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Swinging Effect of Salicylic Acid on the Accumulation of Polyhydroxyalkanoic Acid (PHA) in *Pseudomonas aeruginosa* BM114 Synthesizing Both MCL- and SCL-PHA

RHO, JONG KOOK[2], MUN HWAN CHOI[2,3], JI HOON SHIM[2], SO-YOUNG LEE[1], MYEONG JI WOO[2], BONG-SUNG KO[4], KI-WHAN CHI[4], AND SUNG CHUL YOON[2,3]*

[1]*Nano-Biomaterials Science Laboratory,* [2]*Division of Applied Life Sciences (BK21), Graduate School,* [3]*Environmental Biotechnology National Core Research Center, Gyeongsang National University, Jinju 660-701, Korea*
[4]*Department of Chemistry, University of Ulsan, Ulsan 680-749, Korea*

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Abstract A bacterium, *Pseudomonas aeruginosa* BM114, capable of accumulating a blend of medium-chain-length (MCL)- and short-chain-length (SCL)-polyhydroxyalkanoic acid (PHA), was isolated. Salicylic acid (SA), without being metabolized, was found to specifically inhibit only the accumulation of MCL-PHA without affecting cell growth. An addition of 20 mM SA selectively inhibited the accumulation of MCL-PHA in decanoate-grown cells by 83% of the control content in one-step cultivation, where overall PHA accumulation was inhibited by only ~11%. Typically, the molar monomer-unit ratio of the PHA for 25 mM decanoate-grown cells changed from 46:4:25:25 (=[3-hydroxybutyrate]:[3-hydroxycaproate]:[3-hydroxyoctanoate]:[3-hydroxydecanoate]) at 0 mM SA (dry cell wt, 1.97 g/l; PHA content, 48.6 wt%) to 91:1:4:4 at 20 mM SA (dry cell wt, 1.85 g/l; PHA content, 43.2 wt%). Thus, the stimulation of SCL-PHA accumulation was observed. Growth of *P. aeruginosa* BM114 on undecanoic acid also produced a PHA blend composed of 47.4% P(3HB-*co*-3-hydroxyvalerate) and 52.6% P(3-hydroxyheptanoate-*co*-3-hydroxynonanoate-*co*-3-hydroxyundecanoate). Similar to the case of even-carboxylic acids, SA inhibited the accumulation of only MCL-PHA, but stimulated the accumulation of SCL-PHA. For all medium-chain fatty acids tested, SA induced a stimulation of SCL-PHA accumulation in the BM114 strain. SA could thus be used to suppress only the formation of MCL-PHA in *Pseudomonas* spp. accumulating a blend of SCL-PHA and MCL-PHA.

Keywords: Polyhydroxyalkanoic acid, PHA accumulation inhibitor, salicylic acid, SCL-PHA accumulation stimulator

*Corresponding author
Phone: 82-55-751-5942; Fax: 82-55-753-0765;
E-mail: scyoon@gnu.ac.kr

Polyhydroxyalkanoic acids (PHAs) are accumulated in a wide range of microorganisms in the form of granular inclusion bodies [2, 7, 17]. There are various types of PHA with chemically different monomer compositions, exhibiting many useful physicochemical properties [12, 21]. It is possible to design PHA using many techniques such as homologous PHA synthesis-related gene insertion, combination of different precursor carbon sources, multistep cultures [3], and pathway routing by inhibitors [6, 16, 24] to improve the material properties such as melting point, glass transition temperature, crystallinity, degradation rate, mechanical strength, *etc.* [23].

PHA synthesis-related inhibitors can be used to find the metabolic pathway from which precursors for PHA synthesis are supplied as well as to channel intermediates of a specific pathway to PHA synthesis [6, 16, 24]. In a previous study [15], we have reported that 2-bromooctanoic acid (2-BrOA) very specifically inhibits PHA accumulation of *Pseudomonas fluorescens* BM07 from saccharides without any influence on the cell growth. It was suggested [15, 16] that 2-BrOA inhibits 3-hydroxyacyl-ACP-CoA transferase, PhaG, the enzyme [5, 9, 25] linking between fatty acid *de novo* biosynthesis and biosynthesis of PHA in pseudomonads. The inhibitory effect of 2-BrOA has also been reported in *Pseudomonas putida* CA-3 grown on styrene, phenylacetic acid, or glucose [27, 28]. 2-BrOA was thus suggested to be a very useful inhibitor for efficient pathway routing for the preparation of a specially designed PHA with the cells being in active growth [15, 16]. An apparent competitive relationship between 2-BrOA and octanoic acid or 11-phenoxyundecanoic acid was reported to be present in *P. fluorescens* BM07 [16]. On the basis of the competitive relationship, the inhibitor 2-BrOA was

able to raise the yield of conversion of expensive substituted fatty acids, *e.g.*, 11-phenoxyundecanoic acid into PHA up to 100% [16].

Wautersia eutropha had been known to incorporate only short-chain-length (SCL) monomers into PHA [2, 17]. Acrylic acid, known as a β-oxidation inhibitor for the bacterium, was recently reported to induce incorporation of medium-chain-length (MCL) monomers as comonomers into PHA, when the wild-type cells were cultivated with octanoic acid as the carbon source [6]. Thus, despite their usefulness in the study of the PHA synthesis pathway as well as pathway routing, only a few PHA-synthesis related inhibitors have been reported including 4-pentenoic acid [15].

In this study, we found that salicylic acid, a known inhibitor of a β-oxidation enzyme [22], inhibits only the accumulation of MCL-PHA in *Pseudomonas aeruginosa* BM114, isolated in our lab, which accumulates a mixture of poly(3-hydroxybutyric acid) [P(3HB)] or P(3HB-*co*-3-hydroxyvalerate (3HV)) and MCL-PHA composed of C_6 to C_{11} 3-hydroxyacids from the medium-chain fatty acids such as octanoic, nonanoic, decanoic or undecanoic acid. Salicylic acid specifically blocked only the accumulation of MCL-PHA, with no effect on both the cell growth and simultaneously stimulated accumulation of SCL-PHA. This is the first report that salicylic acid inhibits the accumulation of MCL-PHA in bacteria and modulates the monomer distribution in PHA.

MATERIALS AND METHODS

Microorganism and Culture Media

A sample from an active sludge in a municipal wastewater plant in Jinju City (Korea) was resuspended in distilled water and inoculated to a mineral salts M1 medium containing 0.05% ammonium sulfate and 1% octanoic acid [4]. After 2 days of incubation at 30°C and 200 rpm, the culture was diluted 10^5 folds, and 100 μl of the dilution was inoculated onto octanoate M1 mineral agar plates and cultivated for 24 h at 30°C. Several single colonies were tooth-picked according to their opacity, usually caused by PHA synthesis in cells, and purified by a series of spreading on octanoate agar plates. Among the colonies, a bacterial colony capable of accumulating a blend of SCL- and MCL-PHAs, characterized by gas chromatography, was isolated. The strain was characterized according to Bergey's manual [13] and identified as *Pseudomonas aeruginosa*. The strain was named *Pseudomonas aeruginosa* BM114 and deposited in the Korean Collection for Type Cultures (KCTC 11133BP). The isolated *P. aeruginosa* BM114 was used throughout the experiments. Nutrient-rich medium was used in the seeding, maintenance, and storage of the isolated strain and contained 1% yeast extract, 1.5% nutrient broth, and 0.2% ammonium sulfate. The modified M1 mineral-salts medium of the same composition as that reported earlier [4] was used as PHA synthesis medium.

In a one-step cultivation, the culture (5 ml) grown in nutrient-rich medium at 30°C, 180 rpm, for 12 h was transferred to 500 ml of M1 mineral-salts medium containing an appropriate amount of a carbon source and salicylic acid and 1.0 g/l ammonium sulfate in a 2-l flask and cultivated to maximal growth. Salicylic acid was solubilized by adding sodium hydroxide, and the pH of the solution was adjusted to 7 before adding to the medium. The growth of cells was monitored by the measurement of the optical density at 660 nm. The remaining fructose was measured using the DNS method, and the remaining carboxylic acids were determined gas chromatographically [4]. The cells were isolated by centrifuging (7,000 rpm, 10 min) the cell suspension, washed with methanol, and dried under a vacuum at room temperature for 2 days. The percent inhibition of PHA synthesis and cell growth in the presence of salicylic acid were determined according to the procedure reported earlier [15]. The salicylic acid level was monitored by measuring UV absorption of the culture supernatant at 298 nm using the nonsalicylic-acid-added culture medium as the blank. The remaining NH_4^+ was measured using Nessler's reagent [4]. Minimum of triplicate experiments were carried out and statistically averaged.

Quantitative Assay of PHA in Cells

The monomer composition of the PHA in the cells was determined by analyzing the methyl esters, recovered from a sulfuric acid/methanol treatment of the cells, using a Hewlett-Packard HP5890 Series II gas chromatograph equipped with a HP-5 capillary column and a flame ionization detector [4]. A typical GC run condition was as follows: initial temperature 80°C, 2 min; heating rate, 8°C/min; final temperature 250°C, 1.75 min; carrier (He) flow rate, 3 ml/min; injector temperature, 250°C; detector temperature, 300°C. The standardization of each GC peak was made against the PHA of known structure characterized by quantitative NMR analyses [15].

Characterization of Isolated PHA

The 125-MHz ^{13}C NMR spectra for isolated polymers were recorded at 25°C on a $CDCl_3$ solution of polyester (25 mg/ml) with a 10 μs pulse width, 5 s pulse repetition, 25,000 Hz spectral width, 64 K data points, and 5,000–10,000 accumulations. Tetramethylsilane was used as an internal chemical-shift standard. The integration of the split spectral signals was performed with a standard-software program. The assignment of local sequences such as dyad and triad for SCL-PHA was made according to the literature [1, 10, 29].

Table 1. Characterization of the isolated blend-type-PHA forming bacterium

Test	Result	Test	Result
Cell shape	Rod	Utilization of	
Gram staining	–	Glucose	+
Growth on nutrient agar	+	L-Arabinose	–
Growth on MacConkey agar	+	D-Mannose	–
		Tryptophane	–
Biochemical test		Maltose	–
Cytochrome oxidase	+	Phenylacetate	–
Arginine dehydrolase	+	Mannitol	+
Urease	+	Caprate	+
Nitrate reduction	+	Adipate	+
Indole test	–	Malate	+
		Citrate	+
Hydrolysis of		Gluconate	+
Esculin	–	N-Acetylglucosamine	+
Gelatine	+		
p-Nitrophenyl-β-D-galactopyranoside	–		

RESULTS AND DISCUSSION

Characterization and Identification of the Isolated Strain

Table 1 shows the morphological, biochemical, and nutritional characteristics for the isolated strain. Activities of cytochrome oxidase and arginine dehydrolase were present. Denitrification was observed. Esculin was not hydrolyzed, but gelatin was hydrolyzed. Glucose was utilized for growth but L-arabinose and D-mannose were not utilized. Phenylacetate was not utilized. Thus, from the test result obtained by using the API identification program (ID 32 GN strip test) in accordance with standard methods in Bergey's manual [13], the isolate was identified as a strain of *Pseudomonas aeruginosa* (% id=99.5) and named *P. aeruginosa* BM114. An almost complete 16S rDNA sequence (1,247 nucleotides) was determined and compared with the known 16S rDNA sequence of *P. aeruginosa* K3 (NCBI Accession No. EF064786). The strain was thus identified as *P. aeruginosa* by 99% homology of the 16S rRNA gene sequence.

Accumulation of a blend of SCL-PHA and MCL-PHA in *P. aeruginosa* BM114

The general characteristics of carbon-source (*e.g.*, saccharides) dependent PHA synthesis of the isolated *P. aeruginosa* BM114 was very similar to that of other pseudomonads (Table 2). The PHA accumulated from fructose was composed of ~20 mol% 3-hydroxyoctanoate (3HO) and ~70 mol% 3-hydroxydecanoate (3HD). 3-Hydroxycaproate (3HC), 3-hydroxydodecanoate (C12), 3-hydroxy-5-dodecenoate (C12:1), 3-hydroxytetradecanoate (C14), and 3-hydroxy-7-tetradecenoate (C14:1) constituted only minor components (totally ~ 10 mol%) of the PHA. However, when grown on the medium-chain fatty acids such as octanoic or decanoic acid, in addition to the typical MCL-monomer-units such as 3HC, 3HO, and 3HD, the isolated PHA contained more than ~50 mol% of 3HB-unit (Table 2). Thus, 3HD is the main monomer-unit in fructose-grown cells, but 3HB is the main monomer-unit in decanoate-grown cells. To investigate whether SCL- and MCL-units form a real copolymer or a blend of SCL-PHA and MCL-PHA, fractionation experiments were carried out using acetone because MCL-PHA is highly soluble in acetone. The ^{13}C NMR spectrum of the isolated "whole" PHA clearly revealed the mixed signals for the carbons associated with both P(3HB) and MCL-PHA: typically, two methine -CH-O- carbon signals at 70.0 (associated exclusively with 3HB) and 71.2 (associated exclusively with MCL-monomer-units) ppm and two carbonyl absorptions at 169.5 (attributed to 3HB) and 169.8 (attributed to MCL-3-hydroxyl monomer-units) ppm (Fig. 1). The expanded spectrum did show no further split peaks around 169.5 ppm peak (the upper inserted figure in Fig. 1A), indicative of a P(3HB) homopolymer. In addition, the ^{13}C NMR signals for the acetone-soluble fraction appeared to be exclusively due to the typical "pure" MCL-PHA containing no SCL-comonomer-unit. According to GC analysis, the acetone-insoluble fraction revealed the methyl ester of 3HB only, whereas acetone-soluble fraction displayed the methyl esters of 3HO and 3HD as the major products and the methyl ester of 3HC as the minor product (Table 2 and the upper inserted figure in Fig. 1A). This indicates that the BM114 strain may have both types of PHA synthase; type I (for P(3HB) synthesis) and type II (for MCL-PHA synthesis) [1, 19, 20, 26]. The additional evidence for the suggestion of the probable existence of two types of PHA synthase in the BM114 strain is the fact that in the time-dependent growth profile, the three major monomer-units (3HB, 3HO, and 3HD) were steadily incorporated into PHA throughout the growth period without any preference to one of them, but the resulting polymer was a blend (the growth curve data not shown). The simultaneous incorporation of both SCL- and MCL-monomers clearly indicates the coexpression of the two different synthases in BM114 cells grown with medium-chain fatty acids.

Table 2. Carbon-source dependent PHA synthesis characteristics of *P. aeruginosa* BM114 grown at 30°C and fractionation characterization of the blend-type PHA produced from medium-chain fatty acids.

Carbon source (mM)	Inhibitor (mM)	Culture time (h)	Cell dry weight (g/l)	PHA content (wt%)	PHA composition (mol%)									
					3HB[a]	3HV	3HC	3HH	3HO	3HN	3HD	3HU	C12	C12:1
Fructose (70)	None	144	2.04	30.2	–				19.8		69.8		5.5	4.0
Fructose (70)	SA[b] (2.0)	144	1.93	23.2					20.1		68.7		6.1	4.0
Fructose (70)	SA[b] (10.0)	144	1.42	12.7					19.4		66.1		8.8	4.0
Fructose (70)	2-BrOA[c] (2.0)	144	1.14	6.2	–				37.3		42.7		12.2	7.8
Fructose (70)	37[d]	48	1.75	4.8					50.0		50.0			
Octanoate (60)	None	72	3.23	42.9	70.3		3.5		24.7		1.5			
Nonanoate (40)	None	72	1.89	37.6	24.5	39.2		14.3		22.0				
Decanoate (30)	None	60	2.69	45.0	47.3		4.3		29.4		19.0			
	100 mg[e] polymer	Acetone-soluble		60 mg[f]	-		6.5		53.3		40.2			
		Acetone-insoluble		40 mg	100									
Undecanoate (30)	None	72	2.06	33.7	20.9	26.5		11.7		23.5		17.4		
	200 mg[g] (acetone soluble)	$CHCl_3$/MeOH (1:1.5, v/v) soluble		95 mg	35.8	47.5		4.2		7.8		4.7		
		$CHCl_3$/MeOH (1:1.5, v/v) insoluble		105 mg	0	3.4		21.4		44.2		31.0		

[a]3HB, 3-hydroxybutanoate; 3HV, 3-hydroxyvalerate; 3HC, 3-hydroxycaproate; 3HH, 3-hydroxyheptanoate; 3HO, 3-hydroxyoctanoate; 3HN, 3-hydroxynonanoate; 3HD, 3-hydroxydecanoate; 3HU, 3-hydroxyundecanoate; C12, 3-hydroxydodecanoate; C12:1, 3-hydroxy-5-dodecenoate. The minor components C14 and C14:1 were omitted for simplicity.
[b]SA, salicylic acid.
[c]2-BrOA, 2-bromooctanoic acid.
[d]Culture temperature.
[e]The weight of PHA used for fractionation experiment, isolated from 30 mM decanoic acid-grown cells.
[f]The weight of PHA recovered from organic solvent fractionation.
[g]The weight of PHA recovered from the acetone-soluble fraction in the first fractionation to remove small amount of acetone-insoluble fraction (SCL-PHA) by centrifuging. The PHA dissolved in 10 ml of chloroform was precipitated in 15 ml of methanol with vigorous stirring and the insoluble particulates were removed by centrifugation.
[h]All values are averages of triplicate experiments. The data for dry cell weight and PHA content were reproducible within a maximum of ±10%, and those for monomer composition were reproducible within ±5%.

The PHA synthesized from the medium-chain fatty acids with odd-number of carbons such as nonanoate or undecanoate revealed a slightly different synthesis pattern from the even-fatty-acid-grown cells. Growth on the odd carboxylic acids unexpectedly resulted in the incorporation of ~20 mol% 3HB-unit into PHA as well as odd MCL-monomer-units (Table 2). Fractionation experiments with acetone showed that the PHA produced from nonanoic acid was also a blend of SCL-PHA and MCL-PHA. However, the acetone-insoluble SCL-PHA was composed of ~70 mol% 3HB- and ~30 mol% 3HV-units, whereas the acetone-soluble fraction contained the monomer-units from SCL-PHA as well as MCL-PHA. A careful analysis of the expanded ^{13}C NMR spectrum for the acetone-soluble fraction revealed that the sample was a mixture of P(3HB-*co*-3HV) and MCL-PHA composed of 3-hydroxyheptanoate (3HH), 3-hydroxynonanoate (3HN), and 3-hydroxyundecanoate (3HU), free from 3HV-unit (Fig. 1B). This was confirmed in further careful fractionation of the acetone-soluble fraction with a mixed solvent of chloroform and methanol (1:1.5, v/v), showing 3HV-unit not copolymerized with the MCL-units (Table 2). The mixed C-13 signals due to both SCL- and MCL-units in Fig. 1B(a) were completely resolved into each separate component signal [Figs. 1B(b) and 1B(c)] after fractionation. The expanded carbonyl absorption region for the fractionated SCL-PHA sample clearly revealed the absorption peak attributed to the 3HB-3HV hetero-dyad sequence, in addition to the two homo-dyad peaks attributed to 3HB-3HB and 3HV-3HV. The presence of the hetero-dyad (169.27 ppm), triad (38.81 and 38.65 ppm), and tetrad (not shown) sequences indicates that the SCL-PHA was a chemically linked copolymer of 3HB- and 3HV-units. However, any MCL-unit was not covalently linked to SCL-units, which is evident from the absence of a well-resolved peak at 39.27 ppm reported for the 3HB-(MCL-monomers) copolymer system, slightly downfield of the backbone methylene peak at 39.11 ppm associated with PHA composed of MCL-units only [Ref. 1 and Fig. 1B(c)].

In pseudomonads, the use of medium-chain carboxylic acids with odd-number of carbons as carbon source usually leads to the production of PHA composed of monomer-units with odd-number of carbons, usually the monomer

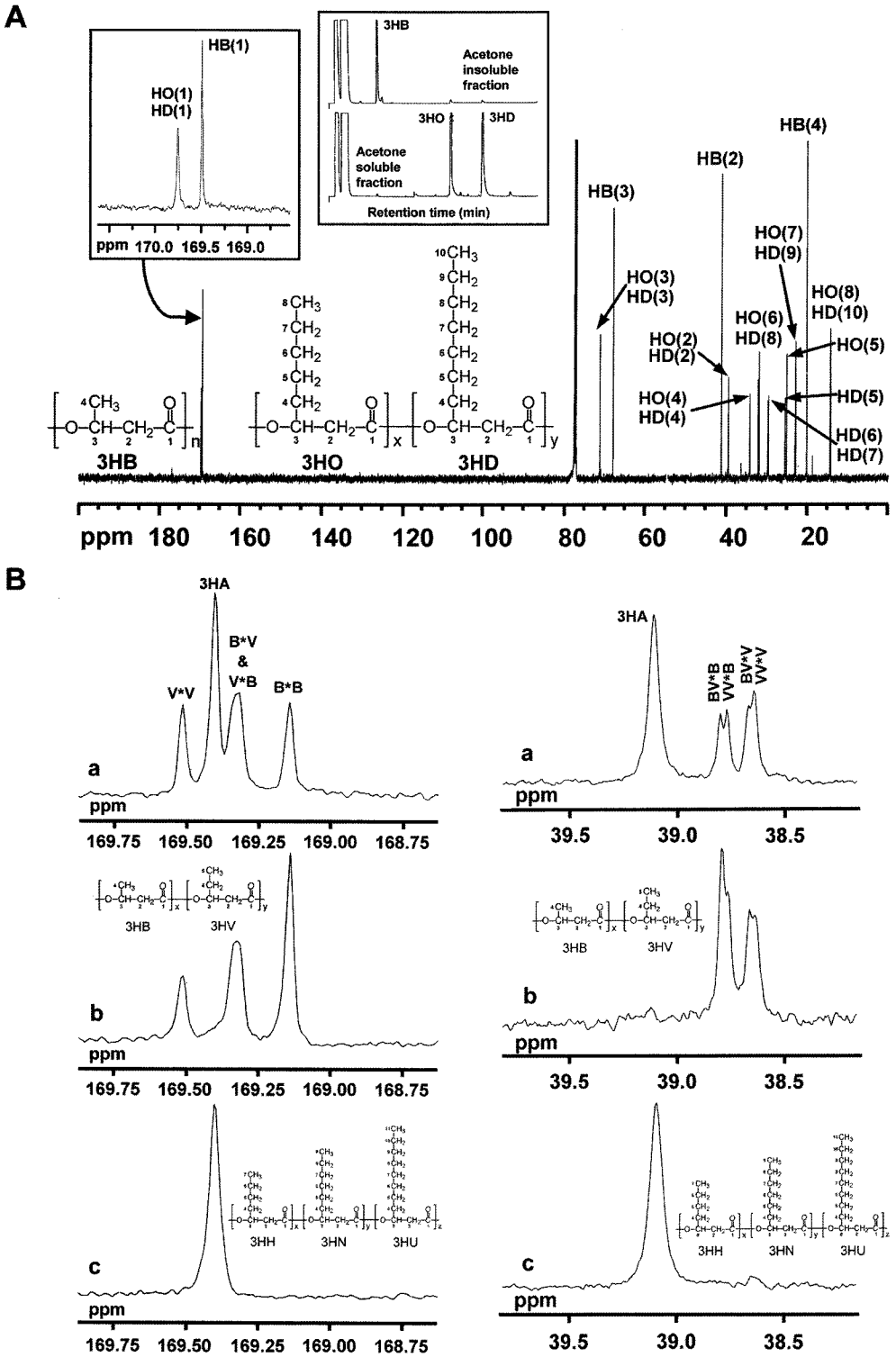

Fig. 1. A. ^{13}C NMR spectrum of the PHA synthesized in *P. aeruginosa* BM114 grown on decanoic acid. The spectrum is for the isolated non-fractionated sample. The insert in the spectrum represents the GC chromatograms for the acetone-insoluble fraction (the upper) and acetone-soluble fraction (the lower), respectively. **B.** Expanded 13C-NMR spectra of the PHA synthesized in *P. aeruginosa* BM114 grown on undecanoic acid. The upper one (a) is for the acetone-soluble fraction mixed with MCL- and SCL-PHAs, the middle one (b) for the acetone-insoluble fraction containing SCL-PHA only, and the lower one (c) for the precipitated fraction in chloroform/methanol (1:1.5, v/v) mixed solvent by further fractionating the acetone-soluble PHA in (a) in the mixed solvent.

The spectra in the region 169–170 ppm represent expanded carbonyl absorptions due to dyad sequences and the region 38.5–39.5 ppm absorptions due to backbone methylene carbon absorptions. The two groups of absorptions 38.81 and 38.65 ppm are due to the C2 carbon in the 3HV-unit in triad sequences.

Table 3. Selective inhibition of MCL-PHA accumulation and stimulation of SCL-PHA accumulation by salicylic acid in *P. aeruginosa* BM114 grown on medium-chain fatty acids for 72 h at 30°C.

Carbon source (mM)	Salicylic acid (mM)	Cell dry weight (g/l)	PHA content (wt%)	SCL-PHA content (wt%)	MCL-PHA content (wt%)	PHA monomer composition (mol%)						
						3HB[a]	3HV	3HC	3HH	3HO	3HN	3HD
Octanoate (60)	0	3.28	48.1	37.8	10.3	71.5		2.6		24.0		1.9
	1.0	3.21	47.5	38.3	9.2	73.3		2.4		22.1		2.2
	2.0	3.34	44.3	39.0	5.3	82.9		1.4		13.7		2.0
	4.0	3.39	40.3	36.2	4.1	85.2		1.3		11.5		2.0
	6.0	3.22	42.9	40.0	2.9	90.0		0.8		7.7		1.5
Nonanoate (40)	0	2.09	33.4	15.6	17.8	15.6	20.6		23.7		40.1	
	5	1.96	35.8	19.6	16.2	18.3	25.5		20.5		35.7	
	10	1.88	36.1	25.4	10.7	22.5	38.0		14.0		25.5	
	20	1.91	39.0	32.9	6.1	28.7	48.9		7.6		14.8	
Decanoate (25)	0	1.97	48.6	28.0	20.6	46.1		3.5		25.0		25.4
	5	1.91	44.9	31.9	13.0	60.9		2.5		20.1		16.5
	10	1.82	50.9	44.3	6.6	80.9		1.1		9.9		8.1
	20	1.85	43.2	40.7	2.5	91.1		0.5		4.1		4.3

[a]3HB, 3-hydroxybutanoate; 3HV, 3-hydroxyvalerate; 3HC, 3-hydroxycaproate; 3HH, 3-hydroxyheptanoate; 3HO, 3-hydroxyoctanoate; 3HN, 3-hydroxynonanoate; 3HD, 3-hydroxydecanoate; 3HU, 3-hydroxyundecanoate.
All values are averages of at least triplicate experiments. The data for dry cell weight and PHA content were reproducible within a maximum of ±10%, and those for monomer composition were reproducible within ±5%.

precursors derived *via* the β-oxidation pathway. Thus, the production of 3HB-unit from odd medium-chain fatty acids may suggest the presence of an additional pathway to produce 3-hydroxybutyryl-CoA in the BM114 strain, similar to that in the typical SCL-PHA-producing *W. eutropha* [2, 17]. In addition, the detection of 3HV-unit only from the SCL-PHA fraction may indicate that the BM114 MCL-PHA synthase is specific against 3HC or higher units, whereas the BM114 SCL-PHA synthase is specific only against 3HB and 3HV.

Selective Suppression of MCL-PHA Synthesis by Salicylic Acid in *P. aeruginosa* BM114 Producing a Blend of SCL-PHA and MCL-PHA

One-step cultivation data showed that salicylic acid did not delay the growth on the three medium-chain fatty acids, even when added up to 20 mM or higher concentration (Table 3). The carbon sources at the indicated concentrations were consumed within 72 h under the cultivation conditions in Table 3. In the inhibition experiments with decanoate-grown cells, too high a concentration of the substrate in medium (*e.g.*, 40 mM) was a problem of causing an insensitive inhibiting effect, resulted probably from the sorption of the hydrophobic inhibitor molecules to the insoluble decanoic acid slurry particles. Therefore, the fed concentration of decanoate in the medium was decreased to 25 mM.

In the case of octanoic acid-grown cells, overall PHA accumulation was not substantially suppressed, but the incorporation of MCL-monomers was effectively blocked at 4 to 6 mM salicylic acid. The accumulation of SCL-PHA was not inhibited at all, rather stimulated, over the level of salicylic acid investigated, but that of MCL-PHA was inhibited by 72% of the control value at 6 mM salicylic acid (Table 3 and Fig. 2). Octanoate-grown cells incorporated more 3HB monomer-units than did decanoate-grown cells at zero salicylic acid concentration. When the cells were grown on nonanoate or decanoate, a higher amount of

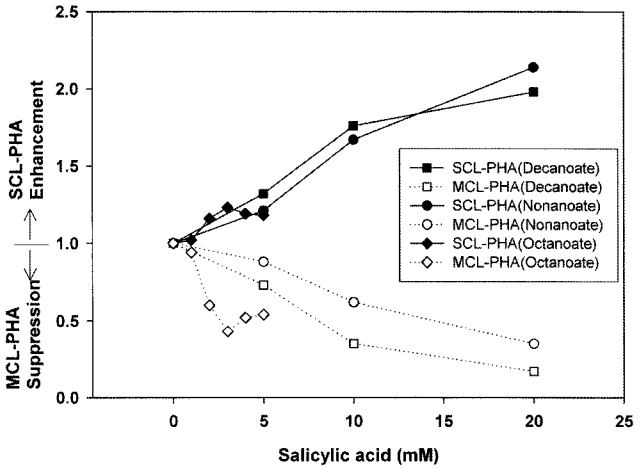

Fig. 2. Suppression of MCL-PHA accumulation and stimulation of SCL-PHA accumulation in *P. aeruginosa* BM114 grown on medium-chain fatty acids by salicylic acid.
The PHA wt% data for salicylic acid-free control cells in Table 3 were used as the reference values in the calculation of relative power (defined as the ratio, $[\text{PHA wt\%}]_{\text{salicylic acid}}/[\text{PHA wt\%}]_{\text{control}}$), of suppression and stimulation.

salicylic acid was required to suppress the MCL-monomer incorporation to the extent similar to that in octanoate-grown cells. Thus, the inhibiting potency of salicylic acid depended on the carbon source used. For the BM114 strain, salicylic acid induced no significant preferential reduction of any specific monomer among the three MCL-monomer-units, contrary to *P. fluorescens* BM07 strain (M. H. Choi, J. K. Rho, J. H. Shim, J. Xu, and S. C. Yoon, submitted for publication).

To check the inhibitor sensitiveness depending on the cultivation methods, we carried out a two-step cultivation. In the two-step cultivation, first-step growth of BM114 on nutrient-rich medium did not lead to accumulation of any PHA. The second-step cultivation of the nutrient-rich-medium grown cells on 20 mM decanoate without additional ammonium sulfate led to a much higher accumulation of P(3HB) (*e.g.*, 78 mol% at 0 mM salicylic acid) than in one-step cultivation (the two-step data not shown). Three to 4 mM of salicylic acid almost completely suppressed the incorporation of MCL-monomer-units (totally ~3 mol% incorporated) to produce P(3HB), ~ 97% of the PHA, but did not affect cell growth at all. Thus, the sensitiveness of salicylic acid to PHA synthesis in the cell depends on the cultivation method.

In nonanoate-grown cells incorporating two different types of SCL-units, 3HB- and 3HV-units, similar to even-carboxylic-acid grown cells, salicylic acid inhibited the incorporation of only MCL-units, 3-hydroxyheptanoate and 3-hydroxynonanoate, not SCL-units. The level of 3HV-unit in PHA was significantly enhanced up to 49 mol% at 20 mM salicylic acid. Different batches of nonanoate cultures exhibited significantly different ratios of [SCL-monomer] to [MCL-monomer] in the resulting PHAs (Table 3). The ratios [3HV-unit]/[3HB-unit] for SCL-PHA and [3HN-unit]/[3HH-unit] for MCL-PHA were 1.3 to 1.9 and 1.7 to 2.0, respectively, which increased with salicylic acid concentration. However, the latter ratio values were not significantly different from batch to batch. The fluctuation in the ratio [SCL-monomers]/[MCL-monomers] was found to be partly related with aeration rate (determined from the shaking rate of the shakers): the slower shaking induced an increase in the level of SCL-monomer-units, principally 3HV-unit. The incorporation of 3HV-unit was further stimulated in the presence of salicylic acid. Thus, salicylic acid is an effective blocker against MCL-monomer-units such as 3HC or higher 3-hydroxyacids in the BM114 strain. It is now possible to suppress the formation of MCL-PHA in the bacterium producing a blend of SCL-PHA and MCL-PHA using salicylic acid inhibitor. This salicylic acid effect also shows that the two different types of monomer-units, 3-hydroxy-SCL-CoA and 3-hydroxy-MCL-CoA, are derived *via* their own separate supplying pathways.

Acrylic acid, known as a β-oxidation inhibitor, was tested to find which synthetic pathway is inhibited by the inhibitor. When BM114 cells were grown on medium-chain carboxylic acids, acrylic acid effectively inhibited only the accumulation of SCL-PHA, not MCL-PHA, as expected from its β-oxidation inhibitory effect reported [2, 6] (Fig. 3). The pattern of linkage between the comonomers constituting each copolymer in the blend was not perturbed by the addition of salicylic acid or acrylic acid, determined from their NMR microstructural analysis (data not shown).

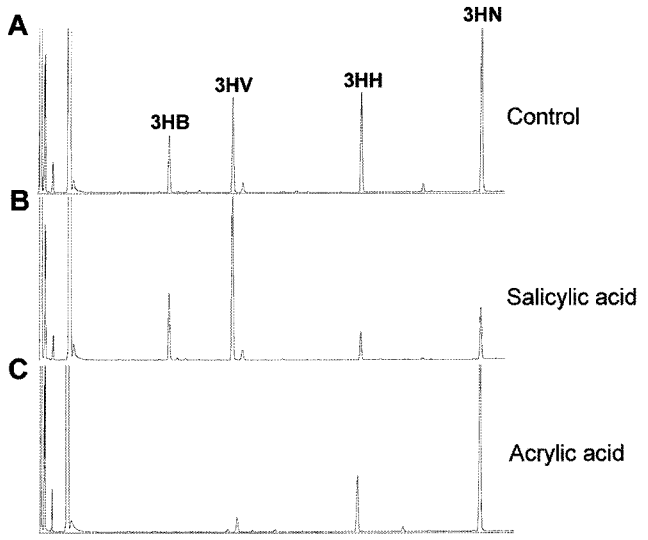

Fig. 3. Gas chromatographic analysis of SCL-PHA and MCL-PHA accumulated in *P. aeruginosa* BM114 grown with 40 mM decanoate (**A**, inhibitor-free control).
The PHA accumulation was inhibited by 5 mM salicylic acid (**B**) or 5 mM acrylic acid (**C**).

Thus, salicylic acid is a stimulator for SCL-PHA accumulation but an inhibitor for MCL-PHA accumulation in medium-chain-fatty-acid-grown BM114 cells (Fig. 2). However, overall PHA production yield was relatively constant irrespective of salicylic acid level (Table 3). Therefore, salicylic acid seemed to play a role in the shift of carbon metabolites leading to MCL-PHA to SCL-PHA. The decrease in MCL-PHA accumulation by salicylic acid was compensated by the increase in SCL-PHA accumulation. However, the situation may be interpreted in an opposite manner. The stimulation of SCL-PHA accumulation caused by salicylic acid resulted in the decrease in MCL-PHA accumulation. At any rate, salicylic acid clearly caused a "swinging phenomenon" in the BM114 strain having the two different pathways to synthesize both SCL- and MCL-PHAs. *Pseudomonas* sp. 61-3 is also known to produce a blend of P(3HB) and P(3HB-*co*-MCL-monomer-units) from sugars [11, 18–20]. Disruption of the chromosomal $phaG_{Ps}$ gene resulted in an increase in the fraction of the SCL-monomer-unit, 3HB-unit in PHA in *Pseudomonas* sp. 61-3 [18]. Salicylic acid thus displays a similar stimulating effect developed by *phaG* gene disruption.

This is the first report that salicylic acid inhibits the synthesis of MCL-PHA in bacteria as well as modulates the monomer distribution of the PHA. Specifically, in the bacteria accumulating a blend type of PHA, the selective specific inhibition of a type of PHA by the specific inhibitor may be an important tool from the biotechnological as well as physiological points of view.

Salicylic acid incurred different physiological reactions among *Pseudomonas* spp. For example, in *P. putida* BM01, salicylic acid was metabolized in the cell and no inhibition of PHA synthesis was observed (data not shown). In octanoate-grown *P. fluorescens* BM07, the addition of 1.5 mM or higher concentration of salicylic acid to medium halted cell growth. Thus, the salicylic acid effect is species specific [14]. Further genetic and molecular biology study is required to understand the strain-specific inhibition by salicylic acid.

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