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Articles

Genome Mining Discovery of Protegenins A–D, Bacterial Polyynes Involved in the Antioomycete and Biocontrol Activities of *Pseudomonas protegens*

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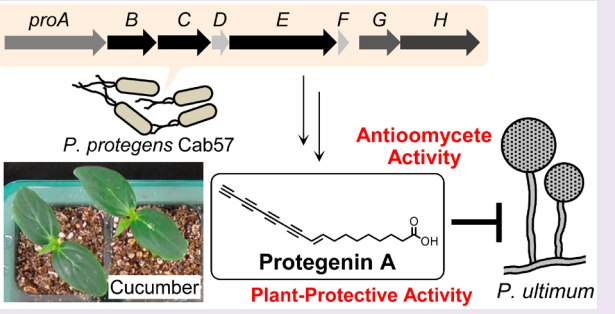
ABSTRACT: Some bacteria uniquely produce "bacterial polyynes", which possess a conjugated C≡C bond starting with a terminal alkyne, and use them as chemical weapons against hosts and competitors. *Pseudomonas protegens* Cab57, a biocontrol agent against plant pathogens, has an orphan biosynthetic gene cluster for bacterial polyynes (named protegenins). In this study, the isolation, structure elucidation, and biological characterization of protegenins A–D are reported. The structures of protegenins A–D determined by spectroscopic and chemical techniques were octadecanoic acid derivatives possessing an ene–tetrayne, ene–triyne–ene, or ene–triyne moiety. The protegenins exhibited weak to strong antioomycete activity against *Pythium ultimum* OPU774. The deletion of *proA*, a protegenin biosynthetic gene, resulted in the reduction of the antioomycete activity of *P. protegens*. The Gac/Rsm system, a quorum sensing-like system of *Pseudomonas* bacteria, regulated the production of protegenins. The production profile of protegenins was dependent on the culturing conditions, suggesting a control mechanism for protegenin production selectivity. *P. protegens* suppressed the damping-off of cucumber seedlings caused by *P. ultimum*, and this protective effect was reduced in the *proA*-deletion mutant. Altogether, protegenins are a new class of bacterial polyynes which contribute to the antioomycete and plant-protective effects of *P. protegens*.



INTRODUCTION

Polyynes are a group of natural compounds with alternating single and triple C–C bonds, reported especially from plants, basidiomycetes, and insects.[1−3] Meanwhile, some bacteria uniquely produce "bacterial polyynes" containing a conjugated polyyne moiety starting with a terminal alkyne (Figure 1A) and use them as potential chemical weapons against hosts and competitors.[4,5] Because of their structural features, bacterial polyynes are unstable and require special caution throughout experiments. Genomic approaches have identified the biosynthetic gene clusters (BGCs) for several bacterial polyynes (e.g., *cay* BGC for caryoynencin) (Figure 1B).[5−7] The BGCs characteristically contain the genes encoding a fatty acyl-ACP ligase, an ACP (acyl carrier protein), three desaturases/acetylenases, and a thioesterase. Our understanding regarding the chemistry and biology of bacterial polyynes has improved in recent years; however, it has been limited to a few compounds that have been identified and characterized.

Various plant-commensal bacteria have been classified into *Pseudomonas* bacteria, including *Pseudomonas fluorescens/protegens*, which have an exceptional capacity to produce a variety of secondary metabolites.[8] Among them, *P. protegens* strains Pf-5 and CHA0 have been evaluated as model biocontrol agents against plant pathogens, including fungi, oomycetes, bacteria, nematodes, and insects.[9,10] Substantial efforts have been devoted to identifying the secondary metabolites (e.g., 2,4-diacetylphloroglucinol, pyoluteorin, and orfamides) (Figure S1) and extracellular enzymes (e.g., AprA protease) involved in the biocontrol activity of the strains Pf-5 and CHA0.[8−10] However, there are still orphan BGCs for secondary metabolites (including possible polyynes) in the genomes of *P. fluorescens/protegens* strains.[5,9,10] A continuous chemical investigation is required to completely unveil the antimicrobial and biocontrol activities of *P. fluorescens/protegens* strains.

Previously, we reported the isolation and chemical/biological characterization of the new bacterial polyynes, collimonins A–D (Figure S2A), from the fungus-feeding bacterium *Collimonas fungivorans* Ter331.[6] Collimonins (previously called collimomycins)[11] were unique derivatives of polyoxygenated hexadecanoic acid containing an ene–triyne

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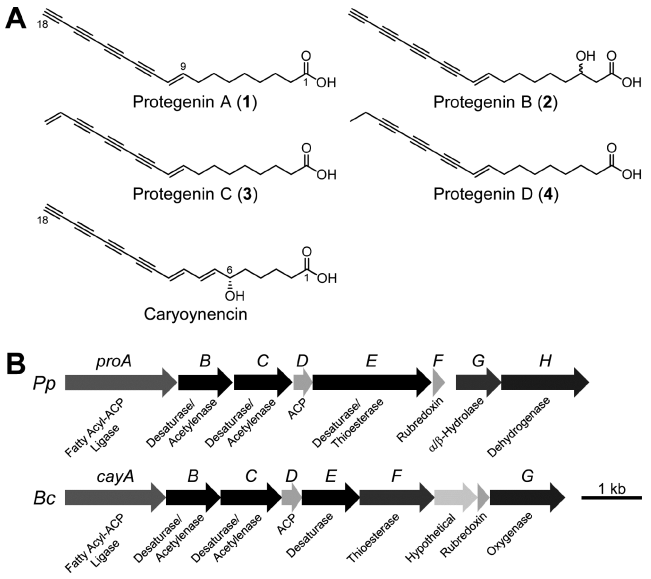

Figure 1. Bacterial polyynes and their BGCs. (A) Structures of protegenins A–D (**1**–**4**) and caryoynencin. (B) *pro* and *cay* BGCs from *P. protegens* Cab57 (*Pp*) and *Burkholderia caryophylli* DSM 50341 (*Bc*), respectively.

moiety, and a *col* gene cluster may be involved in their biosynthesis (Figure S2B). This was the first example reported on the elucidation of the complete structures (including absolute configurations) of bacterial polyynes. In that study, the stable isolation, structural analyses, and derivatization methods were established, enabling the elucidation of the biological activities of collimonins, such as the antifungal activity against *Aspergillus niger*.

P. protegens Cab57 (deposited as MAFF 212077) was identified as a biocontrol strain by Takeuchi et al. in Japan.[12] The results of genome comparison revealed that this Japanese strain shares many biological characteristics with the Pf-5 and CHA0 model strains. In the present study, the genome- and MS-based discovery of protegenins A–D (**1**–**4**) (Figure 1A) from *P. protegens* Cab57 and their chemical/biological characterization are reported. Protegenins are the first natural compounds uniquely harboring an ene–tetrayne, ene–triyne–ene, or ene–triyne moiety. The gene deletion and complementation experiments confirmed that the *pro* gene cluster (Figure 1B) is responsible for the protegenin biosynthesis. The detailed analysis of the biological activity demonstrates that protegenins, mainly protegenin A (**1**), contribute to the antioomycete and biocontrol activities of *P. protegens* strains.

RESULTS

Comprehensive Analysis of BGCs for Bacterial Polyynes. The *P. protegens* strains (including Cab57) whose genomes were sequenced harbor a BGC for possible bacterial polyynes (Figure 1B).[5,12] However, the structures and biological roles of their products (named here "protegenins") have not been elucidated yet. The genes encoding the enzymes/proteins for possible protegenin production were called *proABCDEFGH*. In silico analysis suggested that the *pro* gene cluster starts with a long-chain fatty acyl-ACP ligase (*proA*) containing two desaturases/acetylenases (*proB* and *proC*), an ACP (*proD*), a desaturase/thioesterase (*proE*), a rubredoxin (*proF*), an α/β-hydrolase (*proG*), and a dehydrogenase (*proH*). This gene combination was similar to those of caryoynencin and collimonin biosynthetic genes, except for the *proH* (*cay* BGC: *cayG* encoding a P450 monooxygenase; *col* BGC: no corresponding gene) (Figures 1B and S2B).[5−7] Although the involvement of some additional genes in cepacin biosynthesis was recently suggested,[7] these genes could not be identified in the surrounding region of the *pro* gene cluster. Thus, it was plausible that protegenins are long-chain polyyne fatty acids but different from those of the known polyyne compounds.

Detection and Isolation of Protegenins. Although the secondary metabolites of *P. protegens* strains have been extensively studied, the production of protegenins has not been observed. This implied that the culturing conditions, extraction, and/or analysis methods used were not optimal for the detection of protegenins. The metabolic profiles of Cab57 cultures obtained using various media with special sample preparation treatment were compared. When the common media (King's B and LB) for *P. protegens* were used, *P. protegens* Cab57 actively produced well-known secondary metabolites such as 2,4-diacetylphloroglucinol, pyoluteorin, and orfamide A (Figure S3). The production of possible polyynes **1** and **2** (because they exhibited characteristic UV spectra for polyynes)[5] was observed in the culture extract of WYA plates supplemented with 2 mM *N*-acetyl-D-glucosamine (Figure 2A). These culturing conditions were equal to those

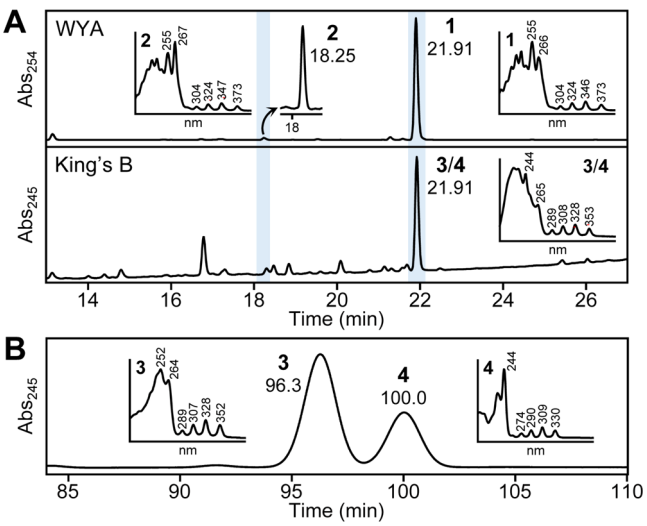

Figure 2. Comparison of the HPLC profiles of the *P. protegens* Cab57 culture extracts prepared using different growth conditions. (A) HPLC analysis of the Cab57 culture extracts prepared from WYA (upper) or King's B (lower) plates. The peaks of protegenins A–D (**1**–**4**) are highlighted in blue. (B) HPLC separation of protegenins C (**3**) and D (**4**). The UV spectra of protegenins are shown as insets.

used for collimonin production by *C. fungivorans* Ter331.[6] After the UV-detector optimization for the polyynes, two additional polyynes (compounds **3** and **4**) were identified in the King's B culture extract by HPLC analysis (Figure 2A). The polyynes **1**, **3**, and **4** exhibited identical chromatographic behavior under the HPLC conditions used. These compounds were named protegenins A–D and isolated from the culture extracts of *P. protegens* Cab57.

The EtOAc extract of 1000 WYA plates, in which *P. protegens* Cab57 was grown for 4 days, was separated on an ODS column by eluting with a mixture of H_2O/MeOH. The

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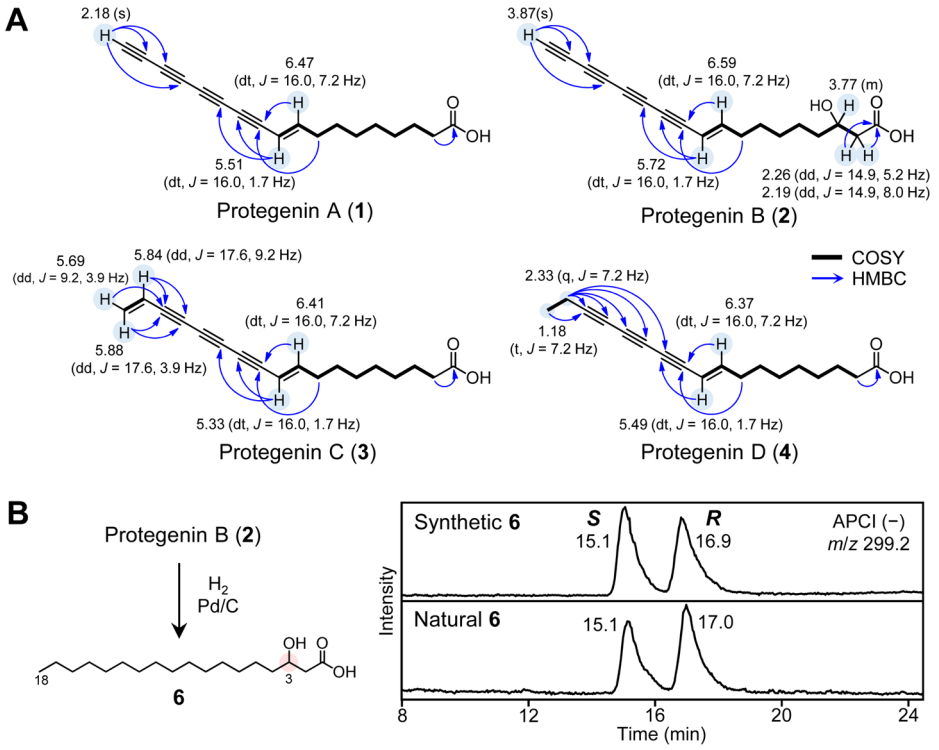

Figure 3. Structure elucidation of protegenins. (A) Key ^{1}H NMR, COSY, and HMBC data of protegenins A–D (**1**–**4**). The difference in the δ_H values of acetylenic protons between **1** and **2** is expected to be due to either the difference in solvents, the effect of residual water and trifluoroacetic acid in the NMR sample of **2**, or both. (B) Preparation of **6** (left) and chiral LC/MS analysis of its absolute configuration (right). SIM detection was performed at *m*/*z* 299.2 (APCI, negative).

fractions eluted with 60, 80, and 100% MeOH showed antioomycete activity against *Pythium ultimum* OPU774 (Figure S4A). Protegenins A (**1**) and B (**2**) were detected in 100% MeOH as the HPLC peaks showed the characteristic UV spectrum for polyynes, although 2,4-diacetylphloroglucinol and pyoluteorin were eluted in 60 and 80% MeOH fractions. The complete evaporation of the fraction containing protegenins frequently caused severe polymerization into insoluble matters and loss of the antioomycete activity. By avoiding complete evaporation and exposure to oxygen with temperatures higher than the ambient temperature and light, compounds **1** (20 mg) and **2** (0.8 mg) were isolated by one-step reversed-phase HPLC separation from the fraction eluted.

The EtOAc extract of 1500 King's B plates, in which *P. protegens* Cab57 was grown for 4 days, was separated on an ODS column by eluting with a mixture of H_2O/MeOH. The fractions eluted with 60 and 80% MeOH showed antioomycete activity against *P. ultimum*; however, protegenins C (**3**) and D (**4**) were detected in 100% MeOH (only weak antioomycete activity) as one major HPLC peak showing a characteristic UV spectrum for polyynes (Figure S4B). After the HPLC separation of compounds **3** and **4** using an ODS column as a single peak, a long-time HPLC purification using a naphtylethyl column allowed the separation of compounds **3** (0.3 mg) and **4** (0.2 mg) (Figure 2B). Protegenins C (**3**) and D (**4**) were more stable than protegenins A (**1**) and B (**2**) during the isolation experiments, but their production was much lower than that of protegenin A (**1**).

Structure Elucidation of Protegenins A–D. Protegenin A (**1**) was obtained as a white solid with a slightly yellow tinge but occasionally changed instantly to another substance with a brown color. Compound **1** originated only some minor ions in the ESI- and APCI-MS spectra; thus, it was difficult to distinguish its $[M + H]^+/[M - H]^-$ ions from the background ions. Probably compound **1** is too hydrophobic to be effectively ionized using these ionization methods. To solve this, compound **1** was converted to cycloadduct **5** by copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC) with benzyl azide (Figure S5).[5,6] Compound **5** strongly exhibited an $[M + H]^+$ ion at *m*/*z* 400 in the positive ESI-MS. The molecular formula of **5** was determined as $C_{25}H_{25}N_3O_2$ by HRESIMS with *m*/*z* 400.2022 $[M + H]^+$ (calcd for $C_{25}H_{26}N_3O_2^+$, 400.2020). That is, the molecular formula of **1** was $C_{18}H_{18}O_2$. The ^{1}H NMR data (in $CDCl_3$) accounted for 17 nonexchangeable protons, composed of one terminal alkyne proton, two (*E*)-olefinic protons ($^{3}J_{H,H}$ = 16.0 Hz), and seven methylene groups (Figure 3A and Table S1). Analysis of ^{13}C NMR, DEPT, and HMQC spectra accounted for 18 carbon signals, and the presence of a carboxyl group, an (*E*)-olefin, a tetrayne, and seven methylene groups were identified. The characteristic UV spectrum suggested the presence of an ene–tetrayne moiety (Figure 2A).[5] Through the COSY and HMBC correlations, the structure of **1** was obtained as an octadecanoic acid derivative possessing an ene–tetrayne moiety (Figure 3A).

The molecular formula of protegenin B (**2**) was elucidated as $C_{18}H_{18}O_3$ by HRESIMS with *m*/*z* 281.1183 $[M - H]^-$ (calcd for $C_{18}H_{17}O_3^-$, 281.1183). Moreover, the UV spectrum indicated that compound **2** also contains an ene–tetrayne moiety (Figure 2A). These results suggested that compound **2** was a hydroxylated derivative of **1**. The presence of the substructure 3-hydroxyaliphatic acid in **2** was confirmed by ^{1}H NMR and COSY spectra (in DMSO-d_6) (Figure 3A and Table S2). The 1D and 2D NMR data analyses showed that the

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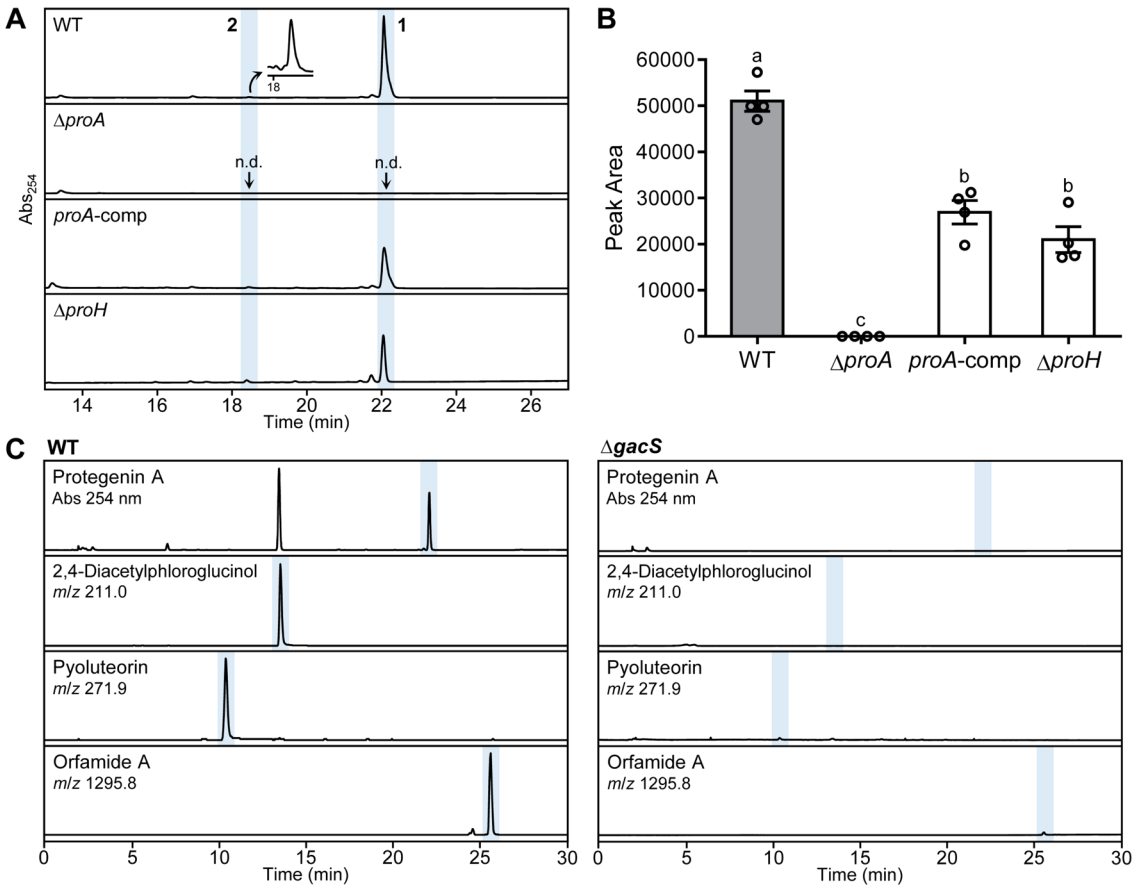

Figure 4. Analysis of the protegenin biosynthesis and its regulation. (A) HPLC comparison of protegenin productivity in the *P. protegens* Cab57 and its mutants. (B) Comparison of protegenin A (**1**) productivity between Cab57 and its mutants. The ordinate represents the HPLC peak area of **1**. Error bars are ±SEM (n = 4). Different letters above the bars indicate significant difference ($p < 0.05$, Tukey's test). (C) HPLC and LC/MS comparison of secondary metabolites produced by Cab57 (left) and Δ*gacS* (right).

planar structure of compound **2** is related to the (*E*)-3-hydroxyoctadeca-9-en-11,13,15,17-tetraynoic acid (Figure 3A). To elucidate its absolute configuration, compound **2** was converted to **6** by hydrogenation with Pd/C under H_2 atmosphere, and its chiral-LC retention time was compared with that of the synthetic 3-hydroxyoctadecanoic acid (Figure 3B). As a result, compound **6** was determined to be a racemic mixture. Consequently, the structure of protegenin B (**2**) was completely determined.

Protegenin C (**3**) was obtained as an amorphous white powder. The UV spectrum of compound **3** was different from those of protegenins A (**1**) and B (**2**) (Figure 2B), indicating that it possesses a different ene−yne substructure. Compound **3** did not react with benzyl azide under CuAAC conditions, suggesting the absence of a terminal alkyne in this compound. The molecular formula of compound **3** was determined as $C_{18}H_{20}O_2$ by HRESIMS with m/z 269.1537 $[M + H]^+$ (calcd for $C_{18}H_{21}O_2^+$, 269.1536). The ^{1}H NMR data (in $CDCl_3$) accounted for 19 protons, composed of three vinyl protons, two (*E*)-olefinic protons ($^3J_{H,H}$ = 16.0 Hz), and seven methylene groups (Figure 3A and Table S3). Analysis of ^{13}C NMR and HSQC spectra accounted for 18 carbon signals, and the presence of a carboxyl group, two pairs of olefinic methines, a triyne, and seven methylene groups were identified. The COSY and HMBC correlations shown in Figure 3A indicated that compound **3** is a derivative of **1**, in which the terminal alkyne was hydrogenated to the corresponding alkene.

Protegenin D (**4**) was also purified as an amorphous white powder, and the UV spectrum of compound **4** was similar to those of collimonins (Figure 2B).[6] The CuAAC reaction of compound **4** with benzyl azide did not form any cycloadduct. Therefore, compound **4** probably has an ene−triyne moiety, but it does not start with a terminal alkyne. The molecular formula of **4** was determined as $C_{18}H_{22}O_2$ by HRESIMS with m/z 271.1692 $[M + H]^+$ (calcd for $C_{18}H_{23}O_2^+$, 271.1693). The 1D and 2D NMR data (Figure 3A and Table S4) analyses revealed that compound **4** is the (*E*)-octadeca-9-en-11,13,15-triynoic acid.

Protegenin Biosynthesis and Its Regulation by Quorum Sensing. To evaluate if the *pro* gene cluster is responsible for protegenin production, the deletion mutant of *proA* (Δ*proA*) was obtained by double crossover recombination using the pK18mobsacB vector.[13,14] The deletion of *proA* resulted in the protegenin production loss (Figure 4A and B). The complementation of the *proA* gene into Δ*proA* (*proA*-comp) rescued the protegenin production in the created mutant.[15] The *pro* gene cluster turned out to be responsible for the biosynthesis of protegenins in *P. protegens* Cab57.

Because *proH* seemed to be characteristic in the protegenin BGC, its deletion mutant was created, and the culture extract was analyzed. The protegenin A (**1**) production of Δ*proH* on the WYA plates was significantly reduced compared with that observed for wild-type Cab57 (Figure 4A and B). Δ*proH* produced protegenins C (**3**) and D (**4**) on King's B plates, but

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their production was also greatly reduced. Furthermore, apparent accumulation of the possible intermediate and shunt product was not observed, indicating that *proH* is not essential but important for protegenin production.

The production of secondary metabolites in *P. fluorescens/protegens* strains is regulated by the Gac/Rsm system, a quorum sensing (QS)-like system of *Pseudomonas* bacteria.[16] This cascade is initiated by the GacS/GacA two-component system. The GacS sensor kinase is known to be phosphorylated when an unidentified signal is received and activates the cognate GacA response regulator. GacA in turn triggers the expression of small regulatory RNA molecules, which titrate translational repressors and relieve the translational inhibition of target mRNAs. The QS system participation in the regulation of the production of protegenins was also evaluated. In contrast to Cab57, Δ*gacS* (a *gacS*-deletion mutant of Cab57) produced only a trace amount of protegenin A (**1**), and also 2,4-diacetylphloroglucinol, pyoluteorin, and orfamide A on the WYA plates (Figure 4C). The time-course experiments revealed that the dynamics of protegenin A (**1**) was similar to those of pyoluteorin (Figure S6). Consequently, these results indicated that the production of protegenins is under the Gac/Rsm system control.

Possible Control Mechanism for Protegenin Production Selectivity. In this study, the evaluation of the regulation mechanism involved in protegenin production selectivity is important. According to previous studies,[17−19] the terminal alkynes of protegenins A (**1**) and B (**2**) may be constructed through the desaturase/acetylenase ProB. Thus, the protegenin production shift (A/B → C/D) was expected to be derived from the inhibition of ProB activity. If this is the case, growing Δ*proB* on a WYA plate should also produce protegenins C (**3**) and D (**4**). To verify this, Δ*proB* was obtained, and its metabolite profile was analyzed. However, as a result, Δ*proB* did not produce protegenins C (**3**) and D (**4**) or protegenins A (**1**) and B (**2**) (Figure S7A). Hence, this production shift was not derived from the ProB enzyme disability.

Certain metabolite(s) could affect the Pro enzyme (including ProB) activity. To verify this, we prepared the EtOAc extract from the King's B media of Cab57 and examined whether it affects the production levels and profiles of protegenins. As a result, the extract inhibited the production of protegenin A (**1**) by *P. protegens* Cab57 on WYA plates (Figure S7B). Furthermore, Δ*proA* strongly inhibited the accumulation of protegenin A (**1**), and this inhibitory effect was reduced in the Δ*gacS* extract. However, while the EtOAc extracts of *P. protegenins* strains (King's B media) exhibited the inhibitory effect on the protegenin A (**1**) production, the production shift from protegenins A/B to C/D was not observed in these Cab57 cultures. Collectively, certain EtOAc-soluble metabolite(s) could affect the activity or expression of Pro enzymes/proteins and an undetermined mechanism might be involved in changing the profile of protegenins.

Biological Evaluation of Protegenins. To investigate the biological activities of protegenins, antimicrobial assays of purified protegenins and confrontation assays of the wild-type Cab57, Δ*proA*, and Δ*gacS* were performed. Protegenin A (**1**) showed a growth inhibition against *P. ultimum* (strong, IC_{50} = 24.5 nmol/disc) and *Fusarium oxysporum* (moderate, IC_{50} = 86.0 nmol/disc) but not against *Aspergillus niger* (Figures 5A and S8A). The antimicrobial activities of protegenin B (**2**) were similar to those of protegenin A (**1**) (*P. ultimum*, IC_{50} = 25.4 nmol/disc; *F. oxysporum*, IC_{50} = 87.5 nmol/disc).

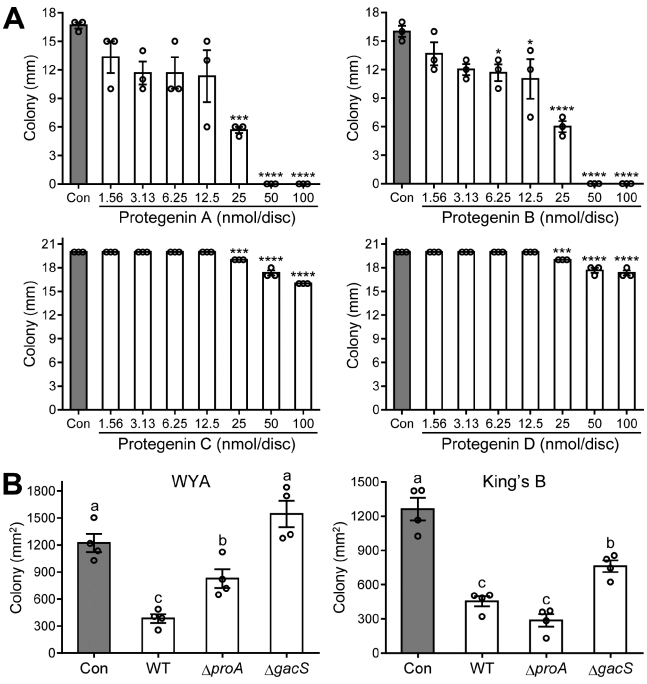

Figure 5. Evaluation of antioomycete activities of protegenins against *P. ultimum* OPU774. (A) Antioomycete activities of protegenins A−D (**1**−**4**). The activities were evaluated by disc diffusion assay, and the colony diameter (mm) was measured. Error bars are ± SEM (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 versus control (Dunnett's test). (B) Antioomycete activities of the *P. protegens* Cab57 and its mutants on WYA (left) and King's B (right) plates. The activities were evaluated by confrontation assay against *P. ultimum*, and the colony area (mm^2) was measured. Error bars are ±SEM (n = 4). Different letters above the bars indicate significant difference (p < 0.05, Tukey's test).

Conversely, among the typical metabolites regulated by the Gac/Rsm system, only pyoluteorin exhibited a strong antioomycete activity (IC_{50} = 7.36 nmol/disc) (Figure S9A−C). 2,4-Diacetylphloroglucinol was more active against the fungi (Figure S9A), while orfamide A was not so effective against the microbes tested in this study (Figure S9C). Confrontation assays of the *P. protegenins* strains on WYA plates also indicated that protegenin production is highly related to the antioomycete activity against *P. ultimum* and slightly related to the antifungal activities against *F. oxysporum* and *A. niger* (Figures 5B and S8B). Therefore, the production of ene−tetrayne-type protegenins is essential for the strong antioomycete activity of *P. protegens* Cab57.

Protegenins C (**3**) and D (**4**) exhibited only a weak growth inhibition against *P. ultimum* and *A. niger* and no inhibitory activity against *F. oxysporum* (Figures 5A and S10A). The low contribution of compounds **3** and **4** to the antioomycete/antifungal activities was also observed in the confrontation assays on King's B agar. Under the conditions used, Δ*proA* exhibited antioomycete/antifungal activities similar to those of Cab57, while Δ*gacS* displayed a lower inhibition compared with Cab57 (Figures 5B and S10B). Furthermore, the antagonism of the tested microbes was still observed in Δ*gacS*, i.e., the antimicrobial activities observed here can be attributed to the metabolites/exoenzymes, which include both the QS-dependent and -independent ones, other than protegenins. Altogether, the protegenins C (**3**) and D (**4**)

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scarcely contribute to the antimicrobial effects of *P. protegens* Cab57.

Suppression of the Cucumber Damping-Off by Protegenins. The contribution of protegenin production to the biocontrol activity of *P. protegens* Cab57 against *P. ultimum* OPU774 was evaluated. For this, a suppression assay of the damping-off of cucumber seedlings was used.[20] The inoculation of *P. ultimum* caused the plant survival rate reduction to 12.8%, and the application of *P. protegens* Cab57 rescued the survival rate to 59.7% (Figure 6A). The plant survival rates of

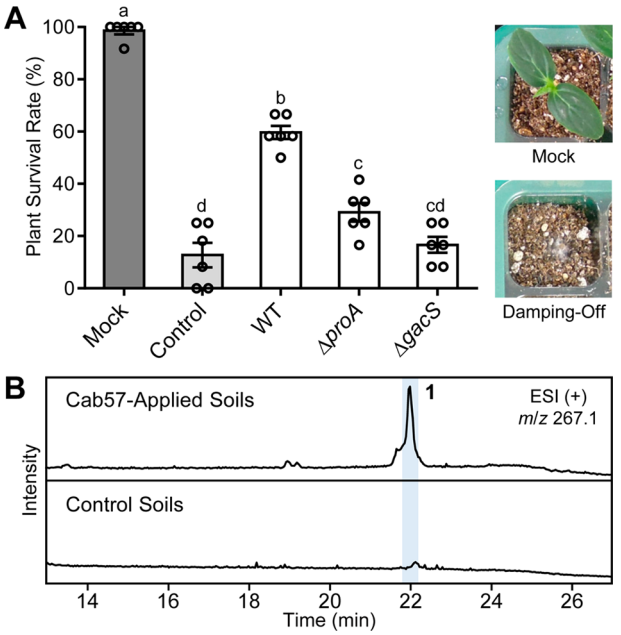

Figure 6. Suppression of the damping-off of cucumber seedlings. (A) Evaluation of the damping-off suppression abilities of *P. protegens* Cab57 and its mutants. Twelve seedlings were used for the trial. Error bars are ±SEM (n = 6). Different letters above the bars indicate significant difference (p < 0.05, Tukey's test). Representative assay images are also shown (right). (B) LC/MS detection of protegenin A (**1**) from the Cab57-applied soils (upper) and control soils (lower). The soil extracts (from 12 wells, ca. 480 g soil) were subjected to preparative HPLC to obtain the fractions in which protegenin A (**1**) was possibly eluted. The detection of protegenin A (**1**) was achieved using SIM mode (m/z 267.1).

Δ*proA* and Δ*gacS* applications were 29.1 and 16.6%, respectively. These results indicated that, although the biocontrol effect of *P. protegens* Cab57 is mainly exerted by the combination of *gacS*-regulated metabolites/exoenzymes, protegenins also exhibit a significant contribution. Furthermore, protegenin A (**1**) from the Cab57-applied soil samples was detected by LC/MS (Figure 6B). Taken together, our data indicated that protegenins play important roles in the biocontrol activity of *P. protegens* Cab57.

Detection of Protegenin A from *P. fluorescens/protegens*. Finally, the conserved production of protegenins in *Pseudomonas* bacteria was evaluated. As a result, the production of protegenin A (**1**) on WYA plates was confirmed in all 11 strains examined, though the production levels of protegenin A (**1**) in *P. fluorescens* strains were low compared to *P. protegens* MAFF 550081 (Figure S11). The *P. fluorescens* strains examined did not produce any protegenins on King's B plates. The production of protegenin A (**1**) may be widely conserved in *P. fluorescens/protegens* strains.

DISCUSSION

Various secondary metabolites have been identified from the *P. fluorescens/protegens* group, and the compounds exhibited antimicrobial, antinematode, and/or insecticidal activities.[8−10] In these studies, rich media (such as King's B media) were generally used, even though the nutrient conditions were different from those of the plant rhizosphere. In this study, it was revealed that *P. protegens* Cab57 produces a high level of protegenin A (**1**) and a moderate level of protegenin B (**2**) on poor WYA media and low levels of protegenins C (**3**) and D (**4**) on rich King's B media. The contribution of protegenins A (**1**) and B (**2**) to the antioomycete and plant-protective properties was demonstrated by disc diffusion and confrontation assays. The possible *pro* BGCs were observed in several data sets of the *Pseudomonas* genome, and the production of protegenin A (**1**) was confirmed in some strains of *P. fluorescens/protegens*. Therefore, protegenins, especially protegenin A (**1**), are key metabolites that have been overlooked for a long time in the biocontrol effects of *Pseudomonas* bacteria.

Although a compound identical to protegenin A (**1**) was previously identified as a possible biosynthetic intermediate of caryoynencin from a *cayG*-deficient mutant of *Burkholderia caryophylli*,[5] the present study is the first to identify it as a natural product and isolated it without chemical derivatization. It is plausible that protegenin A (**1**) or the related compounds are commonly produced by bacterial polyyne BGCs as biosynthetic intermediates for subsequent modifications (e.g., oxidation). This is the second report of a natural compound with an ene−tetrayne assembly, after caryoynencin. Although protegenin B (**2**) is a 3-hydroxylated derivative of compound **1**, the involvement of a further enzyme in protegenin B (**2**) biosynthesis seems to be excluded; rather, compound **2** might be synthesized from (±)-3-hydorxyoctadecanoic acid instead of octadecanoic acid. To the best of our knowledge, the terminal ene−tetrayne−ene moiety identified in protegenin C (**3**) was the first-ever discovered in natural products. The derivatives with terminal C−C bonds ranging from single to triple bonds seem to be extremely rare in a single species.

Additional genes were suggested to be involved in the biosynthesis of cepacins and collimonins, though their genetic and biochemical characterizations have not been performed.[7] Meanwhile, *proABCDEFGH* genes are enough to construct the protegenin structures. The functions of Pro enzymes/proteins were expected to be identical to those of the enzymes of *cay* gene clusters as follows:[5] after the coupling of octadecanoic acid with ACP (ProD) by the fatty acyl-ACP ligase ProA, the desaturase/acetylenase ProB/C/E construct the ene−tetrayne moiety; then, thioester linkage between protegenin and ACP is hydrolyzed by thioesterase ProE and probably α/β-hydrolase ProF, yielding protegenin A (**1**) (Figure S12). In silico analysis indicated that ProH belongs to the family of dihydrolipoyl dehydrogenases, which is usually involved in the recycling of NAD^+/NADH.[21] By considering the significant reduction of protegenin A (**1**) in Δ*proH*, the reaction using ProH might be coupled to the biosynthetic step(s) by other Pro enzymes. Furthermore, *proF* may encode a rubredoxin, which belongs to a class of iron-containing proteins that play an important role in the reduction of superoxide in some anaerobic bacteria and also act as electron carriers in many biochemical processes.[22] However, their involvement in the secondary metabolism has not been well investigated to date. How the substructure ene−

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ACS Chem. Biol. XXXX, XXX, XXX−XXX

tetrayne is biochemically assembled by ProB/C/E also remains unrevealed.

Like 2,4-diacetylphloroglucinol and others, protegenin production was found to be regulated by the Gac/Rsm system in *P. protegens* Cab57. The dynamics of protegenin A (**1**) accumulated in the Cab57 cultures were typical for the QS-controlled secondary metabolites.[23] The natural ligands that activate the Gac/Rsm system have not been identified so far.[16] Meanwhile, the QS signals that are received by such two-component systems have been identified in several bacterial species.[24−28] Because protegenin production is tightly regulated by the QS system, it may be a good indicator for the activity-based ligand-discovery of GacS.

The biological evaluation of protegenins A (**1**) and B (**2**) clearly indicated that they are essential for strong antioomycete activity of *P. protegens* Cab57. The antioomycete activities of protegenins A (**1**) and B (**2**) were higher than those of 4-diacetylphloroglucinol and orfamide A and comparable to that of pyoluteorin. Furthermore, protegenin A (**1**) was detected in the cucumber seedling rhizosphere, in which the *P. protegens* Cab57 actually functioned. Conversely, the biological importance of protegenins C (**3**) and D (**4**) has not been determined. Considering the existence of a possible mechanism that causes metabolic shifts, it is expected that these compounds may have some biological roles. Moreover, the possibility that these compounds are being actively metabolized from protegenin A (**1**) because they are unnecessary cannot be denied. As in the case of collimonins,[6] the terminal alkyne was important for the antioomycete/antifungal activities of protegenins.

Recently, Mullins et al. described cepacin A as a plant-protective metabolite of *Burkholderia ambifaria*.[7] Additionally, de Boer et al. investigated *Collimonas* bacteria, which include collimonin-producing strains, as biocontrol agents against plant pathogens.[29,30] Thus, bacterial polyynes may be the common chemical weapons/defenders of Proteobacteria against competitive eukaryotes. If so, polyyne production and the BGCs may be important for future studies on the selection of plant-protective bacteria.

In conclusion, protegenins A−D (**1**−**4**) were identified from *P. protegens* Cab57 (see the Supporting Information, SI Note), and protegenin A (**1**) was a key metabolite for exhibiting antioomycete and biocontrol effects of *P. protegens* Cab57. Furthermore, insights into why this compound has been overlooked for so long were discussed in this study. The culturing conditions affected the quality and quantity of protegenin production. However, there are still several topics to be elucidated. For example, it is not clear how polyynes exhibit antimicrobial properties and which mechanism is involved for the shift in protegenin production to occur. The discovery of protegenins and identification of their BGC certainly contribute to the future development of research on *Pseudomonas* bacteria as plant-protective agents.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.1c00276.

Supporting figures, tables, methods, note, and NMR data (PDF)

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Notes

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ACS Chem. Biol. XXXX, XXX, XXX−XXX