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Production of Bacterial Quorum Sensing Antagonists, Caffeoyl- and Feruloyl-HSL, by an Artificial Biosynthetic Pathway

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A new series comprising phenylacetyl-homoserine lactones (HSLs), caffeoyl-HSL and feruloyl-HSL, was biologically synthesized using an artificial de novo biosynthetic pathway. We developed an *Escherichia coli* system containing artificial biosynthetic pathways that yield phenylacetyl-HSLs from simple carbon sources. These artificial biosynthetic pathways contained the LuxI-type synthase gene (*rpaI*) in addition to caffeoyl-CoA and feruloyl-CoA biosynthetic genes, respectively. Finally, the yields for caffeoyl-HSL and feruloyl-HSL were 97.1 ± 10.3 and 65.2 ± 5.7 mg/l, respectively, by tyrosine-overproducing *E. coli* with a L-methionine feeding strategy. In a quorum sensing (QS) competition assay, feruloyl-HSL and *p*-coumaroyl-HSL antagonized the QS receptor TraR in *Agrobacterium tumefaciens* NT1, whereas caffeoyl-HSL did not.

Keywords: Artificial biosynthesis, phenylacetyl homoserine lactone, caffeoyl-HSL, feruloyl-HSL

Introduction

Quorum sensing (QS) is a term applied to cell-cell communication that many bacterial pathogens use to control the expression of various genes [1–3]. QS in gram-negative bacteria has been studied extensively over the past 25 years and is typically mediated by diffusible *N*-acyl-homoserine lactone (AHL) signals [4–6]. Since QS is closely related to virulence and biofilm formation, its disturbance by anti-QS agents can provide clues for the development of novel antibiotics. Indeed, inhibition of QS, otherwise known as quorum quenching, is a strategy already employed by some endophytic bacteria to aid survival in host organisms while preventing pathogens from developing resistance. As a result, the development of methods to intercept QS as a potential anti-infective therapy has attracted significant interest [7–10].

A few bacteria, such as *Rhodopseudomonas palustris*, *Bradyrhizobium* sp. BTAi1, and *Silicibacter pomeroyi* DSS-3, produce a natural phenylacetyl-homoserine lactone (HSL), *p*-coumaroyl-HSL [11–13]. It is clear that several photosynthetic bacteria and nitrogen-fixing bacteria respond in complex ways to the presence of exogenous lignin monomers (cinnamic acid, *p*-coumaric acid, caffeic acid, and ferulic acid) and this may drive intercellular signaling for production of metabolites [11–13]. In addition, a series of synthetic phenylacetyl-HSL analogs that are capable of strongly antagonizing or agonizing LuxR-type receptors in a range of gram-negative bacteria have been uncovered [10, 14–17].

RpaI, a LuxI-type synthase that was known as a *p*-coumaroyl-HSL synthase, used *p*-coumaric acid rather than fatty acids [12, 18]. Recently, however, the substrate specificity of the RpaI enzyme demonstrated that four phenolic acids (cinnamic acid, *p*-coumaric acid, caffeic acid, and ferulic acid) are used as a start unit [12]. The reaction of each phenolic acid with broad substrate-specific coumaroyl-CoA ligase (4CL) from *Nicotiana tabacum* in the presence of RpaI led to the formation of each phenylacetyl-HSL product [19, 20]. On the other hand, we already developed an *Escherichia coli* system containing an artificial biosynthetic pathway that yields *p*-coumaric acid, caffeic acid, and ferulic acid from simple carbon sources [21]. In this artificial system, *p*-coumaric acid is converted into caffeic acid, by a hydroxylation step at the 3-position of the benzyl ring, by coumarate 3-hydroxylase (Sam5) from actinomycete *Saccharothrix espanaensis* [22, 23]. Ferulic

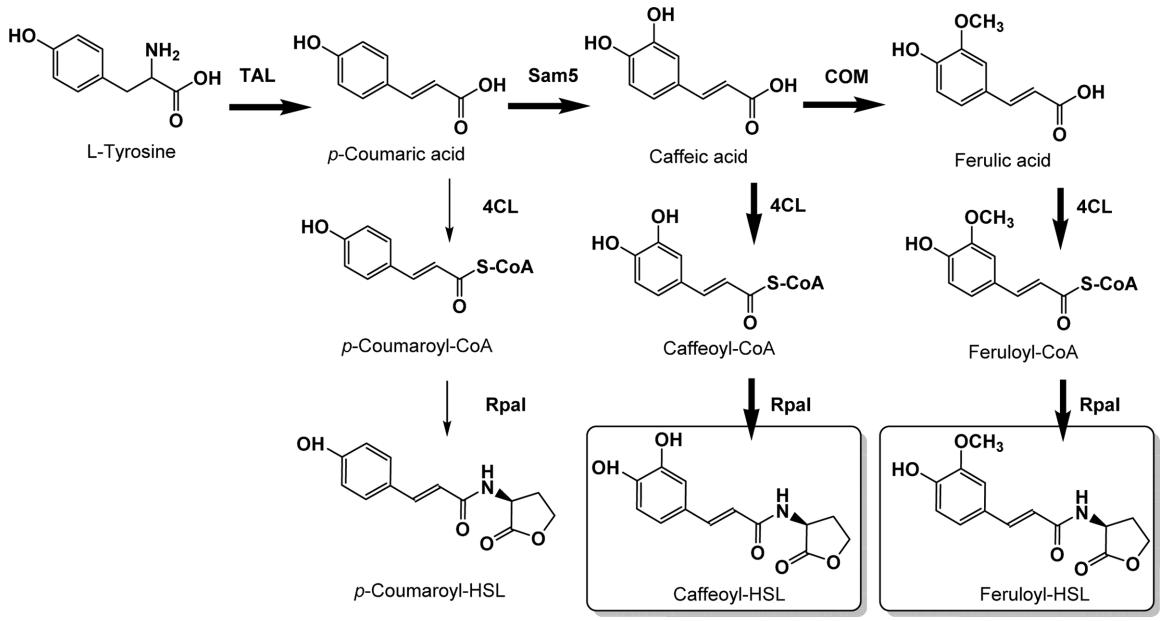

Fig. 1. Engineered biosynthetic pathways for the phenylacetyl-homoserine lectones analogs in *E. coli*.
N-acyl-homoserine lectone synthase (RpaI) from *Rhodopseudomonas palustris*, tyrosine ammonia lyase (TAL) from *Saccharothrix espanaensis*, coumarate 3-hydroxylase (Sam5) from *S. espanaensis*, caffeic acid *O*-methyltransferase (COM) from *Arabidopsis thaliana*, and *p*-coumarate-CoA ligase (4CL) from *Nicotiana tabacum*. The biosynthetic pathways used in this study are marked with bold arrows.

acid is biosynthesized from caffeic acid by the enzyme caffeic acid *O*-methyltransferase, which originates from *Arabidopsis thaliana* (Fig. 1) [24, 25]. Moreover, we recently reported an artificial biosynthetic process for *p*-coumaroyl-HSL in a heterologous host, *E. coli* [20].

Herein, we developed a serial biosynthetic process for caffeoyl-HSL and feruloyl-HSL, using a de novo biosynthesis method in the heterologous host *E. coli* and engineered tyrosine-overproducing *E. coli* containing the coumarate 3-hydroxylase gene (*sam5*), and *sam5* and caffeic acid *O*-methyltransferase (*com*) genes in addition to the previously described artificial *p*-coumaroyl-HSL production vector [20]. The yields for caffeoyl-HSL and feruloyl-HSL were 97.1 ± 10.3 and 65.2 ± 5.7 mg/l, respectively, by the tyrosine-overproducing *E. coli* with a L-methionine feeding strategy. In addition, we tested three phenylacetyl-HSLs (*p*-coumaroyl-HSL, caffeoyl-HSL, and feruloyl-HSL) as QS inhibitors and obtained results suggesting that *p*-coumaroyl-HSL and feruloyl-HSL competed moderately with *N*-(3-oxododecanoyl)-L-homoserine lectone (OdDHL) against TraR in *Agrobacterium tumefaciens* NT1.

Materials and Methods

Chemicals

p-Coumaric acid, caffeic acid, ferulic acid, and *N*-(*p*-coumaroyl)-L-homoserine lactone (*p*-coumaroyl-HSL) were purchased from Sigma-Aldrich (USA) as standards for compound identification by HPLC. Furthermore, L-methionine was purchased from Sigma-Aldrich for increasing the phenylacetyl-HSL production in *E. coli*.

DNA Manipulation

The restriction enzymes (NEB; Takara, Japan), a KOD polymerase (Toyobo, japan), a ligation mix (Takara, Japan), and a plasmid SV prep kit (GeneAll, Korea), were used according to the manufacturer's instructions. The optimized tyrosine ammonia lyase gene (*tal*) from *S. espanaensis*, *p*-coumarate 3-hydroxylase gene (*sam5*) from *S. espanaensis*, caffeic acid methyltransferase gene (*com*) from *A. thaliana*, and optimized *p*-coumaroyl CoA ligase 4CL2 gene (*4cl2nt*) from *N. tabacum* were synthesized previously by DNA 2.0 [21, 22]. The HSL synthase gene *rpaI* from *R. palustris* was codon optimized and synthesized [20]. All genes for this study were synthesized by DNA 2.0 or Bioneer (Korea).

Construction of De Novo Synthesis Vectors, pET-opT54R and pET-opT54MR

Previously, three genes (*tal*, *4cl2nt*, *rpaI*) were cloned into pET-28a(+) vector, which resulted in pET-opT4R [20]. In order to assemble the pET-opT54R vector, a Sam5 coding region was amplified using pET-Sam5 as a template with the primers Npac (the sequence is located upstream of the T7 promoter region of the pET vector and contains the designed PacI site: TTAATTAAT CGCCGCGACAATTTGCGACGG) and Cpac (the sequence is located downstream of the T7 terminator region of the pET vector

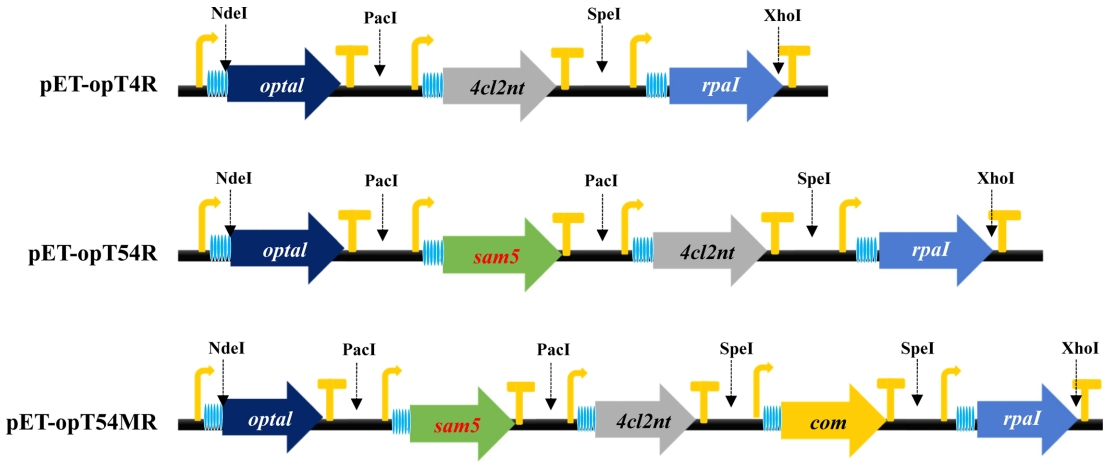

Fig. 2. Organization of the artificial gene clusters used for the production of caffeoyl-homoserine lectone (HSL) and feruloyl-HSL in *E. coli*.

All constructs contained the T7 promoter, RBS in front of each gene, and T7 terminator at the rear of each gene.

and contains the designed PacI site: TTAATTAATGCGCCGCTA CAGGGCGCGTCC), and the amplified fragment was digested with the PacI enzyme and cloned between the PacI site of the pET-opT4R vector, which resulted in pET-opT54R (Fig. 2). Additionally, a COM coding region was amplified using pET-COM [22] as a template with the primers Nspe (the sequence is located upstream of the T7 promoter region of the pET vector and contains the designed SpeI site: ACTAGTAGGTTGAGGCCGTTGAGCACCGCC) and Cspe (the sequence is located downstream of the T7 terminator region of the pET vector and contains the designed SpeI site: ACTAGTTCCTCCTTTCAGCAAAAAACCCCTC), and the amplified fragment was digested with the SpeI enzyme and cloned between the SpeI site of pET-opT54R, which resulted in pET-opT54MR (Fig. 2). The recombinant plasmids were transformed into *E. coli* C41(DE3) and tyrosine-overproducing strain (ΔCOS1) [26] for gene expression. The strains and plasmids used in this study are listed in Table 1.

Production of Phenylacetyl-HSL Analogs by *E. coli*

Recombinant *E. coli* strains were grown in 5 ml of LB medium supplemented with appropriate antibiotic (50 μg/ml kanamycin) at 37°C with shaking at 200 rpm. The overnight cultures of these strains were inoculated (1.5%) into 50 ml of fresh LB medium supplemented with 50 μg/ml kanamycin. The cultures were grown at 37°C until they reached an optical density of 600 nm (OD_{600}) of about 0.6. Then, isopropyl-beta-D-thiogalactoside (IPTG)

Table 1. Plasmids and strains used in this study.

Plasmid or strain	Relevant characteristics	Source
Plasmid		
pET-28a(+)	f1 ori, T7 promoter, Kan^R	Novagen
pET-Sam5	pET-28a(+) carrying a 4-coumarate 3-hydroxylase *sam5* gene	Kang *et al.* [21]
pET-COM	pET-28a(+) carrying a caffeic acid *O*-methyltransferase *comt* gene	Kang *et al.* [21]
pET-opT4R	pET-28a(+) carrying codon-optimized *tal*, *4cl2nt*, and *rpaI* genes	Kang *et al.* [20]
pET-opT54R	pET-28a(+) carrying codon-optimized *tal*, *sam5*, *4cl2nt*, and *rpaI* genes	This study
pET-opT54MR	pET-28a(+) carrying codon-optimized *tal*, *sam5*, *comt*, *4cl2nt*, and *rpaI* genes	This study
Strain		
E. coli C41(DE3)	Derivative strain of *E. coli* BL21(DE3)	
ΔCOS1	*E. coli* C41(DE3); Δ*tyrR*::$tyrA^{fbr}$, $aroG^{fbr}$; tyrosine-overproducing strain	Kang *et al.* [26]
EN1	*E. coli* C41(DE3) harboring pET-opT54R	This study
FN1	*E. coli* C41(DE3) harboring pET-opT54MR	This study
EN2	*E. coli* ΔCOS1 harboring pET-opT54R	This study
FN2	*E. coli* ΔCOS1 harboring pET-opT54MR	This study

was added at a final concentration of 1 mM. The cultures were incubated at 26°C for 5 h. Afterward, cells were harvested by centrifugation, and suspended in 30 ml of synthetic medium (SM) medium (3 g/l KH_2PO_4, 7.3 g/l K_2HPO_4, 8.4 g/l MOPS, 2 g/l NH_4Cl, 0.5 g/l NaCl, 0.1 ml/l trace elements, and 5 g/l $(NH_4)_2SO_4$, 5 g/l $MgSO_4$, and supplemented with 15 g/l glucose, 1 mM IPTG, and 50 μg/ml kanamycin). For the L-methionine feeding experiments, the compound was added at a final concentration of 1 mM into the SM. The cultures were incubated under the same conditions until 24 h. The fermentation broth supernatants were filtered using 0.2 μm cellulose membrane syringe filters (Sartorius) and analyzed by high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS). These experiments were conducted in triplicate. The production of phenylacetyl-HSL analogs in recombinant *E. coli* was analyzed and quantified using Dionex HPLC with a SunFire C18 column (250 × 4.6 mm, 5 μm; Waters, USA) connected to a UV detector (320 nm). A flow rate of 1 ml/min was used with a linear gradient of acetonitrile and 0.05% trifluoroacetic acid buffer by the following method: 10–60% acetonitrile for 25 min. The compounds were further characterized by LC-MS analysis using an LTQ XL linear ion trap (Thermo Scientific, USA) following the previously reported method.

Purification of Caffeoyl-HSL and Feruloyl-HSL

The *E. coli* strains (EN2 and FN2) were cultured via the same method as described earlier, but the culture volume and time were increased to 2 L until 70 h. The resulting cultures were extracted twice with an equal volume of ethyl acetate (EtOAc), respectively. After these extracts were partitioned between EtOAc and water, the extracts were evaporated in vacuum. The EtOAc-soluble materials were further purified by reverse-phase HPLC (Waters Co., USA) using the YMC J'sphere ODS-H80 (10 × 250 mm, 3 ml/min) with a linear gradient from 20% to 100% CH_3CN containing 0.05% TFA in order to yield caffeoyl-HSL and feruloyl-HSL, respectively. The *p*-coumaroyl-HSL was purified by the same method as our described report [20]. The purified caffeoyl-HSL, feruloyl-HSL, and *p*-coumaroyl-HSL were used as standards for quantification of phenylacetyl-HSL production and for QS competition assay.

QS Competition Assay

An AHL-based QS competition assay was carried out using two genetically modified strains, *A. tumefaciens* NT1 [27] and *Chromobacterium violaceum* CV026 [28]. *A. tumefaciens* NT1 contains the *lacZ* reporter gene fused with the gene encoding the TraR receptor that can detect AHLs having a long carbon chain, such as OdDHL, leading to the production of a cyan color. *C. violaceum* CV026 was constructed to make the CviR receptor that is able to sense exogenous AHLs with a short carbon chain length, such as *N*-butyryl-L-homoserine lactone (BHL), resulting in the production of a purple pigment called violacein [28]. Since the TraR and CviR receptors in *A. tumefaciens* NT1 and *C. violaceum* CV026 are homologs of LasR and RhlR receptors, respectively, in *Pseudomonas aeruginosa*, the reporter strains are frequently used to conduct the competitive binding assay of LasR and RhlR receptors in *P. aeruginosa.*

A. tumefaciens NT1 or *C. violaceum* CV026 was cultivated in LB medium overnight at 220 rpm at 30°C and 1 ml of 20-fold diluted overnight culture was dispensed into 15 ml conical tubes. Then, 5 μl of X-gal (20 g/l) and 10 μl of 100 μM OdDHL (Sigma-Aldrich, USA) were added to *A. tumefaciens* NT1, whereas 10 μl of 50 mM BHL (Caymann, USA) was added to *C. violaceum* CV026. Then, 10 μl of test compounds dissolved in DMSO, or DMSO as a negative control, was added to the cultures and incubated at 30°C for 48 h. To see the effect of the test compounds on cell viability, the OD_{600} was observed. The *A. tumefaciens* NT1 cultures were then centrifuged (3,250 ×*g*, 10 min) to collect cells, followed by extraction of the cyan color compound with 500 μl of ethyl acetate. After evaporation of the ethyl acetate layer, the OD of the residue dissolved in DMSO was measured at 545 nm. On the other hand, the violacein compound in 100 μl of the *C. violaceum* CV026 culture was extracted with 900 μl of water-saturated *n*-butanol following addition of 200 μl of 10% SDS to lyse the bacteria. The OD of the butanol layer was measured at 590 nm with a microtiter ELISA reader.

Results and Discussion

Construction of De Novo Biosynthetic Pathways in *E. coli* to Produce Caffeoyl-HSL and Feruloyl-HSL

Previously, we reported the de novo production of *p*-coumaroyl-HSL [20]. In this study, we describe a similar approach for the de novo synthesis of caffeoyl-HSL and feruloyl-HSL in *E. coli* by an engineered artificial biosynthetic pathway. The artificial de novo biosynthesis pathway for production of caffeoyl-HSL and feruloyl-HSL used the addition of *sam5* only, and *sam5* and *com* genes with the previously reported *p*-coumaroyl-HSL biosynthetic genes [21, 22]. The *p*-coumaroyl-HSL biosynthetic pathway genes are the tyrosine ammonia lyase gene (*tal*), *p*-coumaroyl-CoA ligase gene (*4cl2nt*), and LuxI-type phenylacetyl-HSL synthase gene (*rpaI*), which convert tyrosine to *p*-coumaroyl-HSL through *p*-coumaric acid and *p*-coumaroyl-CoA (Fig. 1).

For the de novo synthesis of caffeoyl-HSL in *E. coli*, the final pET-opT54R vector contains the tyrosine ammonia lyase (*tal*), *p*-coumaroyl-CoA ligase (*4cl*), coumarate 3-hydroxylase (*sam5*), and *p*-coumaroyl-HSL synthase (*rpaI*) genes (Table 1 and Fig. 2). The recombinant strain (EN1) that harbors the artificial biosynthetic gene expression vector (pET-opT54R) was cultured in a modified synthetic medium. Moreover, the final pET-opT54MR vector for feruloyl-HSL contains the *tal*, *4cl2nt*, *sam5*, *com*, and *rpaI* genes. The recombinant strain (FN1) that harbors the artificial biosynthetic gene expression vector (pET-opT54MR) was cultured in SM.

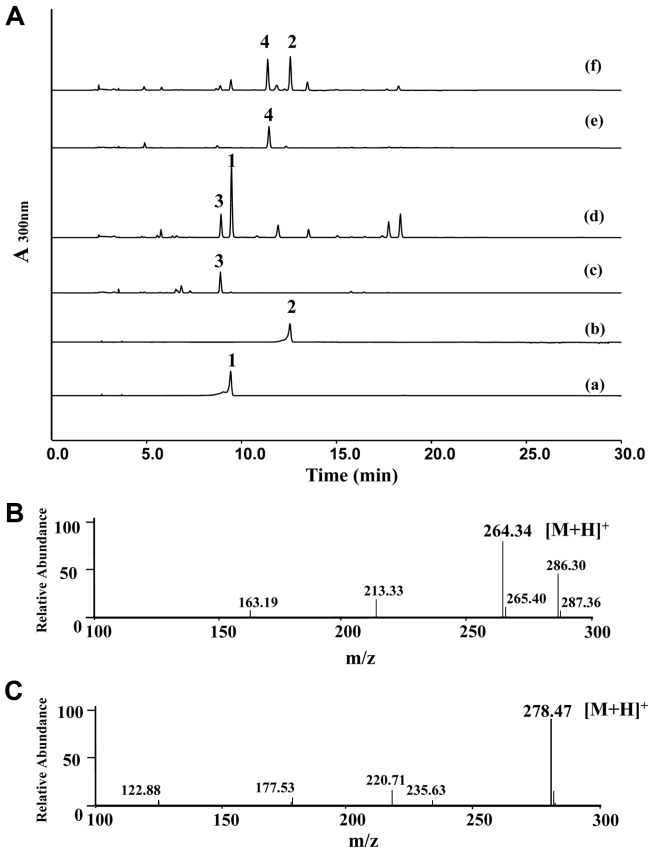

Fig. 3. De novo biosynthesis of caffeoyl-homoserine lectone (HSL) and feruloyl-HSL by engineered strains.

(**A**) HPLC profiles of the standard caffeic acid (a) and ferulic acid (b); the culture broth of EN1 (c), EN2 (d), FN1 (e), and FN2 (f) strains for 24 h. Peak 1, caffeic acid; peak 2, ferulic acid; peak 3, caffeoyl-HSL; peak 4, feruloyl-HSL. The absorbance was monitored at 320 nm. (**B**) Selected mass ion chromatogram of caffeoyl-HSL (*m/z* 264.34 [M + H]$^+$) by strain EN1. (**C**) Selected mass ion chromatogram of feruloyl-HSL (*m/z* 278.47[M + H]$^+$) by strain FN1.

The caffeoyl-HSL peak was detected at 8.9 min in the culture broth of the EN1 strain by HPLC (Fig. 3A). This peak was further analyzed using LC-MS. The peak at *m/z* 264 [M+H]$^+$ corresponded to caffeoyl-HSL (Fig. 3B). Additionally, the FN1 strain with the pET-opT54MR clone exhibited a new peak at 11.4 min in the same culture system compared with the EN1 strain (Fig. 3A). The peak showed a molecular ion at *m/z* 278 [M+H]$^+$, which corresponded to feruloyl-HSL in the mass spectrum (Fig. 3C). In addition, we did not detect intermediate caffeic acid and ferulic acid peaks in each culture condition in wild-type *E. coli* (Fig. 3A(c) and 3A(e)). Thus, we assume that each of the final phenolic acids (caffeic acid and ferulic acid) was efficiently converted into each of the HSL moiety.

Improved Production of Caffeoyl-HSL and Feruloyl-HSL in a Tyrosine-Overproducing *E. coli* Strain

The EN1 and FN1 strains were investigated using metabolite pattern analyses based on the culture times, until the production of each phenylacetyl-HSL was saturated after 24 h. The amount of caffeoyl-HSL and feruloyl-HSL reached 7.1 ± 0.1 mg/l and 13.4 ± 0.7 mg/l, respectively (Fig. 4). As was the case for the previous results in production of caffeic acid and ferulic acid [21], the titer of feruloyl-HSL also increased over caffeoyl-HSL in this artificial de novo biosynthesis. The reason for this titer improvement is still unknown, but accelerated metabolic flow to ferulic acid in the cell [21] and the relative higher 4CL activities than the caffeic acid [19] are possibilities.

To improve the production yield, we used the caffeoyl-HSL and feruloyl-HSL production system in a L-tyrosine-overproducing *E. coli* strain. Recently, we reported engineered L-tyrosine-overproducing *E. coli* ΔCOS1 strains via deregulation of the aromatic amino acid biosynthesis pathway [26]. The tyrosine producer, *E. coli* ΔCOS1, showed a substantial capacity for production of *p*-coumaric acid, caffeic acid, and ferulic acid. Moreover, it is a suitable platform strain for the production of phenylacetyl-HSLs, using phenolic acids as precursors. Using the same experimental conditions described above, the tyrosine-overproducing *E. coli* ΔCOS1 strain harboring pET-opT54R (EN2) and pET-opT54MR (FN2) produced more than 10.5 ± 1.4 mg/l of caffeoyl-HSL and 24.4 ± 8.1 mg/l of feruloyl-HSL, respectively (Fig. 4). These productivities were lower than the previously *p*-coumaroyl-HSL production (60.9 ± 0.5 mg/l) [20]. However, the productivity showed 1.5-fold and 1.8-fold improvement over the titers of the original producers, EN1 and FN1, respectively. These results mean that tyrosine serves as the main precursor for *p*-coumaric acid, caffeic acid, and ferulic acid, and tyrosine-overproducing strains exhibit an enhanced capacity for each phenylacetyl-HSL synthesis from glucose.

In addition, HSLs use *S*-adenosyl methionine (SAM) as the HSL ring donor. SAM is produced from L-methionine and ATP catalyzed by methionine adenosyltransferase in vivo [29]. As L-methionine is the limiting component in SAM biosynthesis, it has always been added to the medium as a supplement [29]. Our study previous showed that the accumulation of *p*-coumaric acid during the *p*-coumaroyl-HSL production in the tyrosine-overproducing cell was resolved by the addition of L-methionine [20]. In this study, we supplied final 1 mM of L-methionine to the culture medium of the EN2 and FN2 strains, respectively, in order to investigate the acceleration of the metabolic flux to caffeoyl-HSL and feruloyl-HSL via the SAM cycle.

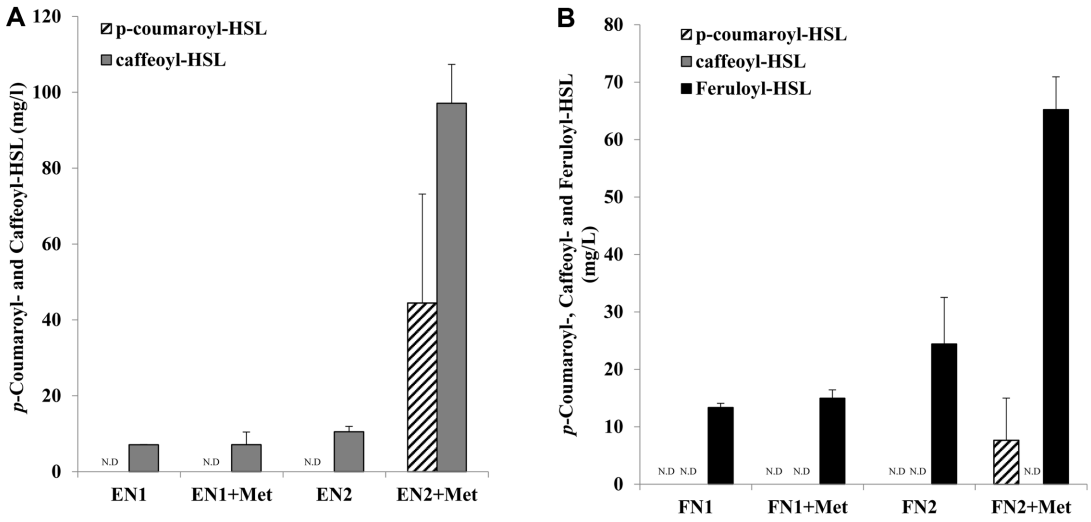

Fig. 4. Effects of L-methionine addition on caffeoyl-homoserine lectone (HSL) and feruloyl-HSL production.
The data were obtained after 24 h fermentation with the addition of 1 mM L-methionine (Met) in the synthetic medium of EN1, EN2, FN1, and FN2 strains, respectively. Error bars represent at one standard deviation from triplicate experiments. ND, not detected on the HPLC profile.

Analysis of the product after 24 h showed that the production of caffeoyl-HSL and feruloyl-HSL reached 97.1 ± 10.3 mg/l and 65.2 ± 5.7 mg/l from the 1 mM L-methionine in the EN2 and FN2 strains, respectively (Fig. 4). However, the fed L-methionine did not affect the production of caffeoyl-HSL and feruloyl-HSL in the parental strains (EN1 and FN1). These results mean that tyrosine and methionine are limiting factors in the production of *p*-coumaric acid and *p*-coumaroyl-HSL, respectively. Finally, the EN2 strain had a 9.2-fold higher production yield of caffeoyl-HSL (97.1 ± 10.3 mg/l) in the culture system with the additional L-methionine medium compared with the original medium (SM). Moreover, the production level of feruloyl-HSL showed 2.7-fold improvement over the FN2 control culture. These results indicate that the elevated L-methionine assimilation pathway allows for metabolic flux improvement of extra phenylpropanoic acids from tyrosine, converting it to phenylacetyl-HSL analogs. However, the accumulation of *p*-coumaroyl-HSL was shown on the L-methionine additional cultures of EN2 and FN2 strain (Fig. 4). We assumed the reason behind the accumulation was due to the lower enzymatic efficiency of the coumarate 3-hydroxylase (Sam5) on relatively short bioconversion times.

In gram-negative bacteria, QS depending on *N*-acyl-HSL autoinducers is the most well-known communication system [1, 6]. The typical core system consists of the production of HSLs, followed by signal recognition by transcriptional regulators that subsequently regulate expression of QS-related genes, including those of HSL synthases. The autoinducer synthases (LuxI homologs) and the LuxR-type regulator often can synthesize and perceive multiple *N*-acyl-HSLs, respectively [2, 3]. However, these phenylacetyl-HSLs are required to activate expression of the LuxR-type regulator (RpaR) in purple nonsulfur bacterium, not to serve as part of the actual QS signal in *E. coli* [12, 16, 18]. Thus, the artificial system in this study successfully demonstrated the de novo synthesis of QS molecules using an artificial biosynthetic pathway in the heterologous host, *E. coli*.

QS Competition Assay

We evaluated three phenylacetyl-HSLs as potential activators or inhibitors of LuxR-type receptor in pathogenic bacteria. We focussed on the QS signaling in *A. tumefaciens* NT1 and *C. violaceum* CV026. Feruloyl-HSL and *p*-coumaroyl-HSL inhibited the binding of OdDHL with its cognate QS receptor TraR in *A. tumefaciens* NT1 (Fig. 5A). They inhibited at rates of 20.6% and 26.4%, respectively, at 100 μM. However, they did not inhibit the binding of BHL with its cognate QS receptor CviR in *C. violaceum* CV026 (Fig. 5B). Neither TraR nor CviR were affected by caffeoyl-HSL. Since QS is closely related to virulence and biofilm formation, its disturbance by anti-QS agents can provide clues for the development of novel antibiotics [30]. To address these challenges, many structurally related synthetic phenyl HSLs can strongly modulate LuxR-type receptors in a range of gram-negative bacteria [9, 10, 14]. In particular, synthetic 4-bromo phenylacetanoyl-HSL was revealed as a potent antagonist of TraR [8]. To best our knowledge, these

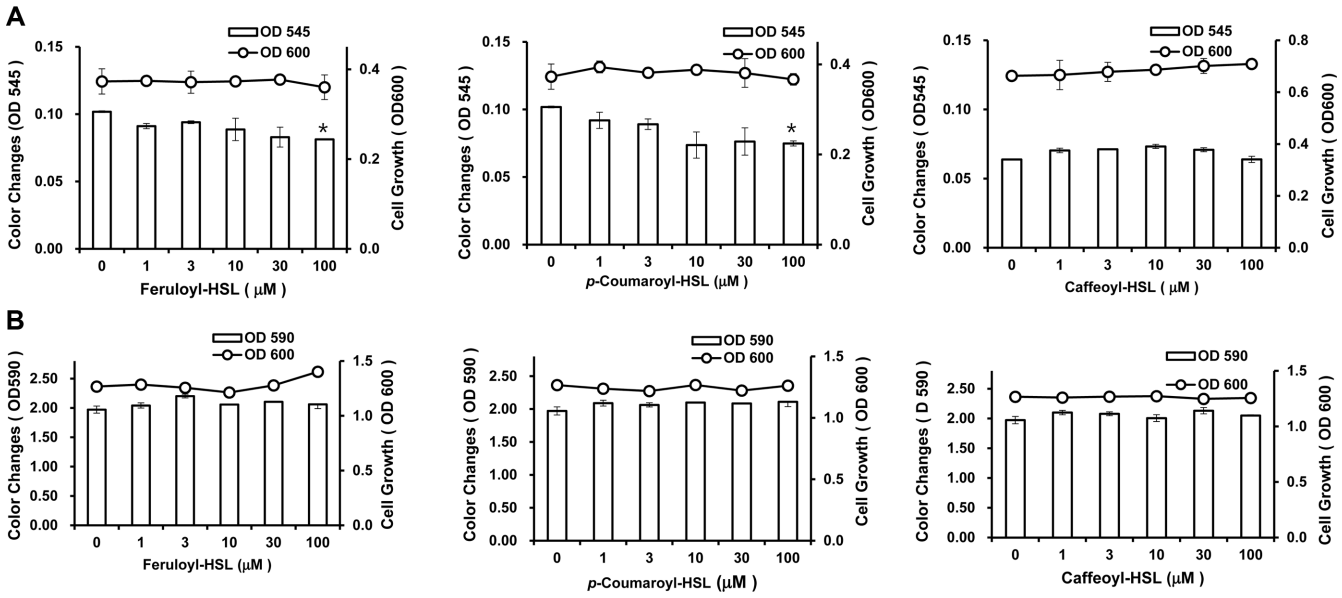

Fig. 5. Quorum sensing competition assay using the reporter strains and *A. tumefaciens* NT1 (**A**) and *C. violaceum* CV026 (**B**).
A. tumefaciens NT1 and *C. violaceum* CV026 were treated with 1 μM *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) and 500 μM *N*-butyryl-L-homoserine lactone (BHL), respectively, and grown in the presence of different concentrations of compounds for 48 h. After cell viability was measured at 600 nm, color changes were assayed. The mean ± SD values are displayed in each bar. *, $p < 0.001$ versus color from cells treated with the same volume of DMSO only.

compounds reported herein represent the first artificial phenylacetyl ligands capable of intercepting QS signals, even if competing moderately with OdDHL against TraR in *A. tumefaciens* NT1.

Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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