J. Microbiol. Biotechnol. (2009), **19**(10), 1142–1149
doi: 10.4014/jmb.0801.465
First published online 2 June 2009

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Effects of Nutrients on Quorum Signals and Secondary Metabolite Productions of *Burkholderia* sp. O33

Keum, Young Soo[1]*, Young Ju Lee[1], Youn Hyung Lee[2], and Jeong Han Kim[1]

[1]*Department of Agricultural Biotechnology, Seoul National University, Seoul 151-742, Korea*
[2]*Department of Horticultural Biotechnology, KyungHee University, Suwon 446-701, Korea*

Received: January 8, 2008 / Revised: May 4, 2009 / Accepted: May 8, 2009

Several bioactive metabolites, including pyrrolnitrin, *N*-acylhomoserine lactones, and polyhydroxyalkanoates were isolated from *Burkholderia* sp. O33. Effects of various nutrients, including sugars, gluconolactone, glycerol, tryptophan, chloride, and zinc were investigated in relation to the production of these metabolites. Logarithmic increase of pyrrolnitrin was observed between 2–5 days and reached a maximum at 7–10 days. Tryptophan concentration reached the maximum at 3 days, whereas 7-chlorotryptophan was gradually increased throughout the studies. Among various carbon sources, gluconolactone, trehalose, and glycerol enhanced pyrrolnitrin production, whereas strong inhibitory effects were found with glucose. Relative concentrations of pyrrolnitrin and its precursors were in the order of pyrrolnitrin >> dechloroaminopyrrolnitrin or aminopyrrolnitrin throughout the experiments. Among three *N*-acylhomoserine lactones, the *N*-octanoyl analog was the most abundant quorum sensing signal, of which the concentrations reached the maximum in 2–3 days, followed by a rapid dissipation to trace level. No significant changes in pyrrolnitrin biosynthesis were observed by external addition of *N*-acylhomoserine lactones. Polyhydroxyalkanoates accumulated up to 3–4 days and decreased slowly thereafter. According to the kinetic analyses, no strong correlations were found between the levels of pyrrolnitrin, *N*-acylhomoserine lactones, and polyhydroxyalkanoates.

Keywords: *Burkholderia* O33, pyrrolnitrin, quorum sensing, *N*-acylhomoserine lactone, polyhydroxyalkanoates

Halogenated antibiotics, pyrrolnitrin and pyoluteorin, are produced from several bacterial species, including *Pseudomonas*, *Burkholderia*, *Serratia*, and *Myxococcus* [12, 15, 24, 28, 29].

**Corresponding author*
Phone: +82-2-880-4654; Fax: +82-2-873-4415;
E-mail: rational@snu.ac.kr

With the increased environmental concerns, the application of biopesticides is considered as promising alternatives to synthetic pesticides. In addition, natural products can serve as synthetic lead compounds of novel fungicides (*e.g.*, fludioxonil and fenpiclonil from pyrrolnitrin). Pyrrolnitrin and pyoluteorin are produced from tryptophan and proline, respectively [14, 17, 29, 35]. Chlorination of corresponding amino acids by novel $FADH_2$-dependent halogenases is the most important biosynthetic step for both antibiotics [8, 20, 22, 25]. Because of their antibiotic activities, various aspects of gene regulations and environmental factors were evaluated to improve the biosynthetic yield or adaptation in a natural environment [*e.g.*, 10, 12, 13, 30, 31]. Biosynthesis of pyoluteorin is under the control of various regulatory genes and antibiotics itself [2, 7, 13]. Although a detailed mechanistic explanation is not available, it was proven that bacterial production of pyrrolnitrin is also dependent on the nutrient status in the culture medium [15, 21].

Among several interesting regulatory aspects, it has to be mentioned that the *N*-acylhomoserine lactone (AHSL)-mediated quorum sensing system regulates numerous primary and secondary metabolisms in several Gram-negative bacteria (*e.g.*, *Pseudomonas*, *Burkholderia*, and *Agrobacterium* spp.) [9, 11, 43].

In addition to antibiotics, *Pseudomonas* and *Burkholderia* produce several additional bioactive metabolites of potential industrial applications (biosurfactants and bioplastics) [16, 26, 34, 39].

From the screening experiments of *Burkholderia* sp. O33, it was found that this strain can produces AHSLs and polyhydroxyalkanoates (PHAs) as well as pyrrolnitrin. In this study, we investigated kinetic aspects of these metabolites from this strain, grown with different nutrients. To identify the possible correlation between quorum sensing signals and other metabolites, the concentrations of AHSLs were also monitored and compared with those of pyrrolnitrin and PHAs at specified intervals.

MATERIALS AND METHODS

Reagents

The following reagents were obtained from Aldrich (Sigma-Aldrich Korea, Korea): pyrrolnitrin, poly(3-hydroxybutyrate-co-3-hydroxyvalerate), 3-hydroxybutyric acid (3HB), sodium chloride, *N*-hexanoylhomoserine lactone (C6-AHSL), *N*-octanoylhomoserine lactone (C8-AHSL), and *N*-decanoylhomoserine lactone (C10-AHSL), sugars (glucose, fructose, sucrose, and trehalose), glycerol, sodium gluconolactone, ammonium chloride, zinc sulfate, *bis* (trifluoromethylacetamide)-trimethylsilyl chloride (BSTFA-TMCS), methoxylamine hydrochloride, and dry pyridine. Solvents, including ethyl acetate, methanol, hexane, dimethyl sulfoxide (DMSO), and methyl *t*-butyl ether (MTBE) were HPLC grade or higher.

Bacterial Culture

Burkholderia sp. O33 was obtained from Korean Agricultural Culture Collection (KACC, No. 12815) and maintained on nutrient agar. Typically, strain O33 was cultured in phosphate-buffered nutrient broth (NBM) medium, supplemented with different nutrients. The NBM comprised Na_2HPO_4 (1.20 g), KH_2PO_4 (0.25 g), and nutrient broth (1.5 g) in deionized water (500 ml), pH 6.8.

To a heat-sterilized culture medium, the following amounts of different nutrients were supplemented aseptically: for sugars, glycerol, or gluconolactone, 1 g/l; for other nutrients, D-tryptophan, L-tryptophan, sodium chloride, or ammonium chloride, 500 mg/l; for zinc sulfate, 100 mg/l. After the addition, the culture was maintained at 30°C, 160 rpm. Three replicates were prepared for each nutrient treatment.

To test the effect of glucose supplementation during active accumulation of pyrrolnitrin, additional culture was prepared as follows: control culture was prepared as described above, and then glucose (300 mg) was added after 4 days. The batch was further cultured at the same condition.

To investigate the external addition of AHSLs, C6, C8, or C10-AHSL (2 μM) was added to the control medium (NBM, alone) and cultured at the same conditions.

For the preparation of large amount of pyrrolnitrin, batch culture (2 l) was prepared in NBM, supplemented with glycerol (10 g/l), and cultured at the same condition for 10 days. Authentic monochloroaminopyrrolnitrin and aminopyrrolnitrin were purified from 4-day cultures (**3** and **4**, Fig. 1).

Extraction of Pyrrolnitrin, Precursors, and *N*-Acylhomoserine Lactones

For quantitative analysis, an aliquot amount of culture (60 ml) was collected after a predefined period and centrifuged (3,500 rpm, 50 min). The supernatant was extracted twice with 80 ml of ethyl acetate and the organic layer was dried over anhydrous sodium sulfate. After removing the organic solvent, the residue was redissolved in ethyl acetate (1.2 ml) and analyzed with a gas chromatograph-mass spectrometer (GC-MS).

For the preparation of large quantities of metabolites, the supernatant from batch cultures (2 l) was saturated with sodium chloride, and extracted with 500 ml of ethyl acetate three times. The combined organic layer was dried over anhydrous sodium sulfate. After removal of solvent, the residue was redissolved in ethyl acetate (2 ml) and purified with preparative silica gel TLC [18].

Extraction and Derivatization of Supplemented Nutrients in Culture Medium

The concentration of supplemented nutrients (sugars, glycerol, gluconolactone, and tryptophan) in the culture medium was analyzed by the following methods. An aliquot of culture medium (10 ml) was centrifuged (13,000 rpm, 50 min). After concentration of the supernatant to dryness, the residue was redissolved in methanol (5 ml) and the insoluble precipitates were removed. The methanol extracts were dried under nitrogen atmosphere. After dissolving the residue in dry pyridine (1 ml), the sample was derivatized with methoxylamine hydrochloride (100 μl, 1 g/10 ml pyridine) at 80°C for 1 h, followed by BSTFA-TMCS (200 μl) at 90°C for 2 h.

Extraction and Derivatization of Polyhydroxyalkanoates

Cell pellets were collected by centrifugation (3,500 rpm, 50 min) and washed with distilled water. To identify the monomeric composition, polyhydroxyalkanoates (PHAs) were purified according to the literature method [1]. For the quantitative analyses of fatty acid and PHAs, cell pellet (200 mg) was suspended in 2 N methanolic NaOH (5 ml) and heated for 1.5 h at 80°C. After cooling to room temperature, the pH was adjusted to 2.0 with concentrated hydrochloric acid. Released fatty acids and PHA monomers were extracted twice with a 50-ml mixture of hexane and MTBE (1:2, v/v). After removing organic solvents, the residue was dissolved in dry pyridine (1 ml) and derivatized with BSTFA-TMCS (150 μl, 80°C, 2 h).

Instrumental Analyses

Pyrrolnitrin and its precursors were analyzed with GC-MS (Shimadzu GCMS QP-2000 and GC-2010), equipped with a DB-5MS column (60 m, 0.25 μm film thickness, 0.25 mm i.d.; Agilent Technologies, U.S.A.). Helium was the carrier gas at a flow rate of 1 ml/min. The column temperatures were programmed as follows: 95°C (10 min) and raised to 295°C at a rate of 2°C/min and held for 20 min. The mass spectra of metabolites were obtained in full-scan mode whereas the quantitation of AHSL was performed at selective ion monitoring (SIM) mode. Ions for SIM analysis were *m/z* 143 (quantitation ion),

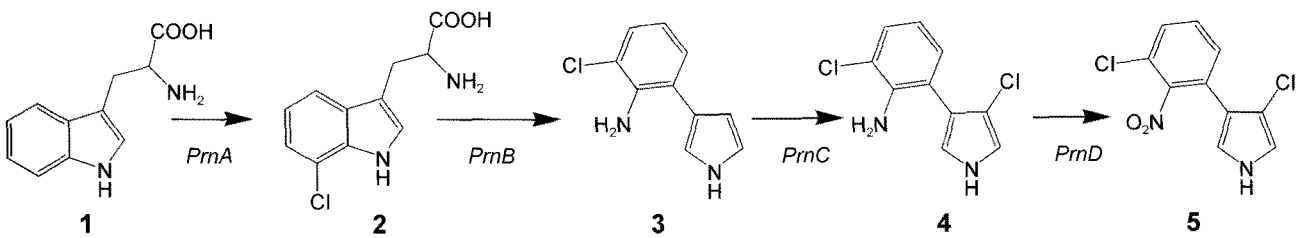

Fig. 1. Biosynthetic pathway of pyrrolnitrin and corresponding enzymes. **1**, tryptophan; **2**, 7-chlorotryptophan; **3**, monochloroaminopyrrolnitrin; **4**, aminopyrrolnitrin; **5**, pyrrolnitrin.

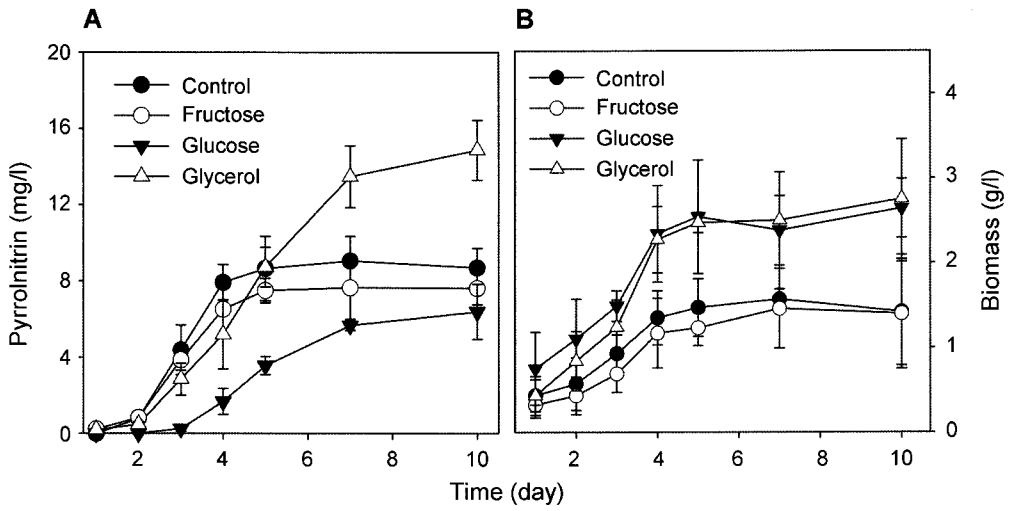

Fig. 2. Change of biomass and production of pyrrolnitrin in nutrient broth, supplemented with selected carbon sources. Data from three replicate experiments are presented with error bar for standard deviation.

125, and 156 (reference ion). Halogenated tryptophans were determined as corresponding indoles in full-scan mode. The retention times (Rts) of indole, 7-chloroindole, and 7-bromoindole were 27.8, 31.2, and 36.8 min, respectively.

Results

Pyrrolnitrin and Precursors in Different Nutrients

Pyrrolnitrin production was initiated after 1–2 days of lag phase, followed by logarithmic increase up to 4 days. In general, the concentration of pyrrolnitrin was almost constant after 4–5 days (Fig. 2A). However, a continuous increase over 7 days was observed in glycerol-supplemented culture, while the amount of biomass reached the maximum after 4–5 days in all test nutrients (Fig. 2B).

Concentrations of pyrrolnitrin in the culture medium ranged from 1.7 to 13 mg/l after 4 days (Table 1). Among the selected nutrients, gluconolactone, glycerol, trehalose, sodium chloride, and ammonium chloride enhanced pyrrolnitrin productions, whereas highly reduced amount of pyrrolnitrin was observed in glucose-, sucrose-, and zinc-supplemented cultures (Table 1). Supplementation of both D- and L-tryptophan did not enhance the accumulation of pyrrolnitrin and its precursors (Table 1). In general, pyrrolnitrin concentrations were far higher than its precursors, regardless of nutrients. However, a higher proportion of monochloroaminopyrrolnitrin was obtained from D- and L-tryptophan, fructose, and trehalose supplementation, whereas the concentration of similar intermediates was much lower in the control and other nutrient-supplemented NBM (Table 1).

In comparison with pyrrolnitrin production, bacterial growth in specific nutrient supplementation gave different responses. For example, biomass productions in glucose- and glycerol-NBM were approximately 2 fold higher than

Table 1. Concentration of pyrrolnitrin and its precursors, and the weight of biomass after 4 days.

Nutrients	Concentrations of metabolites[a] (mg/l medium)					Biomass (g/l)
	MAmPrn	AmPrn	Prn	Trp	Trp-Cl	
Control	0.25±0.02	0.17±0.04	7.90±0.81	0.07±0.01	0.01±0.00	1.35±0.32
Fructose	1.25±0.31	0.15±0.03	6.52±1.53	0.14±0.07	0.52±0.03	1.17±0.41
Glucose	0.02±0.01	ND[b]	1.74±0.71	0.18±0.05	0.01±0.00	2.34±0.57
Gluconolactone	ND	0.28±0.10	9.51±1.21	0.08±0.01	0.11±0.01	1.12±0.20
Sucrose	ND	ND	3.52±1.02	0.01±0.00	0.01±0.00	1.34±0.42
Trehalose	1.12±0.43	0.61±0.08	8.81±0.82	0.09±0.01	0.08±0.01	1.35±0.21
Glycerol	0.01±0.00	0.51±0.07	5.51±0.91	0.18±0.03	0.07±0.01	2.27±0.39
Tryptophan, D	1.78±1.01	0.08±0.01	7.15±0.92	0.52±0.04	0.12±0.04	1.09±0.32
Tryptophan, L	2.34±1.00	0.21±0.12	7.42±1.21	0.57±0.01	0.16±0.05	1.23±0.35
$ZnSO_4$	0.37±0.11	0.28±0.15	6.25±1.17	0.12±0.02	0.13±0.04	0.89±0.25
NaCl	0.43±0.11	0.51±0.14	13.15±0.56	0.03±0.00	0.04±0.01	1.12±0.34
NH_4Cl	ND	0.20±0.05	8.81±0.65	0.11±0.02	0.12±0.07	1.54±0.27

[a]Abbreviations of metabolites: MAmPrn, Monochloroaminopyrrolnitrin; AmPrn, aminopyrrolnitrin; Prn, pyrrolnitrin; Trp, tryptophan; Trp-Cl, 7-chlorotryptophan.
[b]ND, not detected.

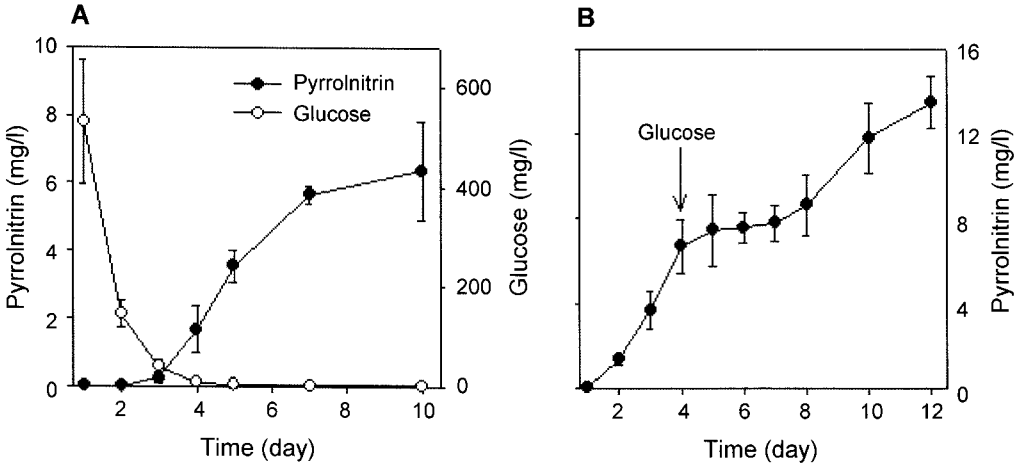

Fig. 3. Time-dependent change of concentrations of pyrrolnitrin and glucose in the culture medium, supplemented with glucose (**A**) and effects of glucose supplementation on pyrrolnitrin production.
Glucose (500 mg/l) was added at 4 days to a control NBM culture (**B**). Data from three replicate experiments are presented with error bar for standard deviation.

other nutrients, whereas slight inhibitory effects were observed with zinc sulfate supplementation (Fig. 2B and Table 1).

Inhibition of Pyrrolnitrin Production by Glucose

Strong inhibition of pyrrolnitrin production was observed in glucose-supplemented culture (Figs. 2 and 3). In comparison with other nutrients, approximately 3 days of lag period was observed in glucose–NBM. The effects were gradually relieved by the depletion of glucose in the culture medium (Fig. 3A). It is noteworthy that the bacterial biomass was approximately 2-fold higher in glucose- and glycerol-supplemented NBM than other nutrients (Fig. 2). Glucose supplementation over control culture (4 days) gave similar effects (Fig. 3B). Rapid increase of pyrrolnitrin was suppressed for 2–3 days, followed by logarithmic increase again (Fig. 3B).

Polyhydroxyalkanoates

PHAs from strain O33 were obtained as white powdery materials. The major monomeric constituents were identified as 3-hydroxybutyric and (HB) and 3-hydroxyvaleric acid (HV), where HB was a dominant component; approximately 9:1, HB vs. HV (Table 2). In general, PHA contents were 20–40% of cellular biomass. The concentrations were dependent on carbon sources and incubation time (Table 2). Among the selected carbon sources, PHA concentrations in the cellular biomass from glucose-, glycerol-, and disaccharide-supplemented NBM were significantly higher than that of control culture, whereas the relative compositions of monomer were almost constant over different carbon sources (Table 2). The relative amount of PHA was decreased during culture. For example, concentrations of PHAs were decreased from 38% to 18% in trehalose-supplemented NBM at 2 and 5 days, respectively.

N-Acylhomoserine Lactones and Pyrrolnitrin

From culture supernatants, several *N*-acylhomoserine lactones were identified through GC–MS analyses. Three peaks (retention times, Rts, 47.8, 54.3, and 61.2 min, respectively) gave similar fragmentation patterns of authentic standards and retention times. The most abundant AHSL was identified as *N*-octanoylhomoserine lactone (C8-AHSL), followed by C10- and C6-AHSLs. Concentrations of C8-AHSL were dependent on nutrients, 0.78 to 2.05 μM (Table 3). The AHSL concentrations reached the maximum at approximately 2–3 days and decreased rapidly to trace level at 4–7 days (Fig. 4 and Table 3). Pyrrolnitrin concentrations in external

Table 2. PHA concentration of *Burkholderia* sp. O33, grown with different carbon sources in NBM.

Carbon source	Days[a]	Concentration (%±SD)[b]	Ratio of HB/HV[c]
Control	2	32.1±7.3	92/8
	5	19.2±9.3	90/10
Glucose	2	45.2±11.2	93/7
	5	31.7±6.5	91/9
Fructose	2	28.5±4.2	93/7
	5	24.3±5.6	95/5
Trehalose	2	38.3±7.7	94/6
	5	18.5±7.9	90/10
Sucrose	2	38.7±4.2	91/9
	5	19.5±10.2	92/8
Gluconolactone	2	32.1±9.4	93/7
	5	24.3±3.9	94/6
Glycerol	2	42.3±8.2	89/11
	5	30.5±2.1	91/9

[a]Days of culture.
[b]Percentage concentration of PHAs over cellular biomass (wet weight). SD denotes standard deviation of three replicate experiments.
[c]Relative ratio of 3-hydroxybutyrate (HB) and 3-hydroxyvalerate (HV).

Table 3. Concentrations of *N*-acylhomoserine lactones in culture medium of *Burkholderia* sp. O33 in NBM with different nutrients.

Nutrients	Days[a]	Concentration (μM)			Biomass (g/l)
		C6	C8	C10	
Control	2	ND[b]	0.89±0.23	0.01±0.00	0.45±0.15
Fructose	3	0.02±0.01	1.44±0.45	0.06±0.02	0.62±0.29
Glucose	3	0.01±0.01	1.33±0.23	0.03±0.01	1.48±0.19
Gluconolactone	2	0.01±0.00	1.31±0.45	0.05±0.01	0.38±0.12
Sucrose	3	ND	0.78±0.25	0.01±0.00	0.49±0.17
Trehalose	2	0.02±0.01	1.37±0.12	0.04±0.02	0.49±0.20
Glycerol	3	0.02±0.00	1.46±0.37	0.04±0.01	1.32±0.47
Tryptophan, D	2	0.02±0.01	1.33±0.25	0.05±0.02	0.56±0.15
Treptophan, L	2	0.04±0.02	1.78±0.36	0.07±0.03	0.47±0.14
$ZnSO_4$	2	0.02±0.01	0.97±0.24	0.03±0.01	0.31±0.08
NaCl	2	0.03±0.01	2.05±0.48	0.05±0.01	0.42±0.19
NH_4Cl	2	0.01±0.01	0.78±0.15	0.06±0.02	0.49±0.23

[a]Incubation time for maximum concentration of AHSLs.
[b]ND, not detected.

AHSL-treated culture showed practically no difference between treatment and control (Fig. 5).

Discussion

Bacterial species in the genus of *Burkholderia* have numerous interesting properties of potential industrial applications, including production of antibiotics, biosurfactant, and bioplastics, and degradation of environmental contaminants [15, 21, 32, 39]. Bacterial biosynthesis of antibiotics is highly dependent on environmental factors, including types of nutrient, pH, and temperature [12, 15, 24, 36]. In consideration of industrial applications, detailed understanding of these factors will provide several advantages, including the improvement of the production yield of specific antibiotics and biocontrol efficiency in agricultural practices.

Among selected carbon sources, glucose supplementation gave the largest difference compared with other nutrients. Although both glycerol and glucose strongly enhanced the biomass production more than other nutrients, initiation of pyrrolnitrin biosynthesis was strongly delayed by glucose, accompanied by the reduction of absolute amount of pyrrolnitrin. A similar phenomenon has been observed in pyoluteorin biosynthesis by *Pseudomonas fluorescens* CHA0 [12]. However, the contradictory effect of glucose was reported in the same strains during pyrrolnitrin production [12]. Duffy and Defago [12, 13] suggested that the inhibitory action of glucose over pyoluteorin biosynthesis may be originated from PQQ-dependent enzyme inhibition, where the corresponding protein is one of the most important sources of energy production. However, no detailed description has been provided for the stimulatory

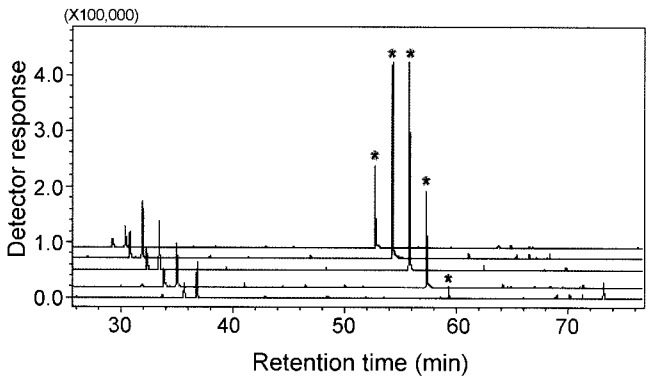

Fig. 4. GC–MS SIM chromatograms of *N*-acylhomoserine lactones in glucose–NBM culture medium of different incubation times.
Chromatograms of culture extracts of 1, 2, 3, 4, and 7 days were listed from top to bottom. Peaks of *N*-octanoylhomoserine lactone are marked with asterisks.

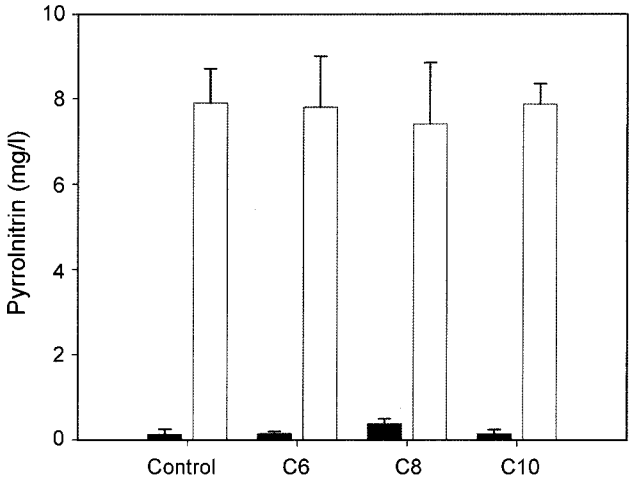

Fig. 5. Time dependent change of concentrations of pyrrolnitrin in the culture medium of control, or the medium treated with C6, C8, or C10-AHSLs at 2 (black bar) and 4 days (blank bar).

effect of glucose over pyrrolnitrin biosynthesis from the same organism. A similar effect of glucose was also reported with some *Burkholderia* spp. [15]. Results from current research suggested that strain O33 may have a different glucose-responsive regulatory mechanism of pyrrolnitrin biosynthesis from other pyrrolnitrin-producing bacteria. Another interesting effect of sugars was found in the disaccharides, sucrose and trehalose. Although both can be metabolized to glucose, trehalose enhanced pyrrolnitrin biosynthesis, whereas an inhibitory effect was observed by sucrose. In addition, gluconolactone strongly improved the pyrrolnitrin production. These results indicate that a highly complex regulatory system is involved in pyrrolnitrin biosynthesis in strain O33, especially for carbohydrates.

Biosynthesis of pyrrolnitrin is initiated by the halogenation of tryptophan (Fig. 1). In general, L-tryptophan is considered as a preferred substrate of tryptophan halogenase of *Pseudomonas* and *Burkholderia* spp. [4, 17, 29]. However, contradictory report is also available [5]. Salcher and Lingens [29] suggested that tryptophan racemase may transform D-isomer to L-form, followed by halogenation. Strain O33 did not show any preference of L- over D-isomers for the production of pyrrolnitrin (Table 1). Further studies with purified enzymes and chiral separation techniques will give better understanding of the preference of D- or L-isomers.

According to previous literature, supplementation of trace minerals, especially zinc, can enhance the biosynthesis of pyrrolnitrin and pyoluteorin in some pseudomonads [12]. However, no effects were observed in strain O33, supplemented with zinc sulfate. Minerals are important constituents of many metaloproteins and gene elements. For example, *prnD* (aminopyrrolnitrin oxidase) contains a catalytic Rieske center, which requires iron for proper bioactivity. However, no zinc-containing enzymes are known in pyrrolnitrin biosynthesis. The effect of zinc supplementation may be related to other enzymes or regulatory elements.

Many rhizobial bacteria produce useful biopolymers, including PHAs. In general, PHAs are heteropolymers of hydroxyfatty acids with chain length of C4–C18 [26]. PHA biosynthesis and degradation are dependent on cellular nutritional status [19, 33, 34]. Although the monomeric compositions of PHAs from strain O33 were almost constant with any carbon source, the concentration was highly dependent on carbon sources and incubation time (Table 2). Differences in PHA concentration with different carbon sources may arise from the relative flux of these carbon sources to PHA monomers versus other metabolic pathways [42]. Recently, it was reported that the phosphoenolpyruvate–carbohydrate phosphotransferase transport system (PEP–PTS) is one of the most critical regulator of PHA homeostasis [37]. PEP–PTS is a well-known regulatory system of bacterial carbohydrate metabolism. In addition, Ruiz *et al.* [27] reported that stationary phase sigma factor *RpoS* enhances PHA depolymerization in stressed condition. Reduction of PHA concentration in strain O33 in the stationary phase may be attributable to the reduction of nutrients and the accompanied stresses.

AHSL concentrations in supernatants showed dynamic changes during the culture period. Some *Burkholderia* species produce AHSLs to modulate their physiology in response to surrounding environments [11]. Because the biosynthesis of AHSLs is population-dependent, active accumulation of AHSLs are commonly observed during high-density cultures. Their concentrations, however, decreased rapidly, when the cellular growth reached to stationary phase [6]. AHSL concentrations in this study also showed similar responses. However, many additional factors may be involved in the regulation of AHSL biosynthesis. For example, the amount of AHSLs in NaCl–NBM was approximately 3-folds larger than those of control culture, of which the biomass productions were almost similar (Table 3). This finding suggested that nutrient characteristics are also important determining factors of AHSL biosynthesis. In the natural environment, quorum signals, including AHSLs, are rapidly inactivated through chemical and/or biological degradation [9, 23, 38, 41]. Nonenzymatic degradation (*e.g.*, abiotic lactonolysis) is another possible route of AHSL inactivation [3, 41].

Bacterial biosynthesis of some antibiotics is under the control of the quorum sensing system [24, 40, 43]. Liu *et al.* [24] reported that pyrrolnitrin production by *Serratia plymuthica* requires a specific AHSL (*N*-3-oxo-hexanoyl HSL), and disruption of the AHSL synthase gene results in complete inhibition of pyrrolnitrin production. Zhou *et al.* [43] reported that biosynthesis of unknown antifungal compounds was under AHSL-mediated regulation in *Burkholderia ambifara* BC-F. However, no studies are available on other bacteria producing pyrrolnitrin. It is noteworthy that the pyrrolnitrin concentration continuously increased even after complete disappearance of AHSLs. For example, no traces of AHSLs were observed after 5 days in glycerol–NBM, whereas the concentration of pyrrolnitrin increased up to the end of the experiments (Fig. 2). In addition, no significant changes of pyrrolnitrin accumulation were observed in the cultures supplemented with synthetic AHSLs (Fig. 5). From the above results, it can be postulated that AHSLs in strain O33 may have no effects over pyrrolnitrin production.

In summary, *Burkholderia* sp. O33 can produce not only pyrrolnitrin but also PHAs and quorum sensing signals. Production of pyrrolnitrin was highly dependent on nutrients. Trehalose, glycerol, and gluconolactone were the preferred substrates for pyrrolnitrin production, whereas glucose strongly inhibited pyrrolnitrin biosynthesis. Both glycerol and glucose were the best substrates for PHAs production. Quorum sensing signals from strain O33 (*N*-acylhomoserine lactones) showed dynamic changes during

the culture period. However, no clear correlation has been found between the production of pyrrolnitrins and PHAs and AHSLs. Information from this study will provide a better understanding to improve the biological characteristics of this strain.

Acknowledgment

This work was supported by the BioGreen 21 Program (20070301034037) from Rural Development Administration, Korea.

References

1. Berger, E., B. A. Ramsay, J. A. Ramsay, C. Chavarie, and G. Braunegg. 1989. PHB recovery by hypochlorite digestion. *Biotechnol. Tech.* **3:** 227–232.
2. Brodhagen, M., M. D. Henkels, and J. E. Loper. 2004. Positive autoregulation and signaling properties of pyoluteorin, an antibiotic produced by the biological control organism *Pseudomonas fluorescens* Pf-5. *Appl. Environ. Microbiol.* **70:** 1758–1766.
3. Byers, J. T., C. Lucas, G. P. C. Salmond, and M. Welch. 2002. Nonenzymatic turnover of an *Erwinia carotovora* quorum-sensing signaling molecule. *J. Bacteriol.* **184:** 1758–1766.
4. Chan, E. C. 1994. Cloning a mutated *trp* operon for the biosynthesis of an antibiotic agent. *Biotechnol. Lett.* **16:** 1021–1026.
5. Chang, C. J., H. G. Floss, D. J. Hook, J. A. Mabe, P. E. Manni, L. L. Martin, K. Schroeder, and T. L. Shieh. 1981. The biosynthesis of the antibiotic pyrrolnitrin by *Pseudomonas aureofaciens*. *J. Antibiot.* **34:** 555–566.
6. Chen, C.-C., L. Riadi, S.-J. Suh, D. E. Ohman, and L.-K. Ju. 2005. Degradation and synthesis kinetics of quorum-sensing autoinducer in *Pseudomonas aeruginosa* cultivation. *J. Biotechnol.* **117:** 1–10.
7. Corbell, N. and J. E. Loper. 1995. A global regulator of secondary metabolite production in *Pseudomonas fluorescens* Pf-5. *J. Bacteriol.* **177:** 6230–6236.
8. De Laurentis, W., L. Khim, J. L. R. Anderson, A. Adam, R. S. Philips, S. K. Chapman, K.-H. van Pee, and J. H. Naismith. 2007. The second enzyme in pyrrolnitrin biosynthesis pathway is related to the heme-dependent dioxygenase superfamily. *Biochemistry* **46:** 12393–12404.
9. Dong, Y.-H. and L.-H. Zhang. 2005. Quorum sensing and quorum-quenching enzymes. *J. Microbiol.* **43:** 101–109.
10. Dubuis, C. and D. Haas. 2007. Cross-species *gacA*-controlled induction of antibiosis in pseudomonads. *Appl. Environ. Microbiol.* **73:** 650–654.
11. Duerkrop, B. A., R. L. Ulrich, and E. P. Greenberg. 2007. Octanoyl-homoserine lactone is the cognate signal for *Burkholderia mallei* BmaR1-BmaI1 quorum sensing. *J. Bacteriol.* **189:** 5034–5040.
12. Duffy, B. K. and G. Defago. 1999. Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescens* biocontrol strains. *Appl. Environ. Microbiol.* **65:** 2429–2438.
13. Duffy, B. K. and G. Defago. 2000. Controlling instability in *gacS–gacA* regulatory genes during inoculant production of *Pseudomonas fluorescens* biocontrol strains. *Appl. Environ. Microbiol.* **66:** 3142–3150.
14. Elander, R. P., J. M. Mabe, R. H. Hamill, and M. Gorman. 1968. Metabolism of tryptophans by *Pseudomonas aureofaciens*. *Appl. Microbiol.* **16:** 753–758.
15. El-Banna, N. and G. Winkelmann. 1998. Pyrrolnitrin from *Burkholderia cepacia*: Antibiotic activity against fungi and novel activities against *Streptomyces*. *J. Appl. Microbiol.* **85:** 69–78.
16. Gunther IV, N. W., A. Nunez, W. Fett, and D. K. Y. Solaiman. 2005. Production of rhamnolipids by *Pseudomonas chlororaphis*, a nonpathogenic bacterium. *Appl. Environ. Microbiol.* **71:** 2288–2293.
17. Hamill, R. L., R. Elander, J. Mabe, and M. Gorman. 1968. Metabolism of tryptophans by *Pseudomonas aureofaciens*. V. Conversion of tryptophan to pyrrolnitrin. *Antimicrob. Agents Chemother.* 388–396.
18. Hammer, P. E., D. S. Hill, S. T. Lam, K.-H. van Pee, and J. M. Ligon. 1997. Four genes from *Pseudomonas fluorescens* that encode the biosynthesis of pyrrolnitrin. *Appl. Environ. Microbiol.* **63:** 2147–2154.
19. Hoffmann, N. and B. H. A. Rehm. 2005. Nitrogen-dependent regulation of medium-chain length polyhydroxyalkanoate biosynthesis genes in pseudomonads. *Biotechnol. Lett.* **27:** 279–282.
20. Holzer, M., W. Burd, H.-U. Reibig, and K.-H. Van Pee. 2001. Substrate specificity and regioselectivity of tryptophan 7-halogenase from *Pseudomonas fluorescens* BL915. *Adv. Synth. Catal.* **343:** 591–595.
21. Hwang, J., W. S. Chilton, and D. M. Benson. 2002. Pyrrolnitrin production by *Burkholderia cepacia* and biocontrol of *Rhizoctonia* stem rot of poinsettia. *Biol. Control* **25:** 56–63.
22. Kirner, S., P. E. Hammer, D. S. Hill, A. Altmann, I. Fisher, L. J. Weislo, M. Lanahan, K.-H. van Pee, and J. M. Ligon. 1998. Functions encoded by pyrrolnitrin biosynthetic genes from *Pseudomonas fluorescens*. *J. Bacteriol.* **180:** 1939–1943.
23. Leadbetter, J. R. and E. P. Greenberg. 2000. Metabolism of acyl-homoserine lactone quorum-sensing signals by *Variovorax paradoxus*. *J. Bacteriol.* **182:** 6921–6926.
24. Liu, X., M. Bimerew, Y. Ma, H. Muller, M. Ovardis, L. Eberl, G. Berg, and L. Chernin. 2007. Quorum sensing signaling is required for production of the antibiotic pyrrolnitrin in a rhizospheric biocontrol strain of *Serratia plymuthica*. *FEMS Microbiol. Lett.* **270:** 299–305.
25. Nowak-Thompson, B., N. Chaney, J. S. Wing, S. J. Gould, and J. E. Loper. 1999. Characterization of the pyoluteorin biosynthetic gene cluster of *Pseudomonas fluorescens* Pf-5. *J. Bacteriol.* **181:** 2166–2174.
26. Reddy, C. S. K., R. Ghai, and R. V. C. Kalia. 2003. Polyhydroxyalkanoates: An overview. *Biores. Technol.* **87:** 137–146.
27. Ruiz, J. A., N. I. López, and B. S. Méndez. 2004. *rpoS* Gene expression in carbon-starved cultures of the polyhydroxyalkanoate-accumulating species *Pseudomonas oleovorans*. *Curr. Microbiol.* **48:** 396–400.
28. Salcher, O. and F. Lingens. 1980. Isolation and characterization of *Pseudomonas aureofaciens* ATCC 15926 with an increased capacity for synthesis of pyrrolnitrin. *J. Gen. Microbiol.* **118:** 509–513.

29. Salcher, O. and F. Lingens. 1980. Metabolism of tryptophan by *Pseudomonas aureofaciens* and its relationship to pyrrolnitrin biosynthesis. *J. Gen. Microbiol.* **121:** 465–471.
30. Schnider, U., C. Keel, C. Voisard, G. Defago, and D. Haas. 1995. Tn5-directed cloning of *pqq* genes from *Pseudomonas fluorescens* CHA0: Mutational inactivation of the genes results in overinduction of the antibiotic pyoluteorin. *Appl. Environ. Microbiol.* **61:** 3856–3864.
31. Schnider, U., C. Keel, C. Blumer, J. Troxler, G. Defago, and D. Haas. 1995. Amplification of the housekeeping sigma factor in *Pseudomonas fluorescens* CHA0 enhances antibiotic production and improves biocontrol abilities. *J. Bacteriol.* **177:** 5387–5392.
32. Seeger, M., M. Zielinski, K. N. Timmis, and B. Hofer. 1999. Regiospecificity of dioxygenation of di- to pentachlorobiphenyls and their degradation to chlorobenzoates by the *bph*-encoded catabolic pathway of *Burkholderia* sp. strain LB400. *Appl. Environ. Microbiol.* **65:** 3614–3621.
33. Shi, H.-P., C.-M. Lee, and W.-H. Ma. 2007. Influence of electron acceptor, carbon, nitrogen, and phosphorus on polyhydroxyalkanoate (PHA) production by *Brachymonas* sp. P12. *World J. Microbiol. Biotechnol.* **23:** 625–632.
34. Stubbe, J. and J. Tian. 2003. Polyhydroxyalkanoate (PHA) homeostasis: The role of PHA synthase. *Nat. Prod. Rep.* **20:** 445–457.
35. Thomas, M. G., M. D. Burkart, and C. T. Walsh. 2002. Conversion of L-proline to pyrrolyl-2-carboxy-S-PCP during undecylprodigiosin and pyoluteorin biosynthesis. *Chem. Biol.* **9:** 171–184.
36. van Rij, E. T., M. Wesselink, T. F. C. Woeng, G. V. Bloemberg, and B. J. J. Lugtenberg. 2004. Influence of environmental conditions on the production of phenazine-1-carboxamide by *Pseudomonas chloraphis* PCL1391. *Mol. Plant Microbe Interact.* **17:** 557–566.
37. Velazquez, F., K. Pfluger, I. Cases, L. I. de Eugenio, and V. de Lorenzo. 2007. The phosphotransferase system formed by PtsP, PtsO, and PtsN proteins controls production of polyhydroxyalkanoates in *Pseudomonas putida*. *J. Bacteriol.* **189:** 4529–4533.
38. Wang, Y.-J., and J. R. Leadbetter. 2005. Rapid acyl-homoserine lactone quorum signal biodegradation in diverse soils. *Appl. Environ. Microbiol.* **71:** 1291–1299.
39. Wattanaphon, H. T., A. Kerdsin, C. Thannacharoen, P. Sangvanich, and A. S. Vangnai. 2008. A biosurfactant from *Burkholderia cenocepacia* BSP3 and its enhancement of pesticide solubilization. *J. Appl. Microbiol.* **108:** 1–8.
40. Wood, D. W., F. Gong, M. M. Daykin, P. Williams, and L S Pierson III. 1997. *N*-Acyl-homoserine lactone-mediated regulation of phenazine gene expression by *Pseudomonas aureofaciens* 30-84 in the wheat rhizosphere. *J. Bacteriol.* **179:** 7663–7670.
41. Yates, E. A., B. Philipp, C. Buckley, S. Atkinson, S. R. Chhabra, R. E. Sockett, *et al.* 2002. *N*-Acylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa*. *Infect. Immun.* **70:** 5635–5646.
42. Yu, J. and Y. Si. 2004. Metabolic carbon fluxes and biosynthesis of polyhydroxyalkanoates in *Ralstonia eutropha* on short chain fatty acids. *Biotechnol. Prog.* **20:** 1015–1024.
43. Zhou, H., F. Yao, D. P. Roberts, and T. G. Lessie. 2003. AHL-deficient mutants of *Burkholderia ambifaria* BC-F have decreased antifungal activity. *Curr. Microbiol.* **47:** 174–179.