and 5% DEG supplementation. The negative control is the same recombinant with heat treatment. The green bar represents 10 μm .

The monomer composition of the oligomers secreted with DEG supplementation was determined by the aforementioned subtracting method (Figure 2.8). The highest production ($8.3 \pm 1.5 \text{ g L}^{-1}$) of D-LAOs containing $86.0 \pm 4.5 \text{ mol } \% \text{ LA}$ was achieved with 5% DEG

supplementation, corresponding to 57% of the theoretical carbon yield. Moreover, with 5% DEG supplementation glucose was fully consumed after 48 h (data not shown). The increase in the total LA production by the supplement of DEG is presumably due to the fact that the D-LAOs, unlike monomeric LA, are not utilized by *E. coli*. The addition of 8% DEG decreased the D-LAO production (Figure 2.8).

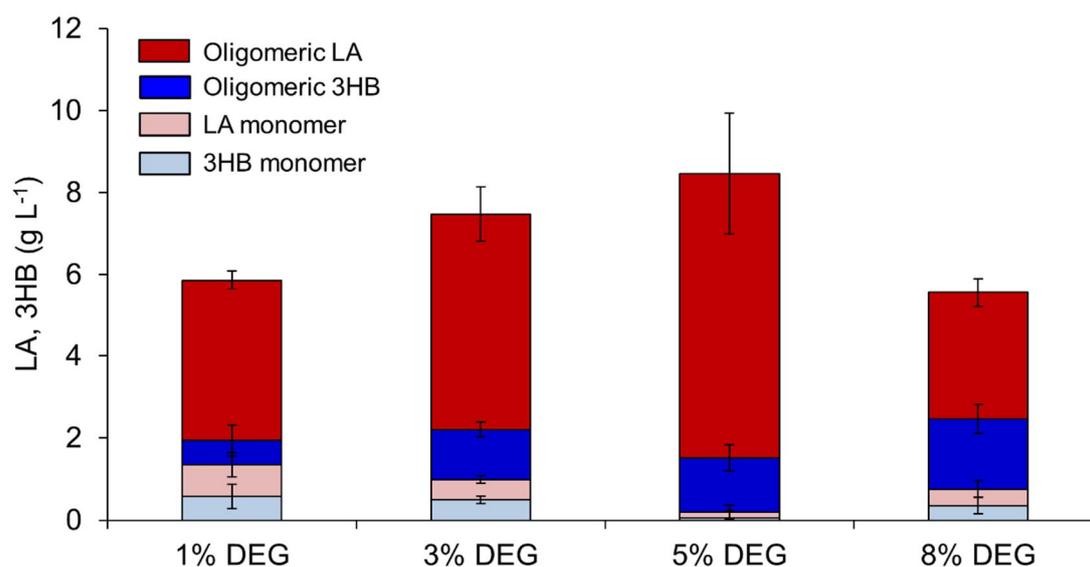
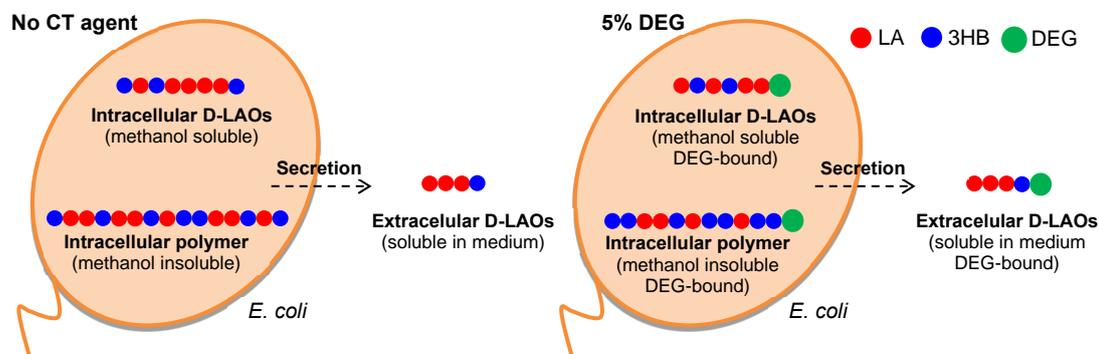


Figure 2.8. Concentrations and compositions of D-LAOs and monomers secreted from recombinant *E. coli* upon the addition of different concentrations of DEG. The LA and 3HB concentrations in the culture supernatant before and after HCl treatment were determined using LC-MS. The difference between the concentrations after and before treatment with HCl is an estimation of the amount and composition of secreted D-LAOs. Values are presented as the mean of biological triplicates. Error bars indicate standard deviation (s.d.).

The two-phase extraction of 5% DEG culture supernatant recovered 40% D-LAOs (3.2 g L⁻¹), described here as extracted D-LAOs (Figure 2.9). The extraction step was needed to remove DEG excess from the D-LAOs. In fact, free DEG was not detected in the extracted D-LAOs, indicating that the use of DEG was not a bottleneck in this system and that it was easily removed from the final product using hydrophilic/hydrophobic interactions. In our future

studies, hydrophobic resins rather than organic solvents will be used toward the greener applications in practical systems.



CT	Intracellular polymer/oligomer						Extracellular oligomer (extracted D-LAOs)		
	Polymer (g L ⁻¹)	LA (mol%)	M _n (×10 ³)	Oligomer (g L ⁻¹)	LA (mol%)	M _n (×10 ³)	Oligomer (g L ⁻¹)	LA (mol%)	DP (mer)
None	3.8 ± 0.12	55 ± 1	29	1.8 ± 0.11	74 ± 1	2.3	0.4 ^b	63 ^b	4-19
5% DEG	0.8 ± 0.07	36 ± 5	6.1	1.7 ± 0.14	59 ± 2	1.7	3.2 ^b	78 ^b	4-10

^a DP: Degree of polymerization. Estimated based on ESI-TOF-MS analysis.

^b Quantified by ¹H NMR.

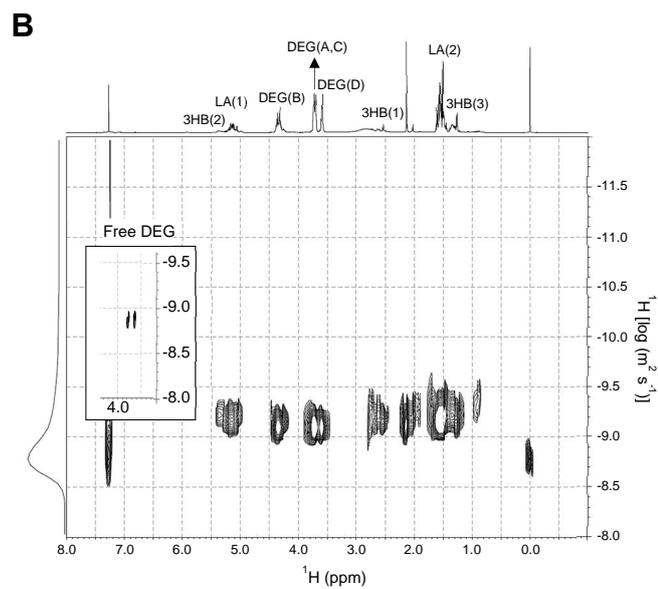
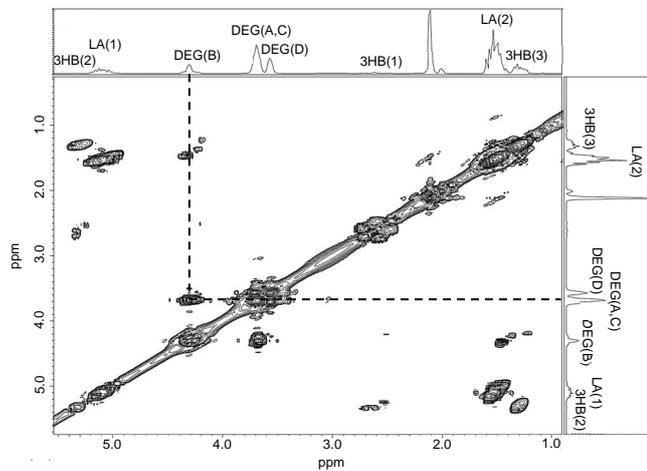
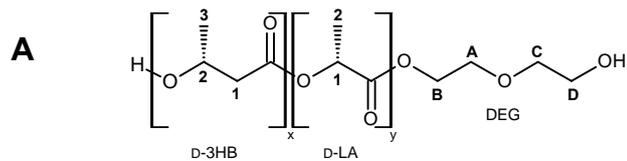
Data are presented as the mean ± standard deviation of three independent biological trials.

Figure 2.9. Whole-cell balance of LA-based products in the D-LAO secretory system. Extracellular extracted D-LAOs, intracellular D-LAOs and the intracellular polymer in cultured recombinant *E. coli* without a CT agent and with the addition of 5% DEG.

2.3.3. Accessing DEG Conjugation at the Terminal of Extracellular D-LAOs

Because DEG was expected to act as an effective CT agent in the synthesis of D-LAOs, extracellular D-LAOs were likely to be capped with DEG at their carboxyl terminal. To evaluate the mode of DEG conjugation, extracted D-LAOs obtained with DEG supplementation were subjected to ¹H ¹H COSY-NMR (in ppm) (Figure 2.10A). The cross signal at 3.7/4.3 ppm indicated that the resonance at 4.3 ppm could be ascribed to the (B) proton in DEG. Therefore, DEG was covalently bound to the carboxyl terminal of D-LAOs. In addition, weak cross signals at 1.5/4.3 and 1.3/4.2 ppm indicated that the resonance of the methine proton at the hydroxyl terminal of LA(1) and 3HB(2) of D-LAOs, respectively, overlapped with the DEG signal. Furthermore, ¹H ¹H DOSY-NMR (Figure 2.10B) revealed

that the diffusion coefficient of DEG was similar to that of D-LAOs but lower than that of free DEG. Therefore, the observed DEG was entirely bound to D-LAOs. ^1H ^{13}C HMQC spectrum (Figure 2.10C) also supported the conclusion that the secreted D-LAOs obtained with DEG supplementation were DEG-bound forms of D-LAOs (termed D-LAOs-DEG). Oligomers secreted without DEG supplementation will hereafter be referred to as free-form D-LAOs. Intracellularly accumulated high molecular weight polymer was also shown to be terminated with DEG at the carboxyl terminal as revealed by ^1H ^1H COSY-NMR (Figure 2.11A) and ^1H ^1H DOSY NMR (Figure 2.11B). On the basis of the ratio between the peak area of the DEG terminal unit and that of the polymeric unit in ^1H NMR, the M_n of the intracellular polymer was estimated to be approximately 5300, which is similar to that obtained by GPC M_n 6100 (Figure 2.9). This result demonstrates that a large fraction of the polymer chains is covalently bound to DEG at the carboxyl terminal. In the previous works, by addition of PEG, the termination at the carboxyl terminal took place in high frequency, and consequently caused reduction in PHA molecular weight (12, 13, 15). ESI-TOF-MS analysis of the extracted D-LAOs-DEG detected periodic m/z values in the range of approximately 400–800 (corresponding to ~4- to 10-mers) (Figure 2.12), indicating that shorter oligomers were synthesized relative to those obtained without DEG. Furthermore, the ESI-TOF-MS analysis detected no series of peaks corresponding to free-form D-LAOs, also supporting that D-LAO produced with DEG supplement should be nearly fully modified with DEG.



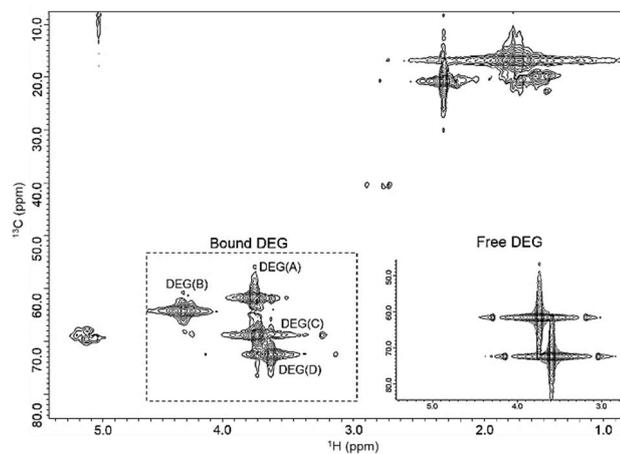
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Figure 2.10. Evaluation of the DEG conjugation to D-LAOs synthesized with 5% DEG supplementation. (A) ^1H - ^1H COSY-NMR; Dashed lines show correlation between the signals at 3.7 and 4.3 ppm, indicating that the signal at 4.3 ppm is ascribed to B proton of DEG. (B) ^1H - ^1H DOSY-NMR. (C) ^1H - ^{13}C HMQC NMR. The four peaks A, B, C and D, which were ascribed to DEG in D-LAOs, differed from those of free DEG, because the DEG bound to oligomers is no longer a symmetric molecule.

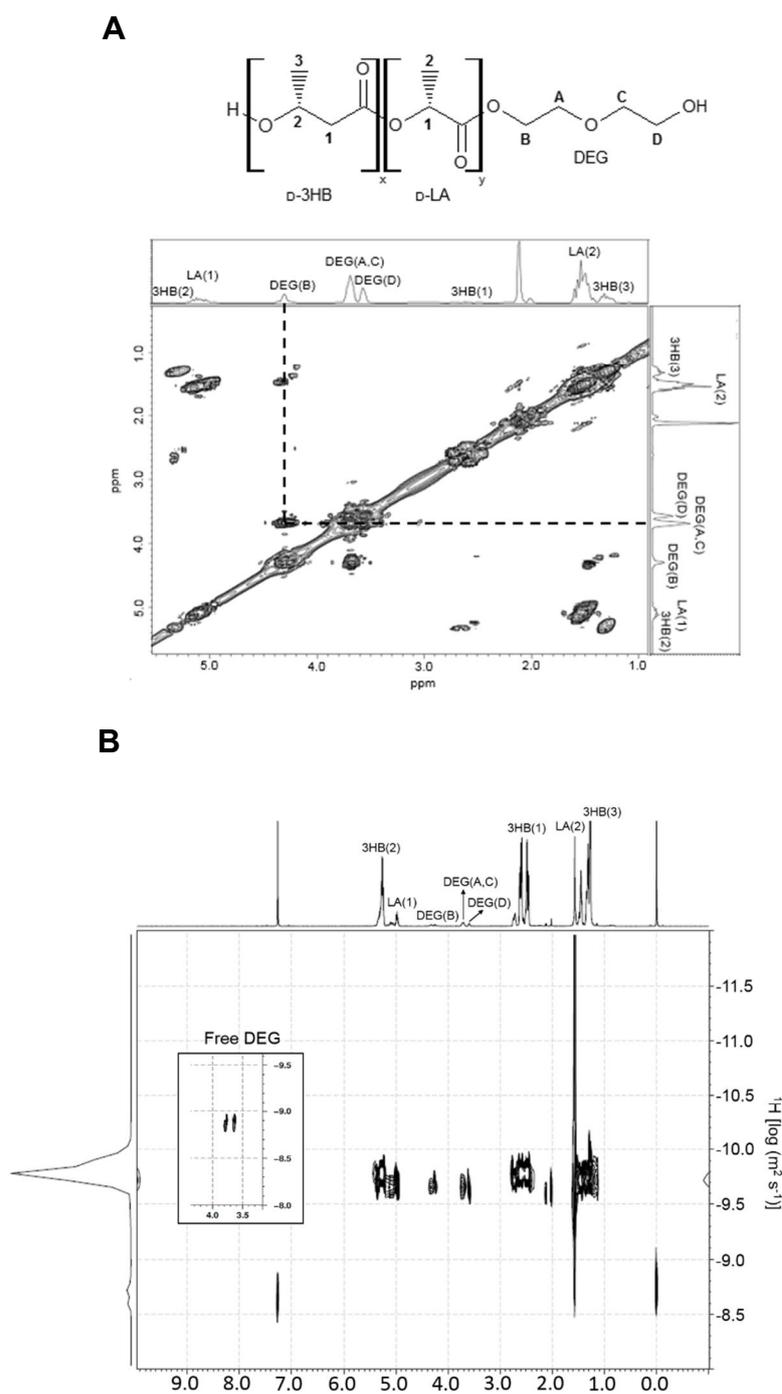


Figure 2.11. Evaluation of the DEG conjugation to intracellularly accumulated high-molecular weight polymer synthesized with DEG supplementation. (A) $^1\text{H}-^1\text{H}$ COSY-NMR. Dashed lines show correlation between the signals at 3.7 and 4.3 ppm, indicating that the signal at 4.3 ppm is ascribed to B proton of DEG. (B) $^1\text{H}-^1\text{H}$ DOSY-NMR.

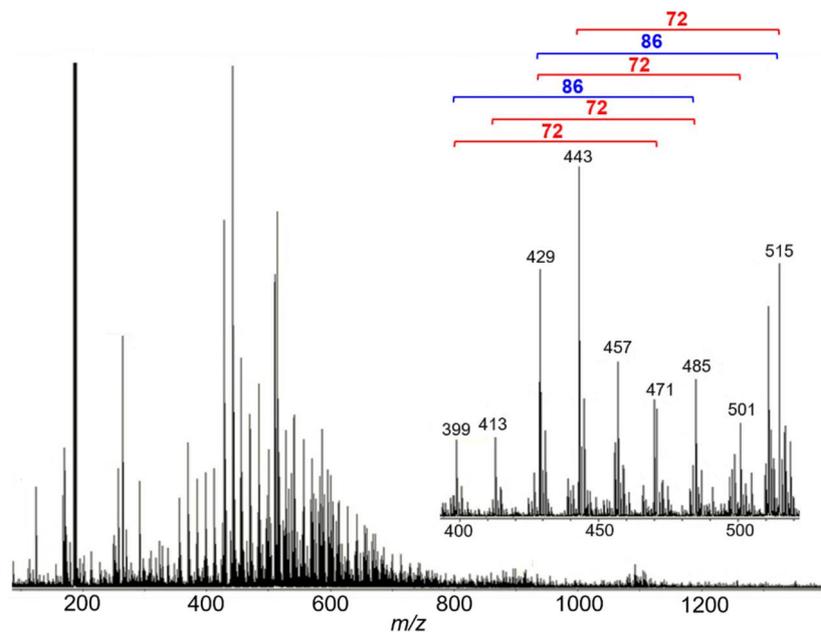


Figure 2.12. ESI-TOF-MS analysis of extracted D-LAOs-DEG. ESI-TOF-MS analysis of D-LAOs-DEG exhibited the periodic peaks, m/z of which were in the range of ~ 400 to 800 , that corresponded to degree of polymerization (DP) of ~ 4 - to 10 -mers.

The efficiency of free-form D-LAOs and D-LAOs-DEG secretion into the medium is an important factor in determining the productivity, and probably, the composition of oligomers. To estimate the secretion efficiency, we focused on the ratio of extracted intracellular and extracellular oligomers (Figure 2.9). In the absence of DEG, the concentration of extracellular free-form D-LAOs (0.4 g L^{-1}) was lower than that of intracellular D-LAOs (1.8 g L^{-1}), indicating the presence of a barrier for secretion. In contrast, DEG supplementation remarkably increased the ratio of extracellular oligomers (3.2 g L^{-1}) to the intracellular fraction (1.7 g L^{-1}), indicating that D-LAOs-DEG were more efficiently secreted than free-form D-LAOs. The enhancement in secretion efficiency by the addition of DEG is presumably due to several factors: concentration, length, and hydrophobicity of the oligomers. The DEG supplement actually increased the frequency of CT reaction and production of oligomers ($8.3 \pm 1.5 \text{ g L}^{-1}$), presumably increasing the mass transfer rate across the cell membrane driven by the concentration gradient of the oligomers. In addition, the DEG supplementation reduces the length of oligomers (Figure 2.3B and Figure 2.12, respectively), and the shorter oligomers

should be secreted more efficiently than the longer molecules. The hydrophobicity of the D-LAOs may also affect the secretion efficiency. D-LAOs-DEG are thought to be more hydrophobic than free-form D-LAOs because of the absence of an ionic carboxyl terminus. The unidentified D-LAO secreting route might favor hydrophobic molecular species over hydrophilic ones. In future, elucidating how D-LAOs are secreted and if specific bacterial transportation systems are responsible for this phenomenon may facilitate enhancing D-LAO secretion. As the related phenomena, bacterial secretion of 3HB oligomers has been reported. The soil isolated *Bacillus megaterium* B-124 secreted 7.6 g L⁻¹ of 3HB trimers into the culture broth (16). Low amounts of methyl esters of 3HB oligomers were extracellularly produced by *Methylobacterium extorquens* DSM13060 (17) and *Roseobacter* clade bacteria (18). These naturally occurring oligomer synthesis is thought to be due to the action of depolymerases responsible for degrading endogenous P(3HB) (17), which is distinguished from the CT-driven D-LAOs synthesis in engineered *E. coli*. These results supported that hydroxyalkanoate oligomers and their end-capped derivatives can be secreted across the cell membrane. However, exporters contributing to the secretion have never been reported.

2.4. Conclusion

Herein, for the first time, we presented the secretion of D-LAOs by *E. coli* expressing a D-specific lactate-polymerizing enzyme. Low amounts of D-LAOs were initially detected in the extracellular fraction of bacterial cells producing P(LA-co-3HB), and the production of D-LAOs was considerably improved by the aid of CT agents. The intracellular LA-based polymer was created by our group, and followed by other researchers (19-21). The limiting factor of the systems in terms of polymer productivity has been the cell volume. The secretion system exhibited here represents a breakthrough of the production limitation and enables the efficient and continuous production of oligomers. In addition, the one-step secretory production of D-LAOs can allow us to establish a shortcut route in the process of PLA production via ROP of lactide, by eliminating the steps of LA purification and polycondensation. Moreover, the D-LAOs-DEG, which possess two hydroxyl ends, have the potential to be used as building blocks for various LA-based polymers production, such as LA-based poly(ester-urethane). Finally, the identification of the D-LAOs secretion route will be an important research target to elucidate the mechanism underlying this newly found phenomenon and to further improve the secretory production of D-LAOs.

2.5. References

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Chapter 3

Creation of a shortcut route in the process of polylactide production:

conversion of D-lactate based oligomers into lactide

3.1. Introduction

Poly lactides (PLAs) are one of the most successful biobased polyesters with diverse applications due to its physical properties, biocompatibility, and processability (1). The conventional process of PLA production from biomass involves (i) a bio-process for the production of lactic acid from biomass sugars, and (ii) a multistep chemo-process containing the oligomerization of lactic acid to generate lactate (LA) oligomers, depolymerization of LA oligomers into lactide (cyclic dimer of LA), and the polymerization of lactide into high molecular weight PLA via ring-opening polymerization (ROP) (2, 3) (Figure 3.1). Although this is the main industrial route, the multistep chemo-bio process is considered complex and expensive relative to petroleum-based polymers (4). Among the steps, the production of lactide from lactic acid, which comprises the oligomerization of lactic acid and the lactide synthesis from LA oligomers, contributes to 30% of the PLA cost (5).

Previously, we have reported the secretory production of D-LA-based oligomers (D-LAOs) (6), which are co-oligomers of D-LA and D-3-hydroxybutyrate (D-3HB), by engineered *Escherichia coli* expressing an evolved D-specific LA-polymerizing enzyme from glucose. The secretory production of D-LAOs is remarkably increased by the supplementation of diethylene glycol (DEG) in the culture medium, leading to the generation of DEG-capped oligomers at the carboxyl terminal (D-LAOs-DEG).

The aim of the present study is to verify the feasibility of the D-LAOs-DEG on the conversion into lactide, since this is a key reaction to construct a shortcut route in the process of poly(D-lactide) (PDLA) production (Figure 3.1). By establishing this new shortcut route, the laborious purification of lactic acid from the microbial culture broth (7) and the lactic acid oligomerization can be eliminated, increasing the sustainability of the material and the cost effectiveness of PDLA. Moreover, the production of optically pure D-LAO is beneficial in

view of the superior thermal properties of poly(L-lactide) (PLLA)/PDLA stereocomplex (8, 9), and the higher production cost of D-LA compared L-LA. Therefore, D-LAOs-DEG were used as substrates for conversion into lactide via metal-catalyzed reaction. To examine the effect of the terminal DEG in D-LAOs-DEG on the lactide formation, free-form D-LAOs (synthesized without DEG addition) were used for comparison. Moreover, to evaluate the influence of 3HB units in D-LAOs-DEG on the lactide synthesis efficiency, we attempted to synthesize LA-enriched oligomers through metabolic and fermentation engineering approaches.

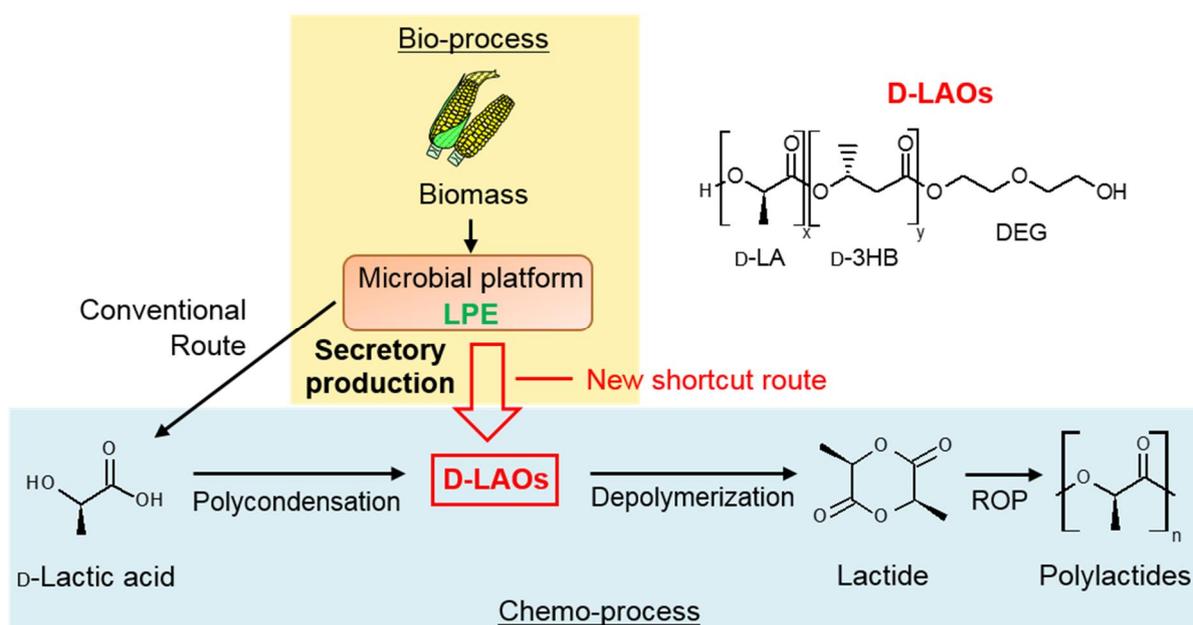


Figure 3.1. Scheme of the conventional route and new shortcut route for PDLA production. In the conventional process, D-lactate (LA) oligomers are chemically prepared from purified D-lactic acid, which is generated by bacterial fermentation, via a polycondensation reaction. In the shortcut route proposed in this study, highlighted in red color, D-LA-based oligomers (D-LAOs) are directly secreted by bacteria from renewable biomass. LPE, Lactate-polymerizing enzyme; ROP, Ring-opening polymerization; DEG, diethylene glycol; D-3HB, D-3-hydroxybutyrate.

3.2. Materials and methods

3.2.1. Bacterial strains and plasmids

E. coli BW25113 (10), and the dual-gene knockout mutant ($\Delta pflA$ and Δdld) JWMB1 (11) were used as the host strains. The expression vector pTV118NpctphaC1_{Ps}(ST/FS/QK)AB harboring *pct*, *phaC1_{Ps}(ST/FS/QK)*, *phaA*, and *phaB* genes (12), was used for the production of D-LAOs.

3.2.2. Culture conditions in test tubes

Cultivations for D-LAOs-DEG production were carried out in 10 mL glass test tubes containing Luria Bertani (LB) medium (1.7 mL) with 20 g L⁻¹ glucose or xylose and 100 mg L⁻¹ ampicillin at 30 °C for 48 h with reciprocal shaking at 180 rpm. The cultivations were performed with the supplementation of 5% DEG (v/v). For observing the aeration effects on D-LAOs production, the cultivations of the dual-gene knockout mutant with xylose as carbon source, were also performed using 2.5, 3.4, and 5.1 mL medium.

3.2.3. Shake flask cultures

Free-form D-LAOs were produced in shake flask cultivation. Seed culture of recombinant *E. coli* was prepared using 2 mL LB medium containing 100 mg L⁻¹ ampicillin in 10 mL glass test tubes and cultured at 30 °C for 12 h with reciprocal shaking at 180 rpm. One milliliter of the seed culture was then transferred into 100 mL LB medium containing 20 g L⁻¹ glucose, and 100 mg L⁻¹ ampicillin in a 500 mL shake flask and cultured at 30 °C for 48 h with reciprocal shaking at 120 rpm.

3.2.4. Measurement of extracellular D-LAOs

The cell-free culture supernatant was analyzed before and after HCl treatment. HCl was added to the supernatant at a final concentration of 2.0 M and incubated at 100 °C overnight to hydrolyse the D-LAOs. Afterward, the hydrolysate was neutralized with 2.0 M NaOH. The estimation of D-LAOs in the culture medium was determined by liquid chromatography-mass spectrometry (LC-MS) (LCMS-8030, Shimadzu, Japan) based on the difference of lactic acid and 3-hydroxybutyric acid concentrations in the samples after and before HCl treatment, as described previously (6).

3.2.5. Extraction of D-LAOs from the culture supernatant

D-LAOs were concentrated from the cell-free culture supernatant by two-phase extraction using chloroform (CHCl_3), with modifications on the previously established method (6). The extraction was performed by adding 1 volume of CHCl_3 to 1 volume of supernatant and mixing vigorously. After the separation of CHCl_3 and water layers, the CHCl_3 phase was transferred to a new test tube. The extraction was performed three times. To remove excess DEG, lactic acid/3-hydroxybutyric acid monomers, and short oligomers, 1 volume of 0.9% NaCl solution pH 8 was added to the resulting CHCl_3 fraction. The washing step was repeated twice. The molecular weight distribution of extracted oligomers was determined by electrospray ionization-time-of-flight-mass spectrometry (ESI-TOF-MS), as described in the literature (6).

3.2.6. Lactide synthesis

The synthesis of lactide occurs via thermal depolymerization of the LA oligomers via a metal-catalyzed backbiting reaction of the OH end groups (13). Approximately 40 mg of vacuum dried extracted D-LAOs containing 68, 78, and 89 mol% LA, were individually weighted together with 40 mg zinc oxide (ZnO, Kanto Chemical, Japan). The sample bottle containing D-LAOs and catalyst was placed inside a rotary type Sibata GTO-350RD glass oven (Sibata Scientific Technology, Japan). The reaction system was heated and kept at 180 °C while being rotated in a circular motion for 1 h under vacuum. The vaporized lactide was condensed into a bottle cooled on ice, and recovered in chloroform. Water-soluble synthetic L-LA oligomers (Glart, Japan) and free-form D-LAO, were also converted into lactide as experimental controls. The lactide yield ($\%$, $2 \times [\mu\text{mol lactide}/\mu\text{mol initial oligomeric LA}]$) was calculated based on the amounts of produced lactide and initial oligomeric LA quantified using the ^1H NMR analysis.

3.2.7. ^1H NMR of D-LAOs and lactide

^1H NMR of extracted oligomers and generated lactides were recorded in CDCl_3 with tetramethylsilane as the internal reference using a JEOL JNM-ECS400 spectrometer (JEOL, Japan) at 400 MHz. Benzoic acid (Wako, Japan) was used as an internal standard to quantify oligomers and lactide based on the integral area of the methyl group ($-\text{CH}_3$); 45° excitation pulse was used and relaxation delay was set to 10 s.

3.3. Results and discussion

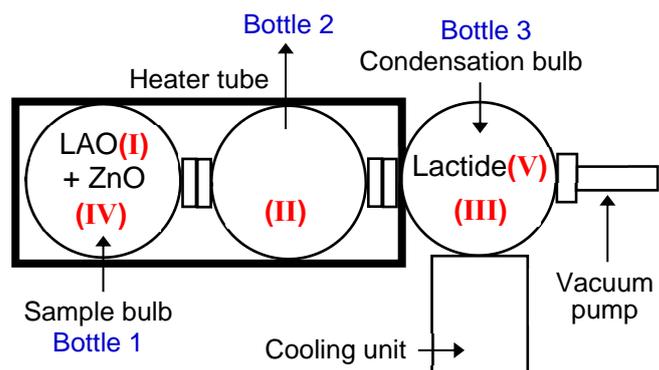
3.3.1. D-LAOs-DEG could be converted into lactide via metal catalyzed backbiting reaction

The synthesis of lactide from the extracted D-LAOs-DEG was undertaken to verify the applicability of the biosynthesized D-LAOs-DEG for subsequent PLA production. These oligomers were produced in the test tube by the wild-type strain BW25113 grown on glucose with 5% DEG supplementation. After extraction of the culture supernatant with chloroform, D-LAOs-DEG with 68 mol% LA and degree of polymerization (DP) of approximately trimer to 7 mer were recovered in chloroform phase (designated as extracted D-LAOs-DEG). As controls, extracted free-form D-LAOs (61 mol% LA, DP ~ trimer to 16 mer) and synthetic L-LA homo-oligomers (L-LAOs) (100 mol% LA, DP ~ trimer to 14 mer), were also used as substrates for lactide synthesis.

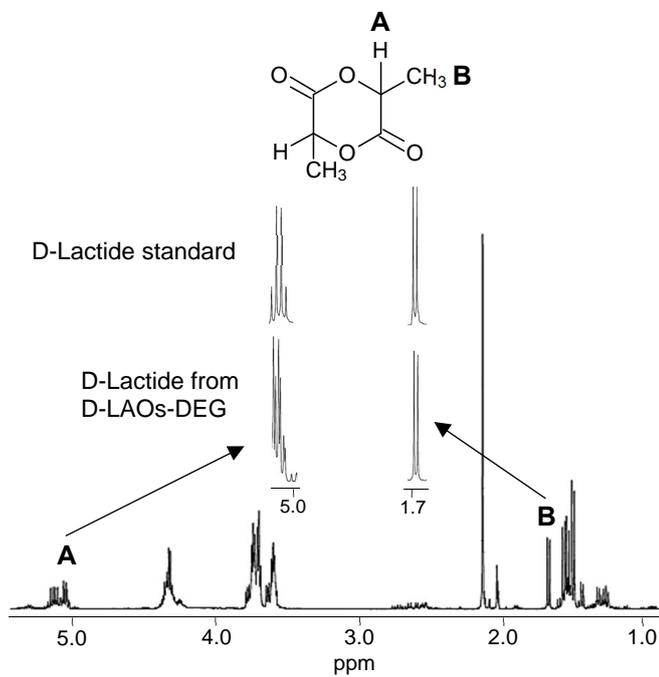
For generating lactides, the LA oligomers were heated with zinc oxide as catalyst, and the vaporized fraction was recovered by condensation in bottle 3 (Figure 3.2A) and subjected to ¹H NMR analysis (Figure 3.2B). The ¹H NMR spectrum (δ in ppm) of the sample generated from D-LAOs-DEG exhibited signals at 5.0 ppm (1H, q, A) and 1.7 ppm (3H, d, B), which were identical to those of the standard D-lactide. The lactide generated from free-form D-LAOs also exhibited the same resonances as the standard D-lactide (Figure 3.3). Synthetic L-LAOs were converted into lactides by using the same procedure. These results proved that the D-LAOs-DEG can be catalytically converted into lactide as well as free-form D-LAOs. The conclusion agrees with the fact that lactide is formed via a backbiting reaction involving the hydroxyl terminal of the oligomer as the active site (14), thus, the carboxyl-terminal structure of D-LAOs should not block the lactide formation. The products condensed in bottle 2 and the residual products in bottle 1 (Figure 3.2A) were also analyzed by ¹H NMR. Unreacted oligomers were found in bottles 1, 2, and 3 after the depolymerization reaction of D-LAOs-DEG, free-form D-LAOs, and synthetic L-LAOs (Figure 3.2C). In addition, the D-LAOs

condensed in bottle 2 had higher 3HB fraction than the oligomers recovered in bottle 3 (Figure 3.2C). The oligomers trapped in bottle 3 should possess lower boiling temperature compared to those in bottle 2, because temperature in bottle 3 is lower than that in bottle 2.

A



B



C

Oligomer samples	Initial oligomer in bottle 1 ($\mu\text{mol unit}$) ^{*, †} (I)	Condensed oligomer in bottle 2 ($\mu\text{mol unit}$) ^{*, †} (II)	Condensed oligomer in bottle 3 ($\mu\text{mol unit}$) ^{*, †} (III)	Residual oligomer in bottle 1 ($\mu\text{mol unit}$) ^{*, †} (IV)	Converted oligomer (%) $1 - [(\text{II} + \text{III} + \text{IV})/\text{I}]$
Free-form D-LAOs					
LA	431	18	253	32	30
3HB	228	22	139	29	16
D-LAOs-DEG					
LA	334	80	87	74	28
3HB	156	37	21	72	16
Synthetic L-LAOs					
LA	495	1	32	248	43

Oligomer samples	Lactide (μmol) [*] (V)	Lactide yield [§] (%) $2 \times (\text{V}/\text{I})$	Lactide formation over substrate consumption (%) $2 \times [\text{V}/(\text{I} - (\text{II} + \text{III} + \text{IV}))]$
Free-form D-LAOs	16	7	25
D-LAOs-DEG	6	4	13
Synthetic L-LAOs	82	33	77

^{*} Quantified by ¹H NMR.

[†] Mole numbers of oligomeric LA and oligomeric 3HB were calculated as LA and 3HB units constituents, respectively.

[§] $2 \times (\mu\text{mol produced lactide}/\mu\text{mol initial oligomeric LA})$.

Figure 3.2. Lactide synthesis from extracted D-LAOs-DEG. (A) Scheme of the lactide synthesis apparatus. The location of the initial oligomer in bottle 1 (I), condensed oligomer in bottle 2 (II), condensed oligomer in bottle 3 (III), residual oligomer in bottle 1 (IV), and lactide (V), in the lactide synthesis apparatus, is represented here. (B) ¹H NMR spectrum of crude lactide synthesized from D-LAOs-DEG 68 mol% LA. (C) Lactide yields from free-from D-LAOs, D-LAOs-DEG, and synthetic L-LAOs as substrates. Refer to Fig. 3.2A for obtaining details about samples localization in the lactide synthesis apparatus.

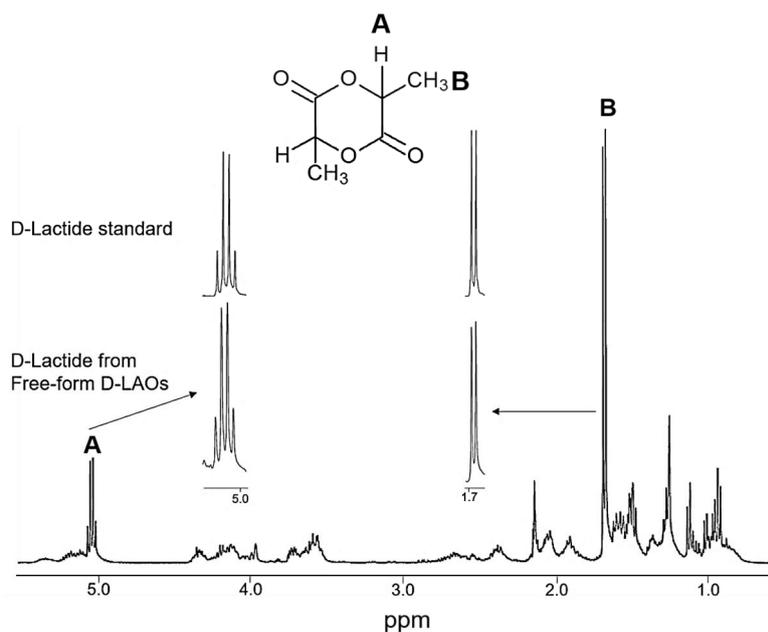


Figure 3.3. ¹H NMR spectrum of crude lactide synthesized from free-form D-LAOs.

3.3.2. LA fraction in D-LAOs-DEG was increased by using xylose as carbon source and a dual-gene knockout mutant as a host strain

Despite the microbial D-LAOs were shown to serve as precursor for lactide, the lactide formation over the substrate consumption obtained from free-form D-LAOs (25%) and D-LAOs-DEG (13%) were substantially lower than that of synthetic L-LAOs (77%) (Figure 3.2C). This result is presumably attributable to the presence of 3HB units in the D-LAOs, which could act as a stopper of the backbiting reaction and decrease the conversion rate of the oligomers into lactide. In addition, the presence of 3HB units decreases the frequency of LA-LA dyad in the oligomers, which is essential for lactide formation. Accordingly, enhancing the LA fraction in the oligomers may further increase the efficiency of the lactide synthesis. Based on this idea, we attempted to produce LA-enriched oligomers by optimizing cultivation conditions such as carbon source, bacterial strain, and culture aeration.

The effect of carbon source, glucose and xylose, on D-LAOs-DEG production and their monomer composition was investigated (Table 3.1). The total production of D-LAOs-DEG in xylose culture was $4.6 \pm 1.1 \text{ g L}^{-1}$, which was lower than that obtained from glucose (6.6 ± 1.2

g L⁻¹). Nevertheless, the D-LAOs-DEG in xylose included a higher LA fraction (97 ± 2 mol% LA) relative to the oligomers secreted in the glucose cultivation (90 ± 4 mol% LA). These results demonstrated that xylose is indeed effective for enhancing the LA fraction in D-LAOs-DEG, although the total production of D-LAOs-DEG was lower compared to the glucose culture. These findings are consistent with the previous report regarding the production of high-molecular-weight P(LA-co-3HB) (15). The LA enrichment in D-LAOs caused by the consumption of xylose might be related to the different capacities of regenerating NADH and NADPH in the metabolism routes of xylose and glucose (16, 17). As a consequence of that, the xylose metabolism is thought to have a higher LA units supplying rate than glucose (15).

Table 3.1. Secretory production of D-LAOs-DEG from glucose and xylose by engineered BW25113 and dual-gene knockout mutant (*ΔpflA* and *Δdld*) JWMB1.

Strain	Carbon source	D-LAOs-DEG			Extracted D-LAOs-DEG		
		CDW (g L ⁻¹)	Oligomer (g L ⁻¹)	LA (mol%) ^a	Oligomer (g L ⁻¹)	LA (mol%) ^b	Recovery (%)
BW25113	Glucose	4.4 ± 0.2	6.6 ± 1.2	90 ± 4	3.5	68	53
	Xylose	5.3 ± 0.1	4.6 ± 1.1	97 ± 2	1.6	62.5	35
JWMB1	Glucose	2.7 ± 0.5	5.9 ± 0.7	78 ± 5	3.0	76.5	51
	Xylose	4.2 ± 0.2	8.1 ± 2.9	97 ± 1	4.5	83	56

^a Determined on the basis of LC/MS analysis.

^b Determined on the basis of ¹H NMR analysis.

In order to address the influence of the host strain, D-LAOs-DEG were produced by the dual-gene knockout mutant (*ΔpflA* and *Δdld*) JWMB1 from glucose or xylose in comparison with the parent strain BW25113 (Table 3.1). The deletion of *pflA* is known to eliminate formate formation from acetyl-CoA, channeling the flux toward lactic acid (18). The *dld* mutation prevents lactic acid oxidation into pyruvate, improving intracellular availability of lactic acid (19). When glucose was used as a carbon source, JWMB1 produced similar amount of D-LAOs-DEG compared to BW25113, and LA fraction in the D-LAOs-DEG obtained using

JWMB1 (78 mol%) was lower than that from BW25113 (90 mol%). In contrast, with the use of xylose, the D-LAOs-DEG production ($8.1 \pm 2.9 \text{ g L}^{-1}$, $97 \pm 1 \text{ mol\% LA}$) was higher than that of BW25113 ($4.6 \pm 1.1 \text{ g L}^{-1}$, $97 \pm 2 \text{ mol\% LA}$). This result indicates that there is a synergy between the dual mutation and use of xylose as a carbon source to increase both the production and LA fraction of the oligomers.

3.3.3. Microaerobic conditions increased the recovery of extracted D-LAOs-DEG

In order to further upregulate the LA fraction in D-LAOs-DEG, the oligomers were produced under microaerobic conditions, which are well-known to promote lactic acid production (20, 21) and LA fraction enrichment in LA-based polyester (22). To control the microaerobic conditions using simplified method, the volume of culture medium in a test tube was increased from 1.7 to 5.1 mL. As the result, recovery of D-LAOs-DEG in chloroform phase was improved by using the microaerobic conditions (Table 3.2). Among them, the highest LA fraction in extracted D-LAOs-DEG was 89 mol% LA (Figure 3.4). The total oligomer productions and their respective LA fractions, were, however, rather decreased (Table 3.2). These results indicated that the hydrophobicity of the oligomers, which is determined by the molecular weight and monomer composition of the oligomers, tends to increase under microaerobic conditions. Here, it should be noticed that the molecular weight of extracted D-LAOs-DEG (DP ~ trimer to 7-mer) was not significantly altered by using microaerobic conditions (Figure 3.5), but the amount of extracted D-LAOs-DEG was increased (Table 3.2). This phenomenon is probably due to the fact that the molecular weight of secreted oligomers is limited by the solubility of oligomer molecules in water.

Table 3.2. Secretory production of D-LAOs-DEG from xylose by engineered *E. coli* JWMB1 under microaerobic condition.

Cultivation volume (mL)	CDW (g L ⁻¹)	D-LAOs-DEG		Extracted D-LAOs-DEG		
		Oligomer (g L ⁻¹)	LA (mol%)	Oligomer (g L ⁻¹)	LA (mol%)	Recovery (%)
1.7	4.2 ± 0.2	8.1 ± 2.9	97 ± 1	4.5	83	53
2.5	3.9 ± 0.1	7.4 ± 1.6	97 ± 2	4.8	84	65
3.4	3.6 ± 0.0	7.7 ± 1.3	93 ± 2	5.0	89	65
5.1	3.0 ± 0.1	6.3 ± 1.1	92 ± 2	3.3	85	52

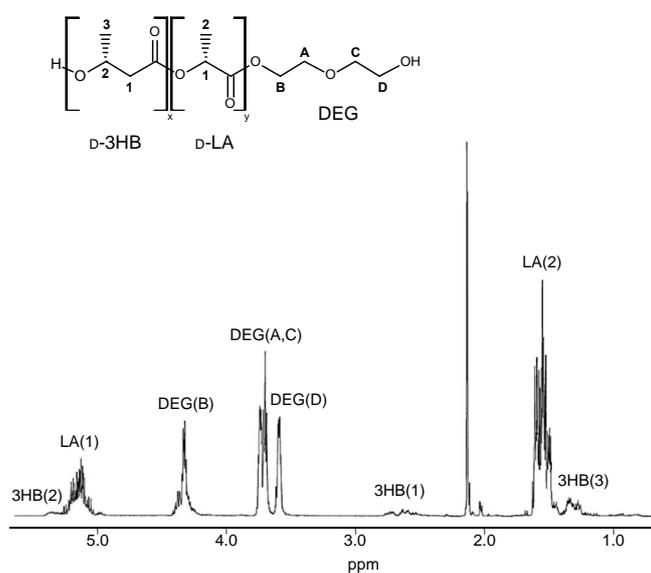
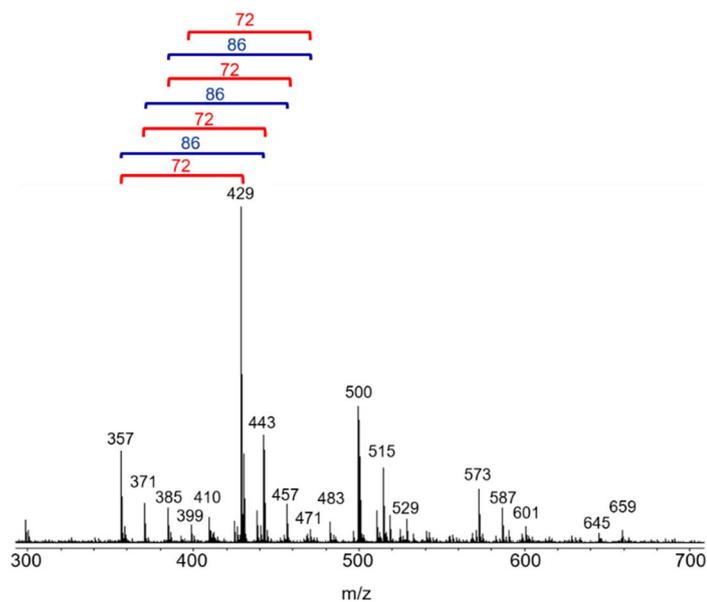


Figure 3.4. ¹H NMR spectrum of D-LAOs-DEG containing 89 mol% LA.

A



B

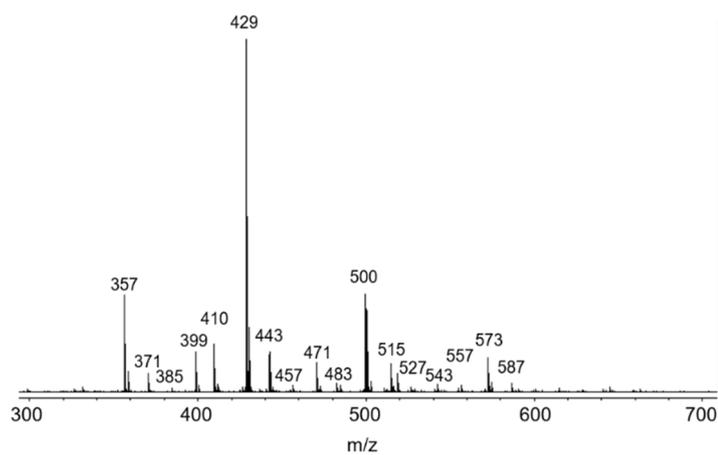


Figure 3.5. ESI-TOF-MS spectra of extracted D-LAOs. (A) D-LAOs extracted from the culture supernatant of JWMB1 using xylose and 5% DEG cultured in 1.7 mL medium. (B) D-LAOs extracted from the culture supernatant of JWMB1 using xylose and 5% DEG cultured in 3.4 mL medium. The intervals between LA (72) and 3HB (86) are represented in the region from 357 to 471 *m/z*.

3.3.4. Lactide yield was enhanced by LA-enrichment in oligomers

The influence of the LA fraction in D-LAOs on the efficiency of lactide synthesis was evaluated using extracted LA enriched D-LAOs-DEG containing up to 89 mol% LA with nearly the same DP of approximately trimer to 7-mer. The LA fraction enrichment in D-LAOs-DEG significantly increased the lactide yields up to 18% (Figure 3.6). Nevertheless, the lactide yield from LA-enriched D-LAOs-DEG was still lower than that obtained from synthetic L-LAOs. Further increase in LA fraction of D-LAOs-DEG seems to be necessary to achieve more efficient lactide synthesis. In addition, an important factor determining the efficiency of lactide synthesis should be the molecular weight of D-LAOs-DEG. During lactide synthesis, significant amount of oligomers was condensed in bottles 2 and 3 (Figure 3.2A and Table 3.3), suggesting that short oligomers were lost by vaporization during heating (23). In the industrial process, oligomers with molecular weights around 400-2500 g/mol, which correspond to DP of approximately 5-mer to 34-mer, are used in the synthesis of lactide (5). Therefore, increasing the molecular weight of the secreted oligomers should be effective to improve the conversion efficiency of D-LAOs-DEG into lactide.

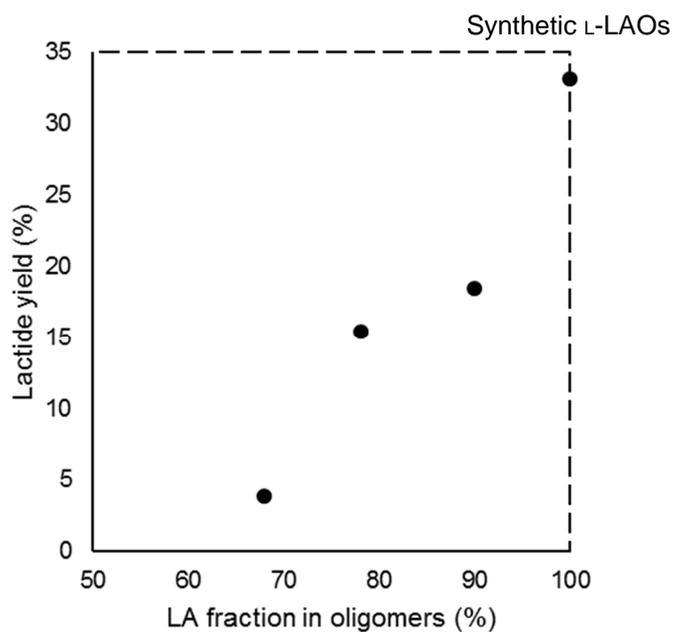


Figure 3.6. Variation of the lactide yield (%) as a function of the LA fraction in extracted D-LAOs-DEG. As a control, lactide was synthesized from synthetic L-LAOs.

Table 3.3. Lactide yield from D-LAOs-DEG varying in LA fraction and from synthetic L-LAOs. Refer to Fig. 3.2A for obtaining details about samples localization in the lactide synthesis apparatus.

Oligomer samples	Initial oligomer in bottle 1 ($\mu\text{mol unit}$) ^{*, †} (I)	Condensed oligomer in bottle 2 ($\mu\text{mol unit}$) ^{*, †} (II)	Condensed oligomer in bottle 3 ($\mu\text{mol unit}$) ^{*, †} (III)	Residual oligomer in bottle 1 ($\mu\text{mol unit}$) ^{*, †} (IV)	Converted oligomer (%) $1 - [(II+III+IV)/I]$	Condensed oligomer (%) $(II+III)/I$
D-LAOs-DEG						
68 mol% LA						
LA	334	80	87	74	28	50
3HB	156	37	21	72	16	37
78 mol% LA						
LA	375	129	79	99	18	55
3HB	105	20	14	71	nd	32
89 mol% LA						
LA	409	116	108	59	31	55
3HB	48	13	6	23	12	39
Synthetic L-LAOs						
LA	495	1	32	248	43	7

Oligomer samples	Lactide (μmol) [*] (V)	Lactide yield (%) [§] $2 \times (V/I)$	Lactide formation over substrate consumption (%) $2 \times [V / (I - (II+III+IV))]$
D-LAOs-DEG (68 mol% LA)	6	4	13
D-LAOs-DEG (78 mol% LA)	29	15	85
D-LAOs-DEG (89 mol% LA)	38	18	60
Synthetic L-LAOs (100 mol% LA)	82	33	77

^{*} Quantified by ¹H NMR.

[†] Mole numbers of oligomeric LA and oligomeric 3HB were calculated as LA and 3HB units constituents, respectively.

[§] $2 \times (\mu\text{mol produced lactide} / \mu\text{mol initial oligomeric LA})$.

nd: not detected.

3.3.5. Potential applications of D-LAOs to produce LA-based polymers

The D-LAOs are featured by their terminal structure and monomer composition, and have the potential to be derivatized into various polymers. The literature provides some examples of the potential use of oligoesters in diverse applications, such as in the synthesis of tailor-made materials (24-26) and as carriers of drugs (27) and bioactive compounds (28). In near future, in order to expand the range of applications, D-LAOs-DEG, which have two hydroxyl ends, will be evaluated as a substrate to produce LA-based poly(ester-urethane)s.

3.4. Conclusion

In conclusion, here, we demonstrated the successful lactide synthesis using D-LAOs-DEG secreted by engineered *E. coli* expressing evolved LPE. Notably, the backbiting reaction-based lactide conversion was not inhibited by the presence of DEG at the carboxyl-terminal of D-LAOs. This finding provides us a proof-of-concept for establishing a shortcut route for PDLA production. Moreover, the improvement in the lactide synthesis efficiency was achieved via LA enrichment in the oligomers by the combination of xylose as carbon source with the use of a dual-gene knockout mutant ($\Delta pflA$ and Δdld) strain.

3.5. References

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Chapter 4

Investigation of the membrane transporters involved in the secretion of D-lactate-based oligomers by *Escherichia coli*

4.1. Introduction

D-Lactate (LA)-based oligomers (D-LAOs) are unusual bacterial oligoesters composed of D-LA and D-3-hydroxybutyrate (3HB) with degree of polymerization (DP) of approximately trimer to 14-mer. D-LAOs are synthesized from glucose and secreted into the culture medium by a recombinant *Escherichia coli* heterologously expressing an engineered polyhydroxyalkanoate (PHA) synthase (PhaC), designated as D-specific-LA-polymerizing enzyme (LPE), together with monomer supplying enzymes (1). D-LAOs are biobased, heavy metal free, water-soluble, moderately hydrophobic (also soluble in chloroform), and enantiopure oligoesters, potentially applicable to a wide range of applications. As a typical example, we have demonstrated the catalytic conversion of D-LAOs into lactide, which is the monomeric unit used for high molecular weight polylactide (PLA) production (2). Therefore, a new shortcut route in the process of PLA production could be created via secretory production of D-LAOs.

The present paper sheds light on the secretion mechanism of D-LAOs by engineered *E. coli*. Molecules can move across the cell membrane to the extracellular environment via simple diffusion and/or via transport system mediated by membrane bound-proteins. In related studies on the transport of organic acids in *E. coli*, the uptake of D, L-lactate and glycolate has been reported to be mediated by the membrane carriers L-lactate-permease (LldP) and glycolate permease (GlcA) (3). The secretion of free fatty acids, on the other hand, has been proposed to occur via both ATP-binding cassette (ABC) transporter MsbA, and simple diffusion (4). Thus, D-LAOs were deduced to be secreted either via intrinsic transporters of *E. coli* and/or simple diffusion. If D-LAOs secretion is mediated by membrane transporters, the respective gene deletions should induce a loss-of-function phenotype of D-LAOs secretion. Based on this hypothesis, in the present study, we designed a screening method to identify D-LAOs secretion route in *E. coli*.

E. coli presumably possesses 1465 membrane proteins according to KEGG database (<http://www.genome.jp/kegg/>). Here, it should be noted that D-LAOs are artificial metabolites, and there is no rational prediction on D-LAOs transporters based on biological machinery, such as operon structure and gene regulation network. Therefore, we selected 209 proteins involved in the transport of organic compounds that were classified into the bacterial secretion system (5), ABC transporters (6), two-component system (7), phosphotransferase system (PTS) (8), and other transporters. The 209 single-gene deletants of membrane proteins, which were transformed to synthesize D-LAOs, were screened to isolate targets that secret less amount of D-LAOs.

4.2. Materials and methods

4.2.1. Bacterial strains and plasmids

E. coli single-gene deletants selected from Keio collection (9), which are membrane protein transporter deficient mutants, and the wild-type strain BW25113 were used. The expression vector pTV118NpctphaCI_{PS}(ST/FS/QK)AB (10) was utilized for the secretory production of D-LAOs. The *pct* encodes propionyl-CoA transferase from *Megasphaera elsdenii*, *phaCI*_{PS}(ST/FS/QK) encodes Ser325Thr/Phe392Ser/Gln481Lys mutated PHA synthase from *Pseudomonas* sp. 61-3, *phaA* and *phaB* encode β -ketothiolase, and NADPH-dependent acetoacetyl-CoA reductase, respectively, both from *Cupriavidus necator* (formerly *Ralstonia eutropha*). For gene overexpression studies, the selected pCA24N derivatives from the ASKA library, a complete set of individual genes from *E. coli* K-12 strain cloned in pCA24N vector under an isopropyl β -D-1-thiogalactopyranoside (IPTG)-inducible promoter (11), and pTV118NpctphaCI_{PS}(ST/FS/QK)AB were introduced into BW25113.

4.2.2. Culture conditions

To produce D-LAOs using, the cells were grown on 1.7 mL of Luria-Bertani (LB) medium containing 20 g L⁻¹ glucose and 100 mg L⁻¹ ampicillin in a 10 mL test tube at 30 °C for 48 h with reciprocal shaking at 180 rpm. Kanamycin (25 mg L⁻¹) and chloramphenicol (30 mg L⁻¹) were added when needed. To overexpress the selected membrane proteins, 1 mM IPTG (final concentration) was added.

4.2.3. Measurement of extracellular D-LAOs and glucose in the culture supernatant

The concentrations of lactic acid, 3-hydroxybutyrate, and glucose in the culture supernatant were measured by HPLC (Jasco, Japan) equipped with an Aminex HPX-87H column (Bio-Rad, U.S.A.). A total of 100 μ l culture supernatant was combined with 100 μ l 4 M HCl and incubated overnight at 100 °C to hydrolyze the D-LAOs. The hydrolysate containing the total amount of lactic acid and 3-hydroxybutyrate (monomers + oligomers) was neutralized with 2 M NaOH and subjected to HPLC analysis. The concentration of D-LAOs in the extracellular fraction was estimated by calculating the difference of lactic acid and 3-hydroxybutyrate concentrations in the samples after (concentration of monomers + oligomers) and before (concentration of monomers) the HCl treatment, as reported previously (1).

4.2.4. Measurement of intracellular D-LAOs

In our previous study, intracellular D-LAOs was defined as a supernatant obtained after methanol precipitation of the chloroform cell extract. In the present study, as a fast method for estimating the intracellular accumulation of D-LAOs, intracellular D-LAOs were directly extracted from the cells with methanol. Lyophilized cells from 1.7 mL culture were soaked in 1 mL methanol at room temperature for 24 h. The extract was filtered by passing through a PTFE filter and the methanol fraction was dried up and weighed (designated as methanol extracted fraction, MEF). The amount of D-LAOs in the methanol extract was determined by HPLC as described above.

4.2.5. Reverse transcription (RT)-PCR analysis of the single-gene overexpressors

The single-gene overexpressors harboring pCA24N derivatives (selected ASKA clones) and pTV118N*pctphaCI*_{Ps}(ST/FS/QK)*AB*, and the parental strain harboring a control plasmid, pCA24N, and pTV118N*pctphaCI*_{Ps}(ST/FS/QK)*AB* were grown on 1.7 mL LB medium containing 20 g L⁻¹ glucose, 100 mg L⁻¹ ampicillin and 30 mg L⁻¹ chloramphenicol at 30 °C with reciprocal shaking at 180 rpm. Overnight culture (2 vol%) was used to inoculate fresh medium containing 1mM IPTG and further cultivated until OD₅₉₅ reached the value of 0.7. The total RNA was extracted using SV Total RNA Isolation System (Promega, U.S.A.). The obtained RNAs were treated with DNase I (NEB, U.S.A.) at 37 °C for 1 h. Ethachinmate (Wako, Japan) was used to improve the recovery of ethanol precipitation of RNA. RNA concentration was determined at 260 nm using spectrophotometer (JASCO V-730, Japan). After which, the RNAs were subjected to PCR and the absence of DNA amplification indicated the effectiveness of the DNase treatment (data not shown). Reverse transcription (RT) and subsequent PCR amplifications were performed using the two-step PrimeScript RT-PCR Kit (Takara, Japan). cDNA was synthesized from 1 µg RNA using random primers provided by the company. Primers used to amplify approximately 400 bp fragments from selected targets are listed in Table 4.1. A housekeeping gene *cysG* encoding a multifunctional uroporphyrin III C-methyltransferase was used as a reference (12).

Table 4.1. Primers used in the RT-PCR.

Primer	Sequence (5' → 3')
ompG F _w	ATGAAAAAGTTATTACCCTGTACCGC
ompG R _v	GTTGAAACGTAAATCGTCAGTCAG
ompF F _w	ATGATGAAGCGCAATATTCTGGC
ompF R _v	CATCGCTGTATGCAGTATCACC
mngA F _w	ATGGTATTGTTTTATCGGGCACACT
mngA R _v	CGAATCTCATCATCCGCAAGG
cysG F _w	GTGGATCATTTGCCTATATTTTGCC
cysG R _v	CAGATGTAACGGCAGCAGTG

4.3. Results and Discussion

4.3.1. Screening of single-gene deletants of membrane transporters to identify D-LAOs secretion routes

Many of the membrane transporters are composed of multiple proteins. To disrupt such multi-component transporters, one gene encoding a putative essential component was deleted. Thus, the screening of 209 deletants was carried out for identifying strain(s) with loss-of-function of D-LAOs secretion (Figure 4.1). Through the screening, 55 strains were found to produce considerably decreased amount of extracellular D-LAOs ($< 0.3 \text{ g L}^{-1}$) compared to parent strain (wild-type strain BW25113 harboring the plasmid for D-LAO production) (Figure 4.2).

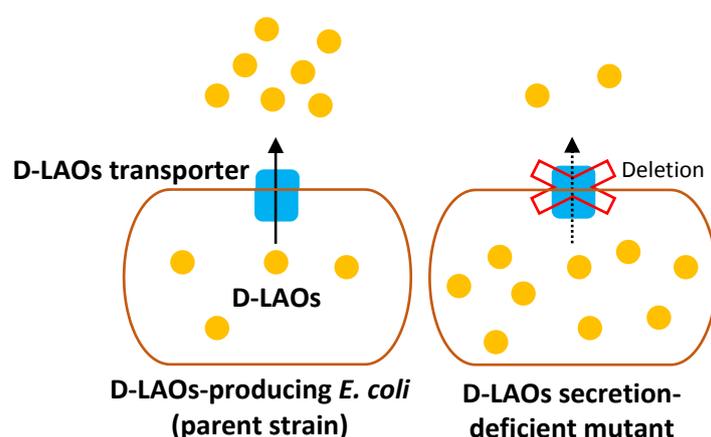


Figure 4.1. Strategy to identify D-LAOs transporter in *E. coli* based on the loss-of-function screening of D-LAOs secretion. The deletion of hypothetical D-LAOs transporter in *E. coli* should lead to a reduction in D-LAOs secretion, and an increase in intracellular D-LAOs accumulation.

To narrow down the range of candidates, the accumulation level of intracellular D-LAOs (wt%) was determined. The strains accumulating greater amount of intracellular D-LAOs were likely to be candidates. Here, we found that the weight of Methanol Extracted Fraction (MEF) was a useful indicator for rapid estimation of the amount of intracellular D-LAOs, because intracellular D-LAOs accounted to approximately 40-70 wt% of MEF (Table

4.2). Figure 4.3 indicates the correlation between the MEF cellular content (wt%), and the cell dry weight (CDW). As seen in the plots, the deletants were categorized into three groups (A, B, and C). Here we focused on deletants in group C, because of their elevated MEF content in the cell, which suggested deficiency in D-LAOs secretion (Figure 4.1). In fact, seven deletants in group C exhibited both diminished secretory D-LAOs production (Figure 4.4A) and greater accumulation of intracellular D-LAOs (Figure 4.4B). Therefore, the seven deletants were chosen as candidates of D-LAOs secretion deficient mutants.

To elucidate the cause of low cell growth of the selected deletants, the strains without plasmid were cultivated under the same culture conditions. In contrast to the recombinant cells harboring the plasmid (Figure 4.2), the selected deletants grew comparably to the parent (Figure 4.5), indicating that the deletion of the membrane proteins alone did not inhibit the cell growth. However, the intracellularly accumulated D-LAOs negatively affected the cell growth. These results supported that the selected membrane proteins could be involved in D-LAOs secretion.

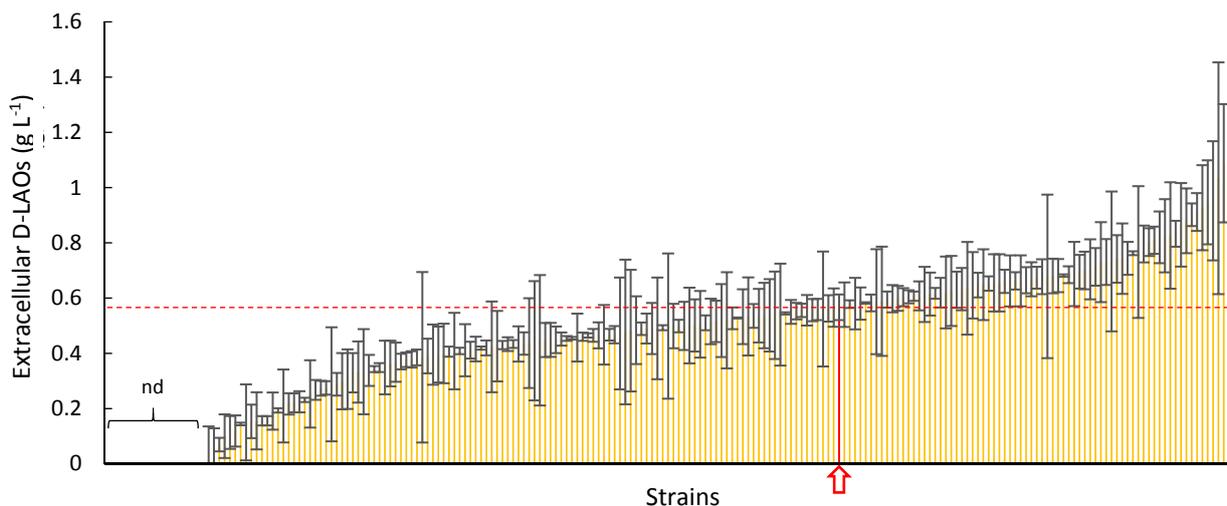


Figure 4.2. Extracellular production of D-LAOs (g L⁻¹) by the screened single-gene deletion mutants. The red arrow represents the parental strain and its production level is indicated by the horizontal dashed l.

Table 4.2. Ratio of intracellular D-LAOs over methanol extracted fraction. Intracellular D-LAOs were measured by HPLC.

	Intracellular D-LAOs relative to methanol extract (%)
Parent	69 ± 10
<i>ompF</i>	48 ± 12
<i>argT</i>	67 ± 5
<i>mngA</i>	68 ± 12
<i>macA</i>	42 ± 7
<i>ompG</i>	48 ± 2
<i>citA</i>	60 ± 9
<i>cpxA</i>	59 ± 14

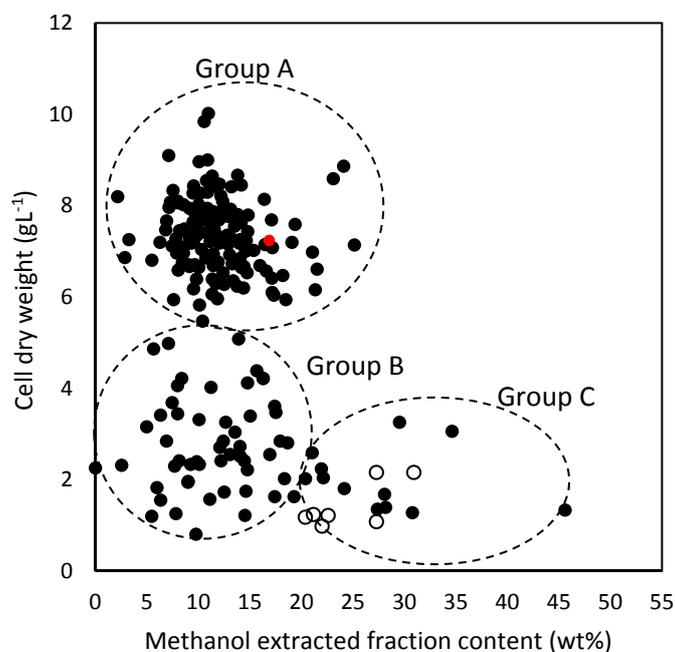


Figure 4.3. Correlation between the cellular content of the methanol extracted fraction (MEF, wt%), which includes intracellular D-LAOs, and the cell dry weight (g L^{-1}) of the single-gene deletion mutants of membrane proteins. The red circle indicates the parent. Group A includes the strains with relatively similar cell growth and MEF cellular content to those of the parent. Group B strains exhibited poor growth and comparable MEF cellular content to that in the parent. Group C strains exhibited poor growth and elevated MEF cellular content. The seven selected D-LAO secretion-deficient mutant candidates are represented by the white circles.

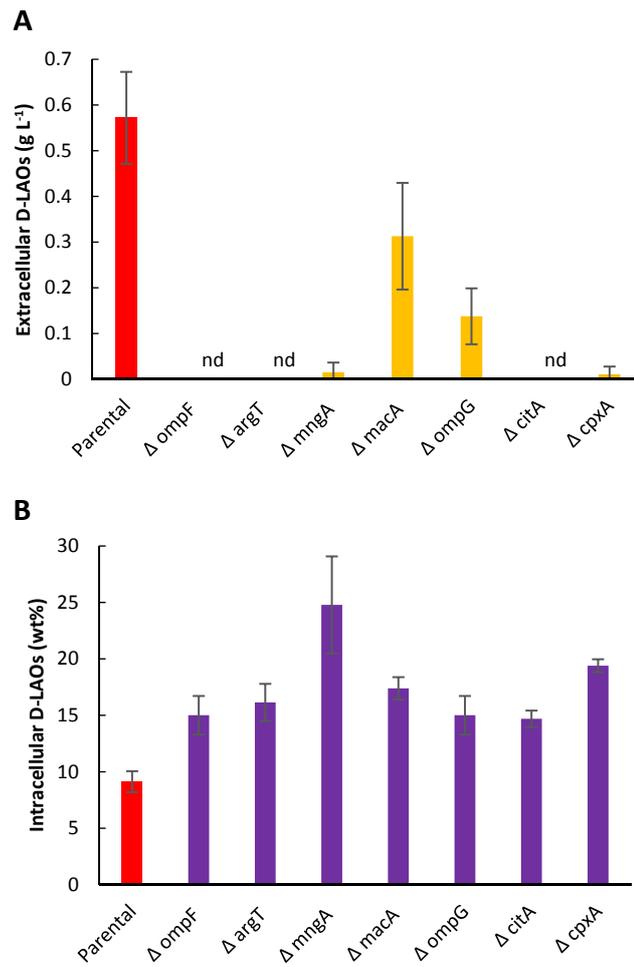


Figure 4.4. Extracellular D-LAO production and intracellular D-LAO accumulation of the selected single-gene deletants harboring the plasmid for D-LAO production. Cells were grown on LB medium containing 20 g/L glucose. (A) Extracellular production of D-LAOs. nd, not detected. (B) Intracellular D-LAO accumulation.

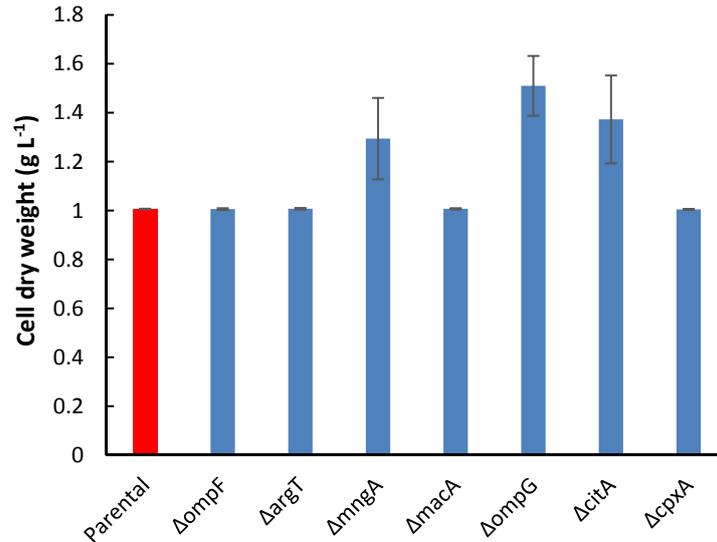


Figure 4.5. Growth of the selected deletants harboring no plasmid (non-D-LAOs-producing conditions) on LB medium containing 20 g L⁻¹ glucose.

4.3.2. The effect of transporter overexpression on D-LAOs secretory production

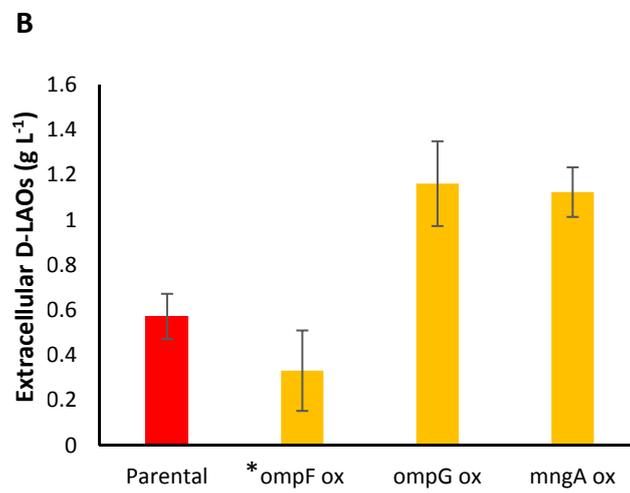
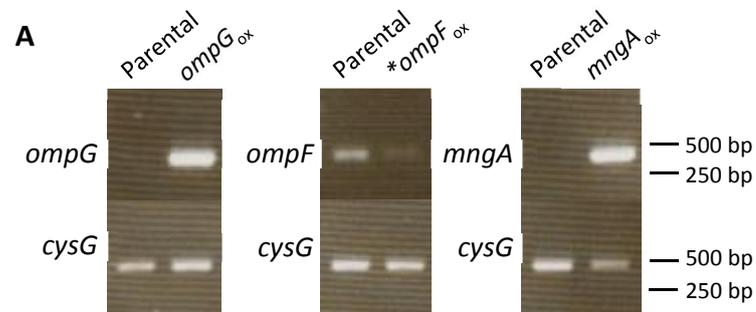
The functions and localization of the selected membrane proteins are listed in Table 4.3. Among the selected candidates, *ompF* and *ompG* encode porins, which are associated with outer membrane, and contain large, open, water filled channels that nonspecifically control and enable the spontaneous diffusion of ions, and small hydrophilic nutrient molecules in bacterial outer membranes (13, 14). At neutral/high pH, OmpG is open and permeable to substrate molecules with size up to 900 Da (14). To address the roles of OmpF and OmpG in D-LAOs secretion, the effect of the genes overexpression on D-LAOs production was evaluated. RT-PCR analysis (Figure 4.6A) demonstrated that the expression level of *ompG*, which was below the detection limit in the parent, was significantly increased in the strain *ompG_{ox}* harboring pCA24N-*ompG*. The *ompG_{ox}* enhanced D-LAOs production compared to parental strain (Figure 4.6B) which suggested that OmpG could function as transporter of D-LAOs. The glucose consumption was similar between the *ompG_{ox}* and the parent (Figure 4.6C), indicating that the increase in extracellular D-LAOs production was not due to the reinforcement in glucose consumption.

Table 4.3. Function of membrane proteins, which were selected as candidates by loss-of-function screening of D-LAOs secretory production.

Orf	Protein function	Localization	Type	References
<i>ompF</i>	Outer membrane porin 1a (Ia;b;F). Participates in the translocation of protein toxins and in the export of YebF protein.	Outer membrane	Other transporters	(18, 19)
<i>citA</i>	Sensory histidine kinase in two-component regulatory system with CitB. Citrate uptake.	Inner membrane (periplasmic and cytoplasmic domain).	Two-component system	(20)
<i>argT</i>	Member of a multi-protein transporter. Binds lysine/arginine/ornithine for uptake.	Periplasm. The multi-protein transporter is located in the periplasm and inner membrane.	ABC transporters	(21)
<i>mngA</i>	2-O- α -mannosyl-D-glycerate (MG) specific enzyme consisting of three domains IIA/IIB/IIC. MG uptake.	Two cytoplasmic domains and one inner membrane domain	Phosphotransferase system (PTS)	(22, 23)
<i>macA</i>	Membrane fusion protein (MFP) component. Macrolide transporter with MacB.	Periplasm (connecting inner and outer membrane components of the transport system). The multi-protein transporter is located in the periplasm and inner membrane.	Other transporters	(24)
<i>cpxA</i>	Sensory histidine kinase in two-component regulatory system with CpxR. Regulates a vast number of genetic loci in response to periplasmic stress.	Inner membrane	Two-component system	(25, 26)
<i>ompG</i>	Outer membrane porin G. Allows the permeability of molecules with up to 900 Da.	Outer membrane	Other transporters	(14)

In contrast, the expression level of *ompF* was detectably high in the parental strain without pCA24N-*ompF*, and when pCA24N-*ompF* was introduced, expression level of *ompF* was relatively decreased compared to parental strain (Figure 4.6A). The unsuccessful overexpression of *ompF* might be due to repression of the high basal expression level of *ompF* in the present of abundant OmpF. In fact, OmpF is known to be one of the most abundant proteins and one of the major porins in *E. coli* (15, 16). Consistent with RT-PCR result, extracellular D-LAO production was decreased in the strain harboring pCA24N-*ompF*, suggesting that OmpF could assist the transporting of D-LAOs as well as OmpG. Accordingly, porins could facilitate the passage of D-LAOs across the outer membrane. In particular, OmpF seems to be one of the major porins responsible for mediating the spontaneous secretion of D-

LAOs by *E. coli*, since its high basal expression level was observed. OmpG, on the other hand, is likely to play a secondary role on D-LAOs secretion.



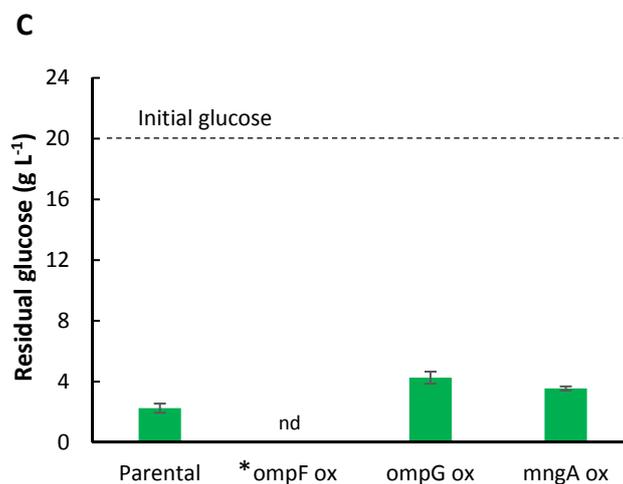


Figure 4.6. D-LAO production by the overexpressors of *ompF*, *ompG* and *mngA* grown on LB medium containing 20 g/L glucose. *E. coli* BW25113 was used as the host. (A) RT-PCR analysis of overexpression of the selected membrane proteins. The parental strain harbored pCA24N. The *ompG*_{ox}, **ompF*_{ox}, and *mngA*_{ox} strains harbored pCA24N-*ompG*, pCA24N-*ompF* and pCA24N-*mngA*, respectively. *ompF*_{ox} is represented as **ompF*_{ox} since its expression was decreased when pCA24N-*ompF* was introduced. *cysG* is a constitutively expressed housekeeping gene. PCR using the *ompF* primer set was carried out for 25 cycles, and the others were performed for 30 cycles. (B) Extracellular production of D-LAOs (g/L). (C) Glucose concentration in the medium (g/L) after 48 h cultivation. nd, not detected.

Regarding to inner membrane-associated proteins, in contrast, there is no common function among the selected candidates (Table 4.3). This result may suggest that there are multiple secretion routes across inner membrane. Among them, *mngA* encodes a single-component transporter, and therefore, D-LAOs production using the strain harboring pCA24N-*mngA* was investigated. The overexpression of *mngA* gene was confirmed by RT-PCR (Figure 4.6A). The *mngA* overexpressor, *mngA*_{ox}, produced greater amount of extracellular D-LAOs compared to the parent (Figure 4.6B), indicating that MngA is related to D-LAOs secretion. It was reported that MngA (formerly HrsA) can be positive porin regulator under some conditions (17). In this study, no considerable upregulation of *ompF* and *ompG* was observed in the strain *mngA*_{ox} (Figure 4.7). Therefore, we concluded that the positive effect of *mngA*_{ox} on D-LAOs production could not be due to elevating porin expression levels, but directly enhanced D-LAOs transport by MngA. To elucidate the precise roles of the other selected candidates, *argT*,

macA, *citA*, and *cpxA*, which function with other membrane components, on D-LAOs secretion, the overexpression of the entire transporter complex would be needed and will be addressed in our future studies.

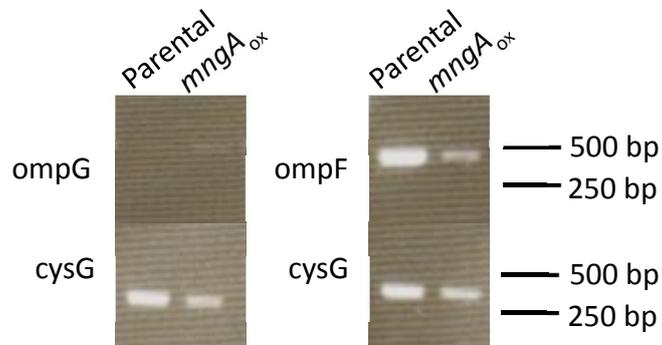
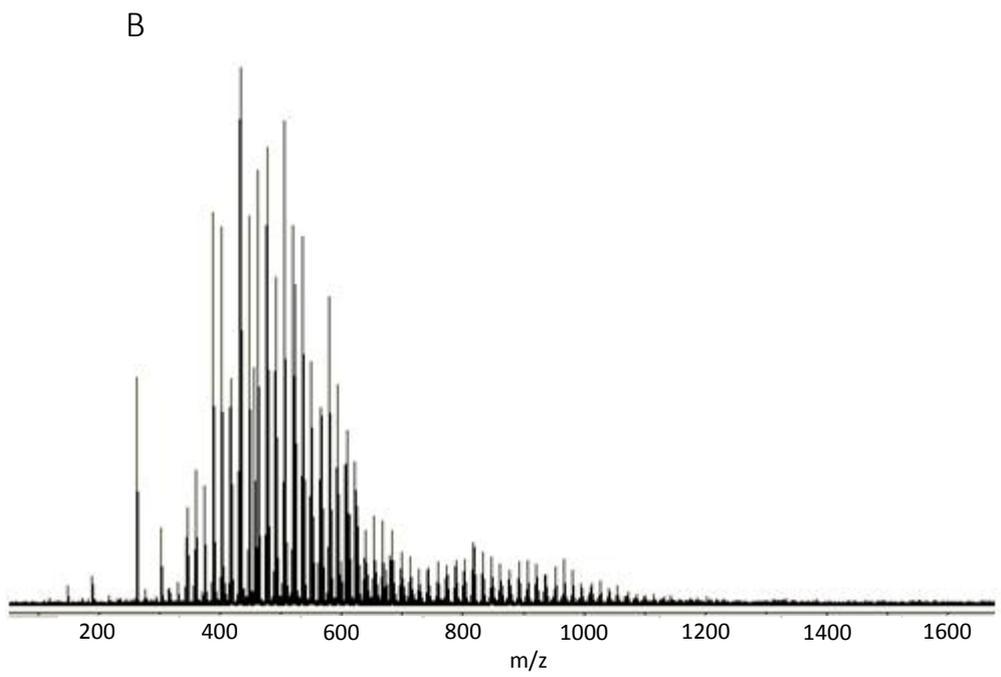
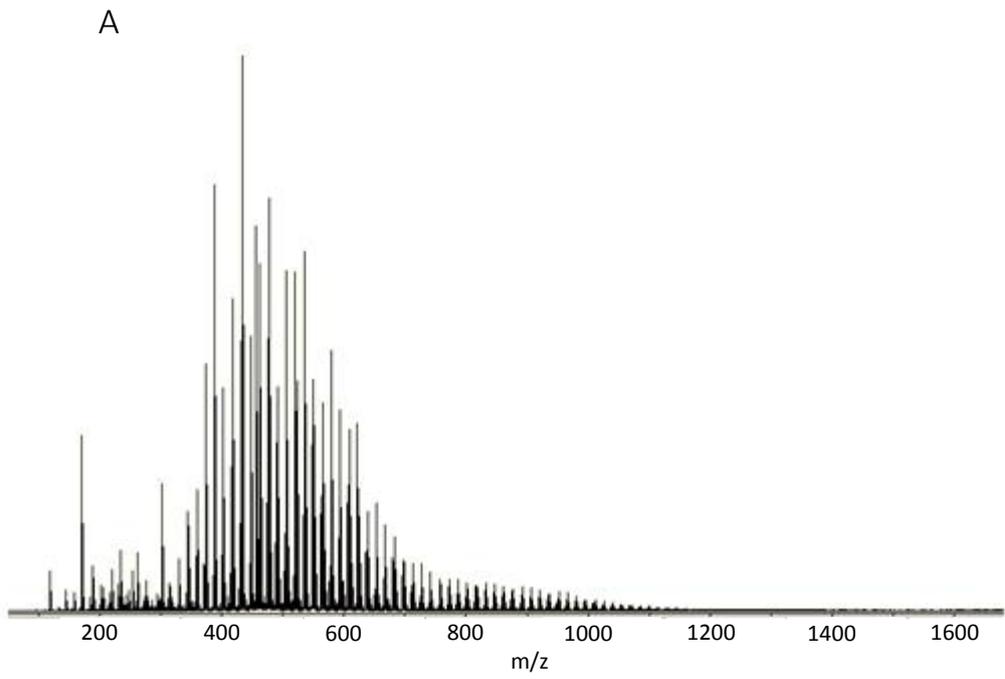


Figure 4.7. Analysis of *ompG* and *ompF* expression in *mngA_{ox}* strain (BW25113 harboring pCA24N-*mngA*). RT-PCR was performed using RNA extracted from *mngA_{ox}*, and *ompG* and *ompF* primers. The *cysG* gene is a housekeeping gene, which is constitutively expressed.

4.3.3. Overexpression of *ompG* and *mngA* had no considerable effect on D-LAOs molecular weight

The increased secretory production of D-LAOs by *ompG_{ox}* and *mngA_{ox}* strains could be caused either by enhanced secretion rate or expansion of the size range of secreted oligomers. Therefore, the DP of D-LAOs produced by these overexpressors was determined. As shown in Figure 4.8, DP values were in the range of approximately trimer to 14-mers which correspond to m/z of 200 to 1000, and there was no significant difference between parent and the overexpressors tested. Therefore, the gene overexpression of *ompG* and *mngA* should reinforce the intrinsic secretion route, which may have limitations on the oligomer size in the range of 3-14 mers, rather than opening a new pathway which would allow the secretion of longer-chain D-LAOs.



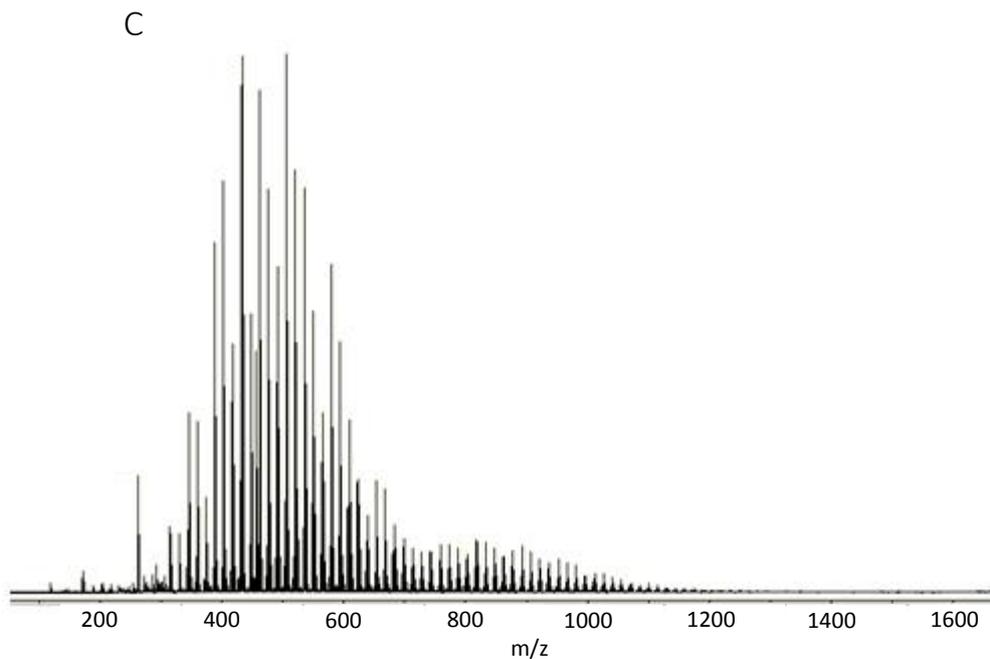


Figure 4.8. ESI-TOF-MS analysis of extracted extracellular D-LAOs. A) Parent B) *ompG*_{ox} C) *mngA*_{ox}.

4.3.4. Proposed model on D-LAOs secretion mechanism

For the extracellular production of compounds by Gram-negative bacteria, the secreted compounds must cross the inner and outer membranes to reach the extracellular environment (5). Thus, based on our results, the passage of D-LAOs across the outer membrane could be mediated by passive diffusion through porins, such as OmpF and OmpG. In case of crossing the inner membrane, inner associated proteins such as MngA, CitA, ArgT, MacA, and CpxA were selected in this study. Therefore, we hypothesize a sequential route for D-LAOs secretion in which porins and these inner membrane proteins could transport D-LAOs in outer membrane and inner membrane, respectively, as illustrated in Figure 4.9. It should be noted that the present result does not exclude the contribution of simple diffusion through phospholipid bilayer, and that some of these routes might work simultaneously.

As related studies, the secretion of 3HB trimer by *Bacillus megaterium* B-124 (27), and 3HB oligomers by *Methylobacterium extorquens* DSM13060 (28), and *Roseobacter* clade

bacteria (29) have been reported, but the secretion systems have never been identified. The three bacteria possess several putative porins based on Uniprot database (www.uniprot.org). The contribution of porins to the secretion of 3HB oligomers will be an interesting subject.

We previously reported that D-LAOs carboxyl-terminated with diethylene glycol (DEG) (defined as D-LAOs-DEG) can be synthesized using *E. coli* by supplementing DEG in the culture medium as a chain transfer agent. D-LAOs-DEG, which are non-ionic and possess hydroxyl group at both terminals, might be secreted via different route(s) from D-LAOs. In fact, D-LAOs-DEG were much more efficiently secreted than D-LAOs (1). The D-LAOs-DEG secretion routes could be identified by using the same strategy reported in this study.

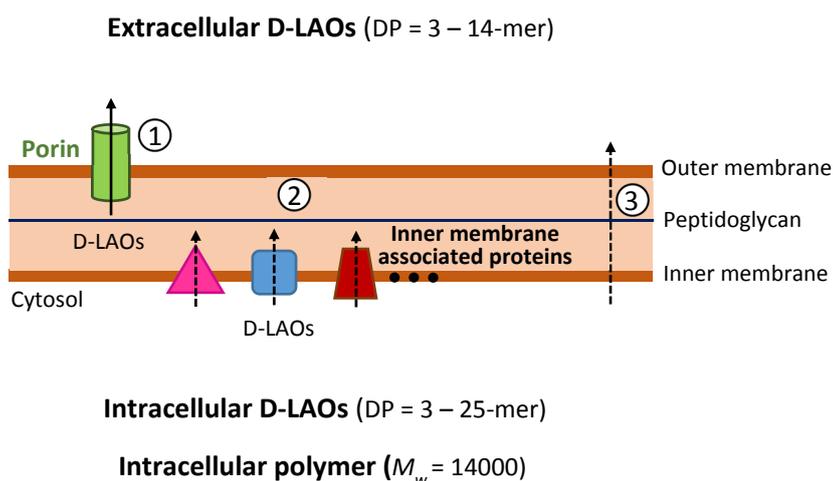


Figure 4.9. Proposed D-LAOs secretion routes in *E. coli*. 1) The passage of outer membrane is mediated by porins such as OmpF and OmpG. 2) The passage of inner membrane may be mediated by multiple inner membrane-associated transporters, such as MngA, CitA, ArgT, MacA, and CpxA. 3) The results demonstrated in this study do not exclude the possibility of simple diffusion through phospholipid bilayer. Dashed lines indicate unidentified routes.

4.4. Conclusion

The D-LAOs secretion route in *E. coli* was investigated based on the loss-of-function screening using a set of single-gene deletion mutants. As the results, porins were proposed to be involved in the passage of D-LAOs through the outer membrane. From deletants of inner membrane-associated proteins, five candidates ($\Delta mngA$, $\Delta citA$, $\Delta argT$, $\Delta macA$, and $\Delta cpxA$) were selected, suggesting the existence of multiple secretion routes across the inner membrane, although the precise roles of these inner membrane-associated proteins in D-LAOs secretion remains uncharacterized. The present study does not exclude the possibility that simple diffusion through phospholipid bilayer partially contributes to D-LAOs secretion. Therefore, the entire D-LAOs secretion phenomenon might be due to the cooperation of these multiple factors. The knowledge obtained in this study should be useful to further engineer bacterial cells towards the development of efficient D-LAOs production system.

4.5. References

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Chapter 5

Conclusion

In this study, we presented the first microbial secretion system of D-LA-based oligomers (D-LAOs), which are random co-oligomers of LA and 3HB. An engineered *E. coli* using sugars as a carbon source and expressing monomer supplying enzymes and an evolved D-specific LA polymerizing enzyme, besides to intracellularly accumulate P(LA-co-3HB), was found to secrete low amounts of D-LAOs into the culture supernatant. The spontaneous secretion of D-LAOs without performing any additional modification in *E. coli*, such as the introduction of exogenous membrane transporters, was an unexpected finding. So far, the possibility of D-LAOs secretion by *E. coli* was uncertain, considering that D-LAOs are unnatural products to *E. coli* and that the limitations caused by factors such as molecular weight and water solubility on the passage of D-LAOs across the cell membrane were unknown. The discovery of the microbial secretion system of D-LAOs should be a breakthrough in this research field since the intracellular accumulation of polyesters and the difficulties for polymer recovery are critical drawbacks in the microbial production of polymers.

The main motivation for the creation of a microbial secretion system of LA oligomers was to utilize them in the process of PLA production. To reach this goal, increasing the secretory production of D-LAOs was mandatory. Among the strategies tested, increasing the frequency of polymer chain transfer (CT) reaction leading to early chain termination by the addition of alcoholic compound as the CT agent in the *E. coli* cultivation, was the most successful. The extracellular production of D-LAOs was substantially increased by the supplementation of diethylene glycol (DEG) in the bacterial cultivation. Also, the D-LAOs were almost fully conjugated with DEG at the carboxyl terminal (designated as D-LAOs-DEG) because of the CT reaction induced by DEG. In addition, the increased extracellular production of D-LAOs-DEG caused by DEG was indicated to take place via secretion rather than cell lysis.

Once the efficient microbial secretion system of D-LAOs was established, the following step was to evaluate the applicability of D-LAOs-DEG in the process of PLA

production, through the conversion of D-LAOs-DEG into lactide. Three factors were likely to influence the lactide synthesis from D-LAOs-DEG: i) DEG conjugation at the carboxyl terminal of D-LAOs, ii) the presence of 3HB units, and iii) the molecular weight of D-LAOs. First, the DEG bond at the carboxyl terminal of D-LAOs containing 68 mol% LA was demonstrated to not hamper the conversion of D-LAOs-DEG into lactide. Second, by increasing the LA fraction up to 89 mol% LA in D-LAOs-DEG through metabolic and fermentation engineering approaches, the lactide yield from D-LAOs-DEG was increased from 4 to 18%. It indicated that 3HB decreases the conversion efficiency, thus the LA fraction in D-LAOs-DEG must be increased for achieving high lactide yield. In addition, the results suggested that the molecular weight of D-LAOs-DEG, which ranges from approximately 3 ó 7 mer, is a limiting factor to further increase the high lactide yield. D-LAOs-DEG produced from the secretory system were successfully converted into lactides, demonstrating the feasibility of the shortcut to the conventional ROP route idealized in this study. By adopting this new D-LA oligomer-providing pathway, the costs, time and energy associated with PLA production steps of LA fermentation, purification and oligomerization can be eliminated. Thus, such biotechnological achievement could greatly contribute to the establishment of cost-effective production of both PDLA and stereocomplexed PLLA/PDLA.

The capacity of engineered *E. coli* spontaneously secrete D-LAOs into the culture supernatant was the most fascinating aspect of this research. Therefore, the investigation of the existence of membrane proteins mediating the transport of D-LAOs across the cell membrane was attempted. It was hypothesized that if the D-LAOs secretion is mediated by membrane transporters, the gene deletion of membrane proteins contributing to D-LAOs secretion should reduce the oligomer secretion. Based on this idea, a loss-of-function screening of the ability to secrete D-LAOs using 209 single-gene deletants of membrane proteins, which are involved in the transport of organic compounds, was performed. Outer and inner membrane-associated

proteins were selected as candidates. The effect of the up- and down-regulation of genes candidates on D-LAOs production was also evaluated. The obtained results suggested that the secretion of D-LAOs by *E. coli* is unspecific and takes place via multiple routes.

Future Work

In Chapter 3, the conversion of D-LAOs-DEG into lactide was successfully performed and improved by increasing the LA fraction in D-LAOs-DEG. Nevertheless, it was observed that during the thermal depolymerization of D-LAOs-DEG, short oligomers were lost during heating decreasing the lactide yield. Thus, for applying the D-LAOs-DEG in the practical production of PDLA, increase and fine-tuning the molecular weight of D-LAOs-DEG will be needed. Moreover, the applications of D-LAOs-DEG can be further expanded, such as for the production of LA-based poly(ester-urethane)s. Therefore, in future, the D-LAOs can also be used as building blocks to be assembled into various LA-based polymers.

Regarding the studies on the secretion routes of D-LAOs in *E. coli*, in Chapter 4 we presented the very first results of this research line. Therefore, the investigation of the candidates of D-LAOs transporters will continue in order to better understand their roles in the microbial secretion system of D-LAOs. Moreover, due to the difference in terminal structure, the secretion route of D-LAOs-DEG might be different from D-LAOs. Thus, the differences and similarities between the secretion systems of D-LAOs and D-LAOs-DEG will be an interesting research target for future studies. Also, the possibility of D-LAOs secretion by simple diffusion need to be investigated.

Lastly, it is expected that the extracellular production reported here can be expanded to various organic acid oligomers. Also, bacterial species, PHA synthases, and types of CT agents can be further explored in order to improve and fine-tuning the microbial secretion systems of organic acid oligomers.

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APPENDIX

Appendix I – Semi-continuous secretory production of D-LAOs-DEG by recombinant *E.*

coli

A potential advantage of the engineered *E. coli* efficiently secreting D-LAOs-DEG is its applicability to a continuous production that would save time and energy for cultivation, and the nutrients for the cell growth. To establish a continuous system, the cells were required to possess long-term productivity of D-LAOs-DEG. Therefore, here I investigated the semi-continuous cultivation, in which the *E. coli* cells were repeatedly used in fresh medium for the secretory production of D-LAOs-DEG

The cells of *E. coli* BW25113 harboring pTV118NpctphaC1_{Ps}(ST/FS/QK)AB were grown in 10 mL test tubes containing 1.7 mL LB with 20 g L⁻¹ glucose, 100 mg L⁻¹ ampicillin and 5% (v/v) DEG at 30 °C for 36 h. The bacterial cultures were centrifuged at 13,000 g for 10 min and the culture supernatant was collected. The cell pellet was resuspended in fresh LB medium (1.7 mL) containing glucose, ampicillin and DEG and further cultivated at 30 °C for 36 h. The same step was repeated one time, followed by a final cultivation at 30 °C for 48 h (Figure S1).

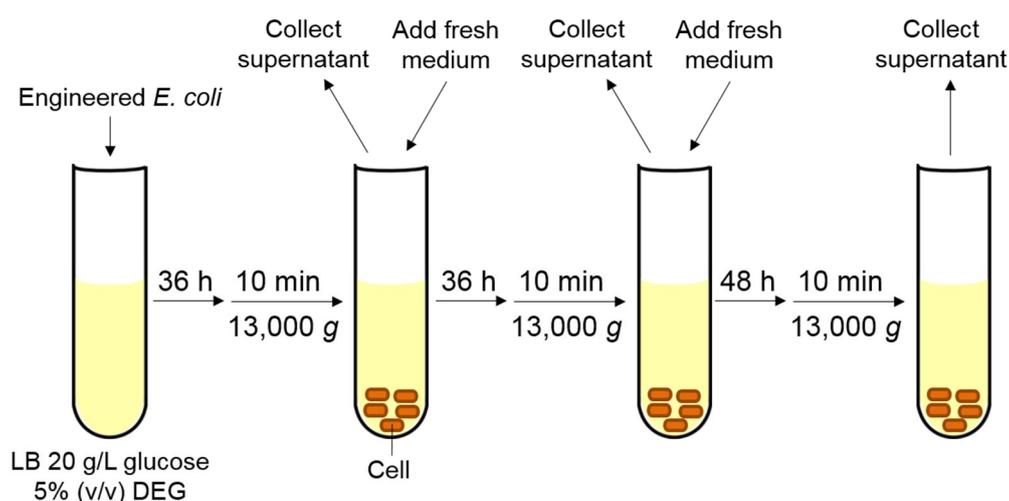


Figure S1. Scheme of the method used for the semi-continuous secretory production of D-LAOs-DEG by engineered *E. coli*.

The concentration of D-LAOs-DEG in the cell-free culture supernatant was estimated by measuring the amount of oligomeric LA using HPLC, as previously (Chapter 2, Section 2.2.5.). The remaining glucose in the culture supernatant after the cultivation was also measured by HPLC (Chapter 4, Section 4.2.3.).

As shown in Figure S2, D-LAOs-DEG can be efficiently produced in the first 36 h cultivation (Supernatant 1). After recycling the cells and incubating for further 36 h (Supernatant 2), the secretory production obtained in the second cycle increased compared to that in the first cycle. The complete consumption of glucose corroborated with the efficient extracellular production of D-LAOs. Nevertheless, by using the cells from a second cell recycling (Supernatant 3), the production and secretion of D-LAOs-DEG was substantially decreased as well as the glucose consumption.

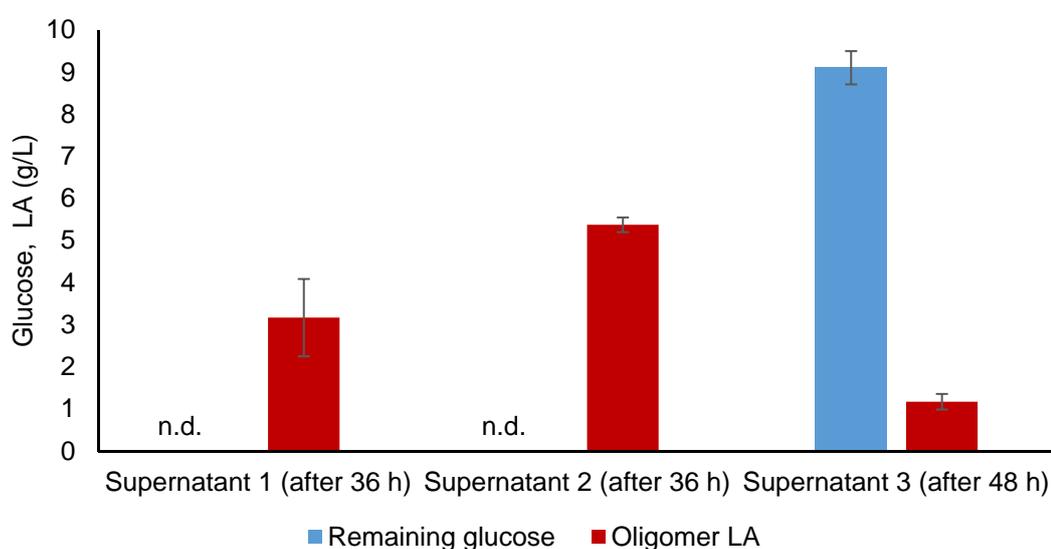


Figure S2. Concentration of oligomer LA and glucose in the culture supernatant along the semi-continuous secretory production of D-LAOs-DEG. n.d., not detected.

Such limitation can be attributable to the polymer accumulation which may saturate the microbial system and inhibit the oligomer production and secretion. Thus, for measuring the polymer content in the cells (% wt), the lyophilized cells were weighted for determining the cell

dry weight (g/L). For the analysis of the polymer production, the cells were treated with concentrated sulfuric acid (H₂SO₄) at 120 °C for 45 min to convert intracellular 3HB units into crotonic acid or LA units into acrylic acid. The samples were subjected to HPLC equipped with an Aminex HPX-87H column (Bio-Rad, U.S.A.) at 60°C eluted with 0.014 N H₂SO₄ at flow rate of 0.7 ml/min.

At the end of the cultivation, the polymer content (51 ± 5 wt%) was relatively high, indicating that the intracellular polymer accumulation would be a limiting factor for the efficient synthesis and secretion of D-LAOs. Cell death after two rounds of cell recycling might be another limiting factor. Thus, the control of the PhaC activity in order to decrease the polymer accumulation and the optimization of the cultivation conditions may contribute to improve the long-term stability in continuous system.

Appendix II – Molecular weight distribution of D-LAOs along a time course cultivation

In Chapter 2, the molecular weight of D-LAOs was described to have a bimodal distribution with peak tops at 7-mer and 12-mer. Here, as an attempt to elucidate such pattern, the molecular weight distribution of oligomers obtained from a time course cultivation was determined.

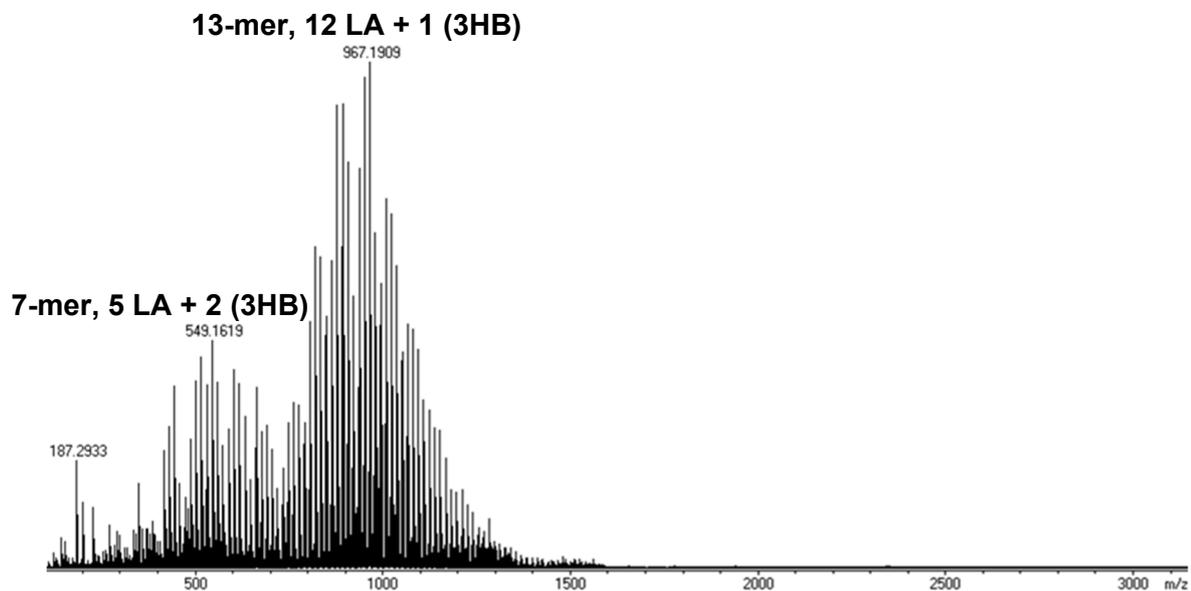
E. coli BW25113 harboring pTV118NpctphaCI_{Ps}(ST/FS/QK) was used for D-LAOs production. The culture was conducted in 10 mL test tubes containing 1.7 mL LB with 20 g L⁻¹ glucose and 100 mg L⁻¹ ampicillin for 48 h at 30 °C. The culture supernatant samples were taken at 12, 24, 30, 36 and 48 h.

The oligomers were extracted from the cell-free culture supernatant by a two-phase extraction using chloroform, as described above (Chapter 3, Section 3.2.5.). The molecular weight distribution of the oligomers was determined by ESI-TOF-MS, as previously (Chapter 2, Section 2.2.7.).

At 12 h (Figure S3A) and 24 h (Figure S3B), the molecular weight distribution with 12-mer as peak top was more abundant. From 30 h (Figure S3C, D, E), the distribution with 7-mer as a peak top started to increase. Furthermore, in all samples of the time course, the peak tops of the molecular weight distribution of D-LAOs are fixed at around 7-mer and 12-mer (Figure S3).

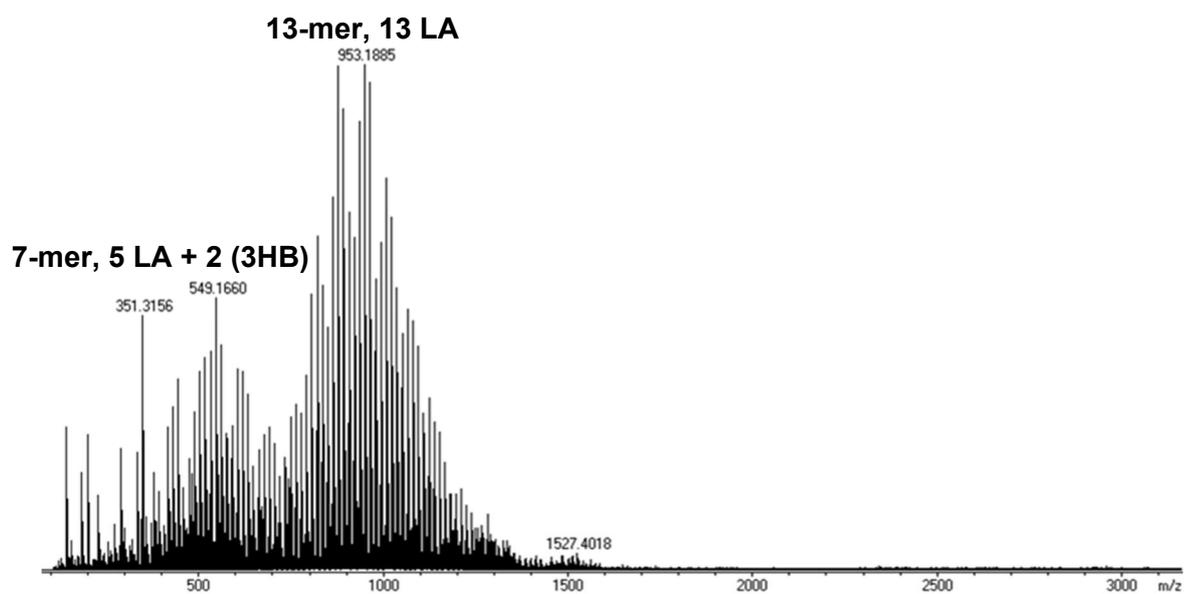
A

12 hours



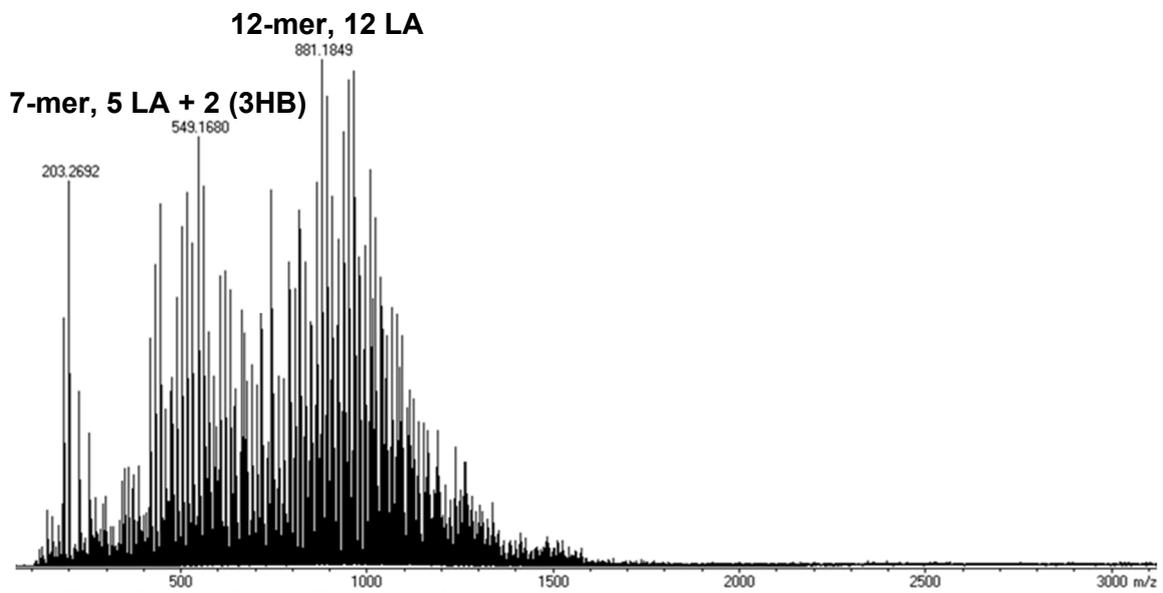
B

24 hours



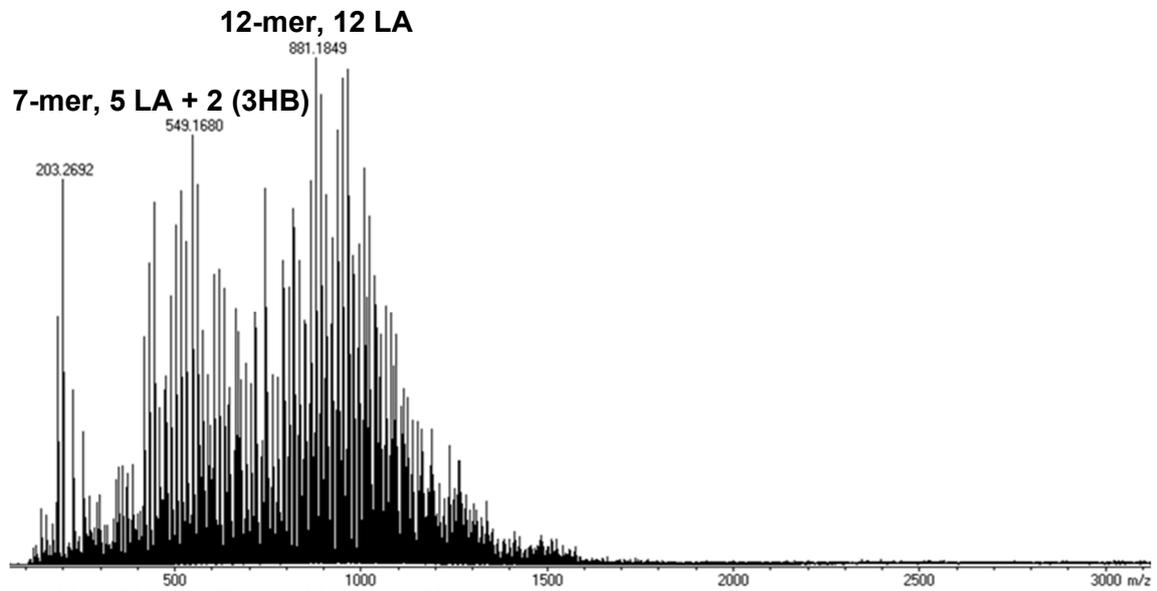
C

30 hours



D

36 hours



E

48 hours

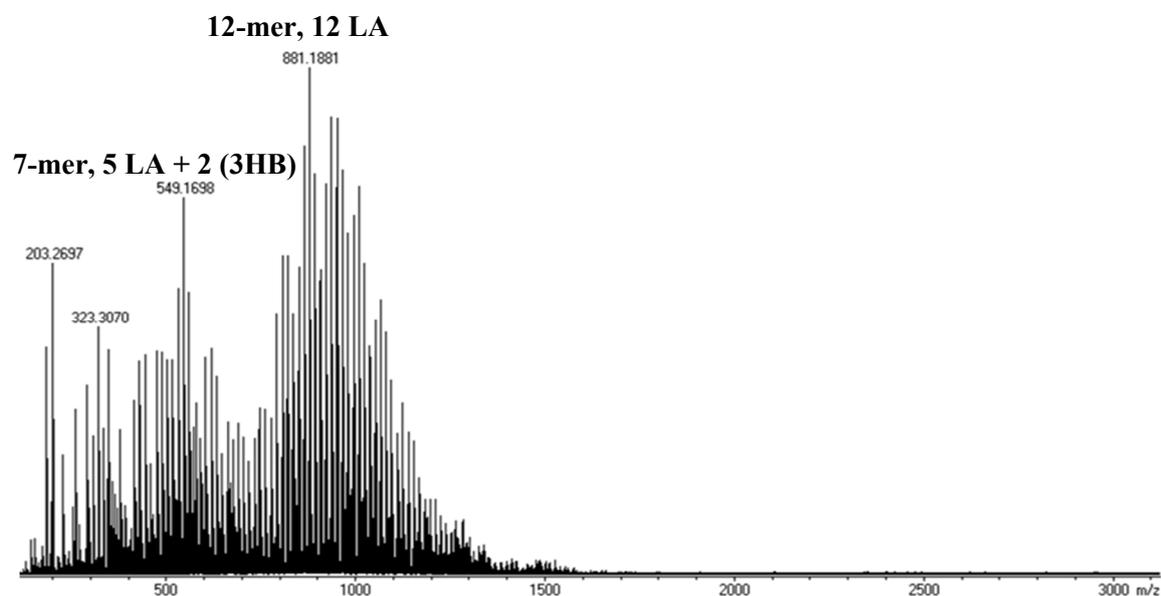


Figure S3. Molecular weight distribution of extracellular D-LAOs along a time course cultivation. (A) D-LAOs secreted at 12 h. (B) D-LAOs secreted at 24 h. (C) D-LAOs secreted at 30 h. (C) D-LAOs secreted at 36 h. (E) D-LAOs secreted at 48 h.

The increase in the distribution of oligomers with lower molecular weights along the time might be due to a random degradation of oligomers in the culture medium at 30 °C. The degradation catalyzed by enzymes is unlikely, since *E. coli* does not produce PHA depolymerases. Thus, a time course detection of the oligomers molecular weight in a culture medium incubated at 30 °C without cells may be useful to address this hypothesis. On the other hand, possible changes in the activity of PhaC during the cultivation may also affect the resulting molecular weight distribution of the oligomers. Therefore, the measurement of the PhaC activity can be useful to elucidate this possibility. Regarding the peak tops of the D-LAOs molecular weight distribution fixed at 7-mer and 12-mer, I hypothesize that the major D-LAOs transporters may have higher affinity for oligomers with DP of 7-mer and 12-mer due to their physical-chemical properties, such as size and hydrophobicity levels (Figure S4). The

investigation of the molecular weight distribution of D-LAOs produced by single-deletants of membrane transporters of D-LAOs may help us to validate this hypothesis.

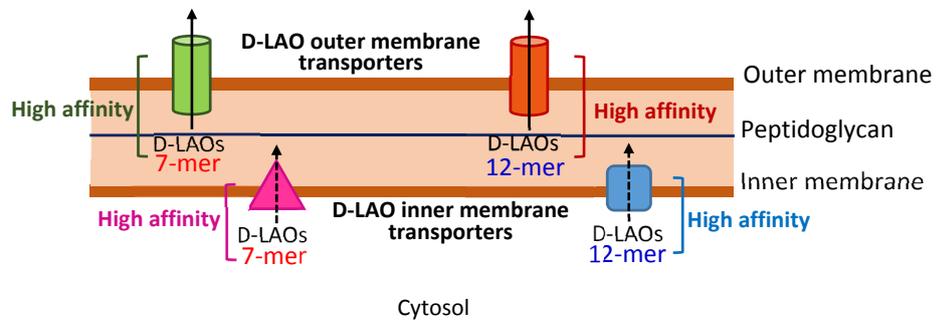


Figure S4. Illustration of the hypothesis that the molecular weight distribution of D-LAOs might depend on the affinity of the D-LAO membrane transporters and the oligomers' physical-chemical properties. In the microbial secretion system of D-LAOs, specific transporters would have higher affinity for D-LAOs with DP of 7-mer and 12-mers.