High-level production of violacein by the newly isolated *Duganella violaceinigra* str. NI28 and its impact on *Staphylococcus aureus*

Seong Yeol Choi1, Sooyeon Kim1, Sungsoo Lyuck1, Seung Bum Kim2 & Robert J. Mitchell1

A violacein-producing bacterial strain was isolated and identified as a relative of *Duganella violaceinigra* YIM 31327 based upon phylogenetic analyses using the 16S rRNA, *gyrB* and *vioA* gene sequences and a fatty acid methyl ester (FAME) analysis. This new strain was designated *D. violaceinigra* str. NI28. Although these two strains appear related based upon these analyses, the new isolate was phenotypically different from the type strain as it grew 25% faster on nutrient media and produced 45-fold more violacein. When compared with several other violacein producing strains, including *Janthinobacterium lividum*, *D. violaceinigra* str. NI28 was the best violacein producer. For instance, the crude violacein yield with *D. violaceinigra* str. NI28 was 6.0 mg/OD at 24 hours, a value that was more than two-fold higher than all the other strains. Finally, the antibacterial activity of *D. violaceinigra* str. NI28 crude violacein was assayed using several multidrug resistant *Staphylococcus aureus*. Addition of 30 μM crude violacein led to a 96% loss in the initial *S. aureus* population while the minimum inhibitory concentration was 1.8 μM. Consequently, this novel isolate represents a phenotypic variant of *D. violaceinigra* capable of producing much greater quantities of crude violacein, an antibiotic effective against multidrug resistant *S. aureus*.

Violacein is produced by numerous bacterial strains spanning various genera, including *Chromobacter*14,15, *Pseudoalteromonas*16,17, *Janthinobacterium*18,19 and *Duganella*20. Moreover, violacein producing bacterial strains have been isolated from diverse environmental locales. *Janthinobacterium*18,21,22,

---

1School of Life Sciences, Department of Biological Sciences, Ulsan National Institute of Science and Technology, Ulsan, S. Korea. 2School of Life Sciences, Chungnam National University, Daejeon, S. Korea. Correspondence and requests for materials should be addressed to S.B.K. (email: sbk01@cnu.ac.kr) or R.J.M. (email: esgott@unist.ac.kr)
for instance, was isolated from a glacier, *Chromobacteria* from a river, *Duganella* from agricultural and forest soils, and *Collimonas* from the sea. In this study, we report on the isolation and initial characterization of a natural soil isolate, *Duganella violaceinigra* str. NI28, obtained from a temperate forest soil sample taken near Ulsan, South Korea. This strain produces violacein at much higher rate and levels than the type strain *Duganella violaceinigra* YIM 31327.

**Results**

**Isolation and Identification of *D. violaceinigra* str. NI28.** Various natural bacterial isolates from a forest soil sample were grown on nutrient agar (NA) and a single colony that had a dark purple hue, suggesting that this strain produced the bisindole violacein, was selected for further characterization. Production of crude violacein by this strain was demonstrated using HPLC analysis (Fig. 1). When the crude violacein extracted from our new isolate was compared with a commercial preparation of crude violacein from *Janthinobacterium lividum* (Sigma-Aldrich, USA), they were basically indistinguishable from each other.

Identification of this strain was next performed using the sequences obtained from three gene loci (16S rRNA, *gyrB* and *vioA*), with each confirming that it was closely related to *Duganella violaceinigra* YIM 31327 (Figs 2 and 3). The level of sequence similarities between NI28 and *D. violaceinigra* YIM 31327 were 98.8% for 16S rRNA gene, 95.9% for *gyrB*, and 88.8% for *vioA*, thus indicating that NI28 is close, but not identical to *Duganella violaceinigra* YIM 31327. This relationship was further supported by a fatty acid methyl ester (FAME) analysis and comparison of this strain and the type strain, *Duganella violaceinigra* YIM 31327, which showed only minor differences between the two strains (Table 1). Based upon the phylogenetic and FAME analyses, this strain was designated *D. violaceinigra* str. NI28 and deposited within the Korea Agricultural Culture Collection (www.genebank.go.kr) (KACC 91951P).

*D. violaceinigra* str. NI28 is Phenotypically Variant from *D. violaceinigra* YIM 31327. Although both *D. violaceinigra* str. NI28 and *D. violaceinigra* YIM 31327 were isolated from forest soils and are related genetically based upon the above results, differences were readily apparent between them. For instance, *D. violaceinigra* str. NI28 was found to have trypsin activity using the API ZYM Kit (bioMerieux, France) while *D. violaceinigra* YIM 31327 was negative for this protease. Furthermore, *D. violaceinigra* str. NI28 grew remarkably well and produced a significant amount of violacein when cultured on NA (Fig. 4A). This was in stark contrast with *D. violaceinigra* YIM 31327, which grew slower and was much less proficient at producing violacein. Figure 4A shows that colonies of *D. violaceinigra* str. NI28 were larger and already producing violacein after 24 hours of growth while those of *D. violaceinigra* YIM 31327 were smaller and still pasty in color. Only after 60 hours did the *D. violaceinigra* YIM 31327 colonies achieve a similar size and hue as 24 hour-old the *D. violaceinigra* str. NI28 colonies (Fig. 4A).

Both of these findings were further evidenced in liquid cultures. As with the colonies, *D. violaceinigra* YIM 31327 was slower to grow in NB liquid media (Fig. 4B). The doubling time for *D. violaceinigra* YIM 31327 was 71 minutes based upon the logarithmic growth stage between 3 and 7 hours. In contrast, *D. violaceinigra* str. NI28 doubled every 53.7 minutes, a value that is 25% faster than *D. violaceinigra* YIM 31327. Not only did the newly isolated *D. violaceinigra* str. NI28 grow faster but the optical density after 24 hours was significantly higher (2.4-fold), as shown in Figs 4B and 5A. We also noticed that *D. violaceinigra* str. NI28 tended to form flocs when grown in liquid cultures while *D. violaceinigra* YIM 31327...
cells generally remained suspended (data not shown). It is not clear what benefit *D. violaceinigra* str. NI28 gains from forming these aggregates, but it was reported that flocs may help protect against predation 28.

**D. violaceinigra** str. NI28 is a Prolific Violacein Producer. All three of these growth characteristics (rate, yield and floc formation) may contribute to the higher violacein production seen with this new strain. As shown in Fig. 5B, *D. violaceinigra* str. NI28 was a much more proficient violacein producer than *D. violaceinigra* YIM 31327 under these conditions, generating as much as 18.9 mg/L of crude violacein after 24 hours. Further incubation for an additional 24 hours did not improve upon this yield with 18.6 mg/L from the two day culture. *D. violaceinigra* YIM 31327, on the other hand, produced only 0.42 mg/L during the first 24 hours, and this dropped to 0.38 mg/L after 48 hours. Considering both time points, the new strain produced approximately 45-fold more violacein than the type strain.

Although *D. violaceinigra* str. NI28 is clearly better than *D. violaceinigra* YIM 31327 at producing violacein, the literature also lists several other violacein producing strains, including *Chromobacter* species and *Janthinobacterium lividum* (Table 2). Consequently, we cultivated each of these strains in NB media and determined both the optical density (OD) and crude violacein yields after 24 and 48 hours (Fig. 5). The 24 and 48 hour *D. violaceinigra* str. NI28 optical densities were similar with most of the other violacein producing strains but both *C. piscinae* and *C. subtsugae* grew to higher ODs (Fig. 5A). However, of all the strains tested, the new isolate gave the highest crude violacein yields, particularly when grown for 24 hours (Fig. 5B). This figure also shows that it was one of only two strains, other than *D. violaceinigra* YIM 31327, which reached a maximum violacein concentration in 24 hours. This rapid and high level production by *D. violaceinigra* str. NI28 in NB clearly sets this strain apart from the other strains, particularly its closest relative, *D. violaceinigra* YIM 31327, which was the poorest violacein producer tested in this study.

When the quantity of violacein produced by all six strains was normalized using the OD, i.e., the specific productivity, the newly isolated *D. violaceinigra* str. NI28 gave the best results (Fig. 5C). This was particularly true for the 24 hour sample where the specific productivity was 6.0 mg crude violacein/OD, a value that was more than twice that obtained for the next closest strain, *C. piscinae* (2.6 mg crude violacein/OD). In comparison, the specific productivity from the type strain, *D. violaceinigra* YIM 31327, was the lowest of all the strains tested (0.33 and 0.34 mg/OD at 24 and 48 hours, respectively). As such, *D. violaceinigra* str. NI28's high level production of violacein implies that this strain contains more violacein per cell than the other strains, a finding which may contribute to making downstream purification of violacein easier.

**Duganella violaceinigra** str. NI28 Violacein is Bacteriocidal Towards *Staphylococcus aureus*. Several recent articles highlighted the activity of violacein against the human pathogen *Staphylococcus aureus* and showed that a crude violacein concentration of 17 μM was capable of inhibiting growth of this strain5,6. Similarly, we tested the activity of *D. violaceinigra* str. NI28 violacin extracts with two *S. aureus* isolates that were obtained from culture repositories (ATCC 25923 and the CCARM strains) and from a patient infected with a multidrug-resistant *S. aureus* (Clinical) (Table 3). As shown in Fig. 6, the...
crude violacein prepared from *D. violaceinigra* str. NI28 was capable of blocking growth of both *S. aureus* strains when added to a final concentration of 15 μM, or approximately 5 mg/L. Moreover, this concentration led to a 70% loss in viability after 24 hours when compared with the initial *S. aureus* colony-forming unit (CFU) values while higher concentrations led to comparably greater losses in viability. For instance, 60 μM violacein reduced both *S. aureus* populations by an average of 99.5% when compared with the initial CFU numbers.

We next tested the efficacy of the *D. violaceinigra* str. NI28 crude violacein against a selection of bacterial strains, including several multidrug resistant *S. aureus* strains that have diverse antibacterial resistance footprints according to the EUCAST breakpoints (Table 3). Table 4 lists the minimal inhibitory concentrations (MICs) of violacein found for each of the strains tested in this study. Growth of all *S. aureus* cultures was inhibited by a violacein concentration of 15 μM (Table 4) regardless of the antibiotic resistant nature of the pathogen. Furthermore, MIC tests performed using *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* found both of these pathogens were resistant to violacein. Parallel tests performed with a commercially available crude violacein from *J. lividum* gave identical results (Table 4), demonstrating that the bioactivity of the crude violacein from *D. violaceinigra* str. NI28 against these pathogens is equivalent to that of the commercially available *J. lividum* crude violacein.

**Discussion**

As a bacterial secondary metabolite, violacein has garnered renewed interest lately. A vibrant violet-hued bisindole derivative of tryptophan, violacein has been shown to have multiple biological activities, including as an anticancer and antibiotic, particularly against *S. aureus*. It is this multifaceted character of violacein, and the fact that resistance to this antibiotic has not yet been described, that makes it an attractive product for bioprocesses.
Table 1. Fatty acid methyl ester (FAME) comparison between *D. violaceinigra* str. NI28 and *D. violaceinigra* YIM 31327. *Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. The fatty acids included in Feature 3 consist of C<sub>16:1</sub>ω7c and/or iso-C<sub>15:0</sub> 2-OH.

| Fatty Acid (%) | NI28 | YIM 31327 |
|----------------|------|-----------|
| **Straight Chain** |      |           |
| C<sub>10:0</sub> | -    | 0.4       |
| C<sub>12:0</sub> | 5.05 | 6.54      |
| C<sub>14:0</sub> | 2.99 | 1.04      |
| C<sub>16:0</sub> | 33.28| 31.42     |
| **Branched**    |      |           |
| Iso-C<sub>15:0</sub> | 0.12 | -         |
| **Unsaturated** |      |           |
| C<sub>14:1</sub> | -    | 0.26      |
| C<sub>18:1</sub> | 3.8  | 3.69      |
| **Hydroxy**     |      |           |
| C<sub>10:0</sub> | -    | 3.82      |
| C<sub>12:0</sub> | 2.66 | 1.74      |
| C<sub>14:0</sub> | 3.41 | 3.6       |
| **Sum in Feature 3** | 48.68 | 47.49 |

Figure 4. *D. violaceinigra* str. NI28 grows faster than *D. violaceinigra* YIM 31327. (A) Image of each strain grown on NB agar plates for 24 and 60 hours. The upper and bottom regions are from the same plate. Note the more rapid colony development and violacein production by *D. violaceinigra* str. NI28. (B) Growth of both strains in NB media confirming that *D. violaceinigra* str. NI28 grows faster. The data for each strain was obtained from three independent cultures.
This study reports on the isolation and characterization of a novel high-level violacein-producing bacterial strain. This strain was identified as *Duganella* using both FAME and phylogenetic analyses and found to be similar based upon these tests with the type strain *D. violaceinigra* YIM 31327, which also produces violacein. However, when these two strains were compared with each other, we found that they were quite distinct from each other. For instance, the new strain grew faster and produced significantly more violacein (45-fold) than *D. violaceinigra* YIM 31327. Moreover, this new isolate also has a tendency to form flocs, another attribute that has been noted as being more economical when harvesting microbial biomass. When this strain, *D. violaceinigra* str. NI28, was compared with several other well-known violacein producers, it grew faster, produced more violacein and had the best yields per biomass. Both

![Figure 5. Growth and violacein production by several different bacterial strains.](A) Optical density values for each culture at 0, 24 and 48 hours. (B) Violacein concentration within each culture at 24 and 48 hours, showing the rapid and high level violacein generation by *D. violaceinigra* str. NI28 as compared with the other strains. (C) The relative concentration of violacein based upon the culture density, illustrating that *D. violaceinigra* str. NI28 gave the greatest yields.
the rapid, high-level production by *D. violaceinigra* str. NI28 and its higher concentration within the cells are clearly advantageous and desirable from an economic viewpoint since the first would reduce the time needed for production of violacein while the second minimizes the biomass needed for its subsequent extraction and purification. These characteristics of *D. violaceinigra* str. NI28 make it a potentially attractive and alternative strain for the commercial production of violacein.

When the violacein from *D. violaceinigra* str. NI28 cultures was purified and analyzed, we found that it was similar with a commercially available preparation from *J. lividum*, as well as that extracted from the various strains employed in this study. As noted above, this bisindole is quite effective against *S. aureus* and, as such, this pathogen was selected for this study due to its overwhelming presence within nosocomial infections. Previous studies reported the minimum inhibitory concentration (MIC) of violacein was 5.7 μg/ml, or approximately 17 μM, with *S. aureus*. The preparation from *D. violaceinigra* str. NI28 was equally potent with an MIC of 5 μg/ml, or approximately 15 μM. This concentration was not only inhibitory but also bactericidal as it led to a 69% reduction in the viability with the ATCC strain and a 78% decrease with the clinical isolate. Both of these findings clearly illustrate that the crude violacein purified from cultures of *D. violaceinigra* str. NI28 is capable of not only inhibiting *S. aureus* growth but also killing this pathogen.

Moreover, the multidrug resistant nature of the *S. aureus* strains did not contribute to their survival when exposed to violacein. Tests using a total of five *S. aureus* strains with different antibiotic resistance

| Strains                          | Description       | Referencea |
|---------------------------------|-------------------|------------|
| *Duganella violaceinigra* str. NI28 | Violacein producer | This study |
| *Duganella violaceinigra*       | Violacein producer | YIM 31327  |
| *Janthinobacterium lividum*     | Violacein producer | ATCC 12473 |
| *Chromobacterium pseudoviolaceum* | Violacein producer | ATCC 7461  |
| *Chromobacterium piscinae*      | Violacein producer | LMG 3947   |
| *Chromobacterium subtsugae*     | Violacein producer | DSM 17043  |
| *Staphylococcus aureus*         | Type strain       | ATCC 25923 |
| *Staphylococcus aureus*         | Multidrug Resistant (MRSA) | Clinical Isolate |
| *Staphylococcus aureus*         | —                 | CCARM 0201 |
| *Staphylococcus aureus*         | Multidrug Resistant (MRSA) | CCARM 3090 |
| *Staphylococcus aureus*         | Multidrug Resistant (MRSA) | CCARM 3840 |
| *Klebsiella pneumoniae*         | Human pathogen    | ATCC 13883 |
| *Pseudomonas aeruginosa* PAO1   | Human pathogen    |            |

Table 2. Strains used in this study. aYIM (Yunnan Institute of Microorganisms); ATCC (American Type Culture Collection); LMG (Belgian Coordinated Collection of Microorganisms); DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH); CCARM (Culture Collection of Antimicrobial Resistant Microbes).

Table 3. Antibiotic susceptibility results. Tests were performed using disc-diffusion plates with the results listed representing the range of diameters (mm) measured for the inhibitory zones from three independent tests. Strains showing resistance are listed in bold. aThe antibiotics tested were vancomycin (Van), rifampin (Rif), ciprofloxacin (Cip), clindamycin (Clin), oxacillin (Oxa), erythromycin (Ery), gentamycin (Gent) and tobramycin (Tob). bRange of diameters (mm) seen for the inhibitory zones. (S) – susceptible; (R) – resistant. cNot done.
footprints, showing that several were multidrug resistant (Table 3), found that each was equally sensitive to the crude violacein from \textit{D. violaceinigra} str. NI28, with MICs of 15 \( \mu \)M (Table 4). These values are basically identical with that reported by Subramaniam et al. (2014), where they found the MIC for \textit{S. aureus} to be 5.7 \( \mu \)g/ml (17 \( \mu \)M). When we performed further MIC tests in a modified M9 media, the effective concentration dropped to only 0.9 or 1.8 \( \mu \)M, a value that is significantly lower than those found with the Mueller-Hinton media and is likely attributable to the nutrient poor nature of the medium used. Similar impacts of the media richness and bacterial responses has been documented previously 33, where the authors found the use of a nutrient poor medium made the bacteria more susceptible to chemicals. Consequently, the use of a minimal media within this study increased the susceptibility of the bacteria to violacein and does not indicate a greater activity against these pathogens. Further evidence of this is seen in the similar MICs obtained with the commercially available \textit{J. lividum} crude violacein, which was also significantly lower (Table 4). The HPLC analyses of these two crude violacein preparations, shown in Fig. 1, found them to be similar. The second peak was not identified but is presumed to be deoxyvio -

\textit{lae}

lacein since this compound is also known to be generated alongside violacein. Although it may also play a role and contribute to \textit{S. aureus} inhibition, a recent study found that deoxyviolacein was less effective against this pathogen than violacein 34.

Two additional pathogens were also tested, \textit{P. aeruginosa} PAO1 and \textit{K. pneumoniae}, but neither of these was sensitive to either of the violacein samples (Table 4). This was not unexpected as several articles previously highlighted the tendency for Gram-positive strains to be more sensitive than Gram-negative strains 6,25. These findings suggest that the presence of the outer membrane within these bacteria provides protection against violacein and its activity and is an area for further evaluation in a later study.

Table 4. Minimum inhibitory concentration results. The concentration listed is that which showed no growth after 24 hours in the resazurin microplate tests using the modified M9 media. The highest crude violacein concentration tested was 60 \( \mu \)M.

![Figure 6. Inhibition and killing of \textit{Staphylococcus aureus} by \textit{D. violaceinigra} str. NI28 violacein. Two \textit{S. aureus} strains were tested: ATCC 25923 and a clinical isolate. The initial CFU was determined just prior to addition of the violacein. The viability of the other samples was determined after a 24 hour exposure to the violacein at the concentrations listed, showing that the addition of 15 \( \mu \)M or greater violacein was bactericidal towards both pathogenic strains. The samples were prepared so that each had the same amount of DMSO (1.3%) added. The concentrations listed are based upon the commercial standard.](image-url)
In conclusion, a violacein-producing strain was isolated from a forest soil sample in Ulsan, South Korea. This strain, *D. violaceinigra* str. NI28, produces violacein at quicker and at higher levels than other comparable strains, particularly the type strain *D. violaceinigra* YIM 31327, when grown under the conditions used in this study. Although the violacein concentrations produced in this study are low, a group recently reported achieving as much as 0.82 g/L with *C. violaceum* by optimizing the conditions for fermentation and the media used35. As *D. violaceinigra* str. NI28 is a new strain and, based upon the violacein production and yields found in this study, is generally equivalent to or better than the other strains tested, expanding its growth and violacein production through similar techniques would likely increase the yields. Moreover, other recent studies have shown the successful expression of the *vioABCDE* operon within other bacterial hosts to produce even higher levels of violacein. The characteristics of *D. violaceinigra* str. NI28 make its *vioABCDE* operon an interesting addition to these studies and, as such, cloning of these genes for their heterologous expression may likewise be beneficial.

**Methods**

**Isolation of *Duganella violaceinigra* str. NI28.** The novel strain characterized in this study, *i.e.*, *Duganella violaceinigra* str. NI28, was isolated using soil obtained from a temperate forest nearby the campus. For this, a 50 g sample of soil was mixed with 500 ml of sterile tap water and shaken at 30 °C for 2 hours. Afterwards, the soil particulates were centrifuged at 500 × g for 5 minutes and the supernatant sampled. Aliquots (100 µl) of this solution were spread out on nutrient agar (NA) (1.6% agar; Difco, USA) plates. A total of forty plates were prepared and these were then incubated at 30 °C for 48 hours. A single colony showing a violet hue on one of the plates was selected for further evaluation.

**Bacterial Strains and Growth.** The bacterial strains used in this study are listed in Table 2. All of the strains were grown from −80 °C freezer stocks that were prepared individually. For the violacein producing strains, the stocks were prepared by adding DMSO (Sigma-Aldrich, USA) to a final concentration of 10%. The *Staphylococcus aureus* strains were stored using glycerol (Sigma-Aldrich, USA) at a final concentration of 25%.

Each violacein producing strain was struck out on NA plates and grown at 30 °C overnight. A single colony was transferred to a sterile 15 ml conical tube (SPL, Korea) containing 3 ml of nutrient broth (Difco, USA). This culture was grown at 30 °C and 250 rpm overnight. To 20 ml of fresh NB medium in a 100 ml baffled flask to increase aeration, 2 ml (10% v/v) of the overnight culture was added and this was again shaken at 30 °C and 250 rpm for 48 hours. At set times during this cultivation (24 and 48 hours), aliquots were taken to determine the culture optical density and violacein concentration as described below. For the growth curves shown in Fig. 4, 100 ml cultures were cultivated within 250 ml flasks. All the other strains were grown using the same protocols except at 37 °C and in TSB media (Difco, USA). Other media was used for the antibiotic resistance characterization and minimum inhibitory concentration determination as described below.

**Identification of *D. violaceinigra* str. NI28 by 16S using phylogenetic markers.** The genus of isolate NI28 was identified using three genes, namely 16S rRNA, gyrB and vioA. The primers for PCR amplification of the genes were 27f (5′-AGA GTT TGA TCM TGG TTC AG-3′) and 1492r (5′-GTC TAC CTT GGT ACG ACT T-3′) for the 16S rRNA gene, Up-1G (5′-YGC GGG CGG YAA GTT CTA-3′) and Up-2G (5′-TCC TCG ACC AGC GCR TGG GT-3′) for *gyrB*, and VPA3 (5′-GCR CAG CTS CAY CCG CAT TGC CAG-3′) and VPA4 (5′-GCG GCC GGC CTC CAT CCA GCC RCA-3′) for *vioA*. The PCR conditions for the *gyrB* gene were: 95 °C for 5 minutes followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 60 s. The final extension was at 72 °C for 15 minutes. The *vioA* gene was amplified in the same manner but with an annealing temperature of 62 °C. The phylogenetic analysis between NI28 and the related taxa including *Duganella* was performed using MEGA 6.0. Neighboring-joining trees were inferred based on Jukes-Cantor evolutionary distances for each gene. The trees were evaluated using bootstrap analysis with 1,000 resampled datasets. The sequences of each gene from strain NI28 were deposited within the GenBank database under the accession numbers (KM087998, KM087999 and KM088000).

**Fatty Acid Methyl Ester Preparation and Analysis.** Colonies of *D. violaceinigra* str. NI28 and *D. violaceinigra* YIM 31327 grown on NA plates were used for this analysis. To prepare the samples, several colonies were transferred to a test tube and 1 ml of Reagent #1 (45 g NaOH (ACS Grade, Sigma-Aldrich, USA), 150 ml HPLC Grade methanol (Sigma-Aldrich, USA) and 150 ml deionized distilled water) was added. After vortexing to suspend the bacteria, this sample was placed at 100 °C for 5 minutes, vortexed once more and then incubated at 100 °C once more for 25 minutes. Afterwards, the tubes and samples were cooled down in a room temperature water bath. To initiate methylation of the fatty acids, 2 ml of Reagent #2 (325 ml 6N HCl (Sigma-Aldrich, USA) mixed with 275 ml HPLC Grade methanol (Sigma-Aldrich, USA)) was added to each tube, which was vortexed and then incubated at 80 °C for 10 minutes. Afterwards, the tubes were quickly cooled by gently shaking them in a room temperature water bath. To each tube, 1.25 ml Reagent #3 (prepared using an equal volume of HPLC Grade hexane (Sigma-Aldrich, USA) and HPLC Grade methyl tert-butyl ether (Sigma-Aldrich, USA)) was added and the contents were gently mixed end-over-end for 10 minutes using a rotator. The aqueous phase was...
removed from the tubes and discarded. To remove any free fatty acids, the samples were washed with 3 ml of Reagent #4 (10.8 g ACS Grade sodium hydroxide in 900 ml deionized distilled water) by gently mixing them as above for 5 minutes. Once more, the aqueous phase was removed and discarded. The organic phase was used for gas chromatography (GC) analysis. For the GC analysis, the column was an Ultra 2 (25 m, 0.2 mm, Agilent, USA). The protocol used was the same as used previously for the D. violaceinigra type strain34.

Phenotypic Assessment of D. violaceinigra str. NI28. A comparative phenotypic analysis of D. violaceinigra str. NI28 and YIM 31327 was performed using the API 20NE and API ZYM test kits (bioMerieux, France) according to the manufacturer's suggested protocol.

Extraction and HPLC Analysis of the Violacein Produced by D. violaceinigra str. NI28. After growth for 24 and 48 hours, 1 ml of the culture was pelleted by centrifugation for one minute at 16,200 × g and room temperature. After aspirating off the media, the pellet was resuspended in an equal volume of ethanol and shaken at 37°C and 250 rpm for 24 hours. The solution was then transferred to another centrifuge tube and the absorbance measured at 575 nm. This value was used to calculate the violacein concentration using an extinction coefficient of 73.4 L mg⁻¹ cm⁻¹ 12. The crude violacein concentration within the ethanol extracts was determined using standards prepared with a crude violacein purchased from Sigma-Aldrich (USA, Cat. No. V9389).

The crude violacein sample was also analyzed using HPLC 1200 (Agilent, USA) as described previously12. For this, 10 μl samples of the ethanol extracted violacein were analyzed using a C-18 column (Hypersil ODS, 5 μm, 250 × 4.6 mm) at 30 °C. The mobile phase was 50% denatured ethanol (HPLC Grade, Sigma-Aldrich, USA) and detection was performed at 575 nm using an Agilent 1260 Infinity ELSD. The violacein concentration within the ethanol extracts was determined as described above using crude violacein purchased from Sigma-Aldrich (USA, Cat. No. V9389) as a known standard. For the viability and MIC tests, the ethanol was dried off and the crude violacein powder solubilized within DMSO at a known concentration.

Antibiotic Disc Diffusion Assay. Each of the strains characterized is listed in Table 3. Each strain was grown on tryptone soy agar plates overnight and several colonies were transferred to 5 ml fresh sterile tryptone soy broth (TSB). They were then grown for overnight (250 rpm, 37 °C), diluted into fresh sterile TSB (0.1%) and grown until the optical density (OD) was equivalent to a 0.5 McFarland standard, which was provided with the API tests used above. After reaching this OD, the culture was spread out on Mueller-Hinton agar plates using sterile, non-toxic cotton swabs. The plates were air-dried for 10 minutes before applying the antibiotic discs (BBL Sensi-Disc, BD, U.S.A.). The antibiotics tested were ciprofloxacin, clindamycin, erythromycin, gentamycin, oxacillin, rifampin, tobramycin and vancomycin. The zones of growth inhibition were determined after 18 hours of incubation at 37°C for all the antibiotics except vancomycin and oxacillin, which were determined after 24 hours. Strain resistance was determined using the latest breakpoint tables available at the European Committee on Antimicrobial Susceptibility Testing (EUCAST) website (http://www.eucast.org/clinical_breakpoints/).

Violacein Killing of Staphylococcus aureus. The extracted crude violacein was concentrated by drying down multiple preparations under nitrogen gas and resuspending it in DMSO. The concentration was once more determined as described above.

Two different strains of S. aureus (ATCC 25923 and a clinical isolate) were used to evaluate the activity of the violacein extracted from P. sp. NI28 cultures. After growth overnight, the S. aureus cultures were diluted 1:100 into 3 ml M9 modified minimal media containing 1 g/L tryptone and 0.2% glucose. We chose this media as it is slightly modified from one that was published recently and used for MIC testing38. Violacein (M.W. 343.33) in DMSO was added to these cultures at a final concentration of 0, 15, 30 or 60 μM. The final DMSO concentration in all cultures was 1.3%. The cultures were then incubated at 37°C and 250 rpm for 24 hours, after which the viable S. aureus numbers were determined using serial dilutions of the cultures that were spread out on TSB agar plates and grown overnight at 37°C.

Minimum Inhibitory Concentration Determination. The crude violacein samples were prepared as above and the commercial violacein was purchased from Sigma-Aldrich (USA). Determining the minimum inhibitory concentration was performed as described previously using resazurin39, with some modification. Briefly, the bacteria were grown overnight in cation adjusted Mueller-Hinton broth (10 mg/L of both Ca²⁺ Mg²⁺) medium at 37°C and 250 rpm and then sub-cultured into fresh media and grown to an OD₆00ₚₚ of 0.1 under the same conditions. When they reached this OD, the cultures were diluted 1:100 into either cation adjusted Mueller-Hinton broth or modified M9 minimal media (containing 1 g/L tryptone and 0.2% glucose).

The media for the plate was prepared separately. Briefly, 3 ml of a sterile 0.2% resazurin solution prepared using distilled water was diluted into 12 ml of the modified M9 media. A 1.5 ml sample of this was then taken and crude violacein was added to a final concentration of 60 μM. Within the first row of the plate (row A), 200 μl of either cation adjusted Mueller-Hinton broth or modified M9 media containing 60 μM violacein was added. Each of the other wells had 100 μl of the same media containing resazurin
added to them. The violacein containing media was then diluted two-fold sequentially from rows A-G but not into the final row (H). To the wells, 100 μl of the prepared bacterial cultures were added, giving a final cell concentration of 4 × 10^5 CFU/well, the highest violacein concentration of 30 μM and a resazurin concentration of 0.02% in each well. The final row (H) was used as a positive control to demonstrate that each bacterium was capable of growing. Each strain was tested independently in triplicate. The control wells had only the media and the violacein added to them to test for contamination. All the wells had a final DMSO concentration of 1%.

Data Analysis. Each of the experiments was performed in triplicate for error analysis. The standard deviations are presented as error bars in each graph where appropriate.

References
1. Hoshino, T., Kondo, T., Uchiyama, T. & Ogasawara. N. Studies on the Biosynthesis of Violacein 1. Biosynthesis of Violacein - a Novel Rearrangement in Tryptophan-Metabolism with a 1,2-Shift of the Indole Ring. Agr Biol Chem Tokyo 51, 965–968 (1987).
2. Bromberg, N. et al. Growth inhibition and pro-apoptotic activity of violacin in Ehrlich ascites tumor. Chem Biol Int 186, 43–52, doi: 10.1016/j.cbii.2010.04.016 (2010).
3. Brucker, R. M. et al. Amphibian chemical defense: antifungal metabolites of the microsymbiont Janthinobacterium lividum on the salamander Plethodon cinereus. J Chem Ecol 34, 1422–1429, doi: 10.1007/s10886-008-9555-7 (2008).
4. Andriggelli-Frohner, C. R., Antonio, R. V., Creczynski-Pasa, T. B., Barardi, C. R. & Simeos, C. M. Cytoxicity and potential antiviral evaluation of violacein produced by Chromobacterium violaceum. Mem Inst Oswaldo Cruz 98, 843–848 (2003).
5. Cazoto, L. L., Martins, D., Ribeiro, M. G., Duran, N. & Nakazato, G. Antibacterial activity of violacin against Staphylococcus aureus isolated from bovine mastitis. J Antimicrob Chemother 64, 395–397, doi: 10.1093/jac/dkq131 (2011).
6. Subramaniam, S., Raví, V. & Sivasubramanian, A. Synergistic antibacterial activity of violacin with commercial antibiotics against pathogenic micro-organisms. Pharm Biol 52, 86–90, doi: 10.3109/13880209.2013.815634 (2014).
7. Nakamura, Y., Sawada, T., Morita, Y. & Tamiya, E. Isolation of a psychrotrophic bacterium from the organic residue of a water tank keeping rainbow trout and antibacterial effect of violet pigment produced from the strain. Biochem Eng J 12, 79–86 (2002).
8. Sanji, N. et al. Antibiotic Sensitivity of the E. coli, Staph. aureus, Klebsiella, Acinetobacter, Pseudomonas and Enterococi (Eskae) Organisms Isolated From the Endotracheal Tubes From Intensive Care Units of a Tertiary Care Hospital. Indian J Pharmacol 45, S194–S195 (2013).
9. Boucher, H. W. et al. Bad Bugs, No Drugs: No ESKAPE! An Update from the Infectious Diseases Society of America. Clin Infect Dis 48, 1–12, doi: 10.1086/595011 (2009).
10. Jiang, P. X., Wang, H. S., Zhang, C., Lou, K. & Xing, X. H. Reconstruction of the violacein biosynthetic pathway from Duganella sp B2 in different heterologous hosts. Appl Microbiol Biotech 86, 1077–1088, doi: 10.1007/s00253-009-2375-z (2010).
11. Jiang, P. X. et al. Pathway redesign for deoxyviolacein biosynthesis in Citrobacter freundii and characterization of this pigment. Appl Microbiol Biotech 94, 1521–1531, doi: 10.1007/s00253-012-3960-2 (2012).
12. Rodrigues, A. L. et al. Microbial production of the drugs violacein and deoxyviolacein: analytical development and strain comparison. Biotechnol Lett 34, 717–720, doi: 10.1007/s10529-011-0827-x (2012).
13. Rodrigues, A. L. et al. Systems metabolic engineering of Escherichia coli for production of the antitumor drugs violacein and deoxyviolacein. Metab Eng 20, 29–41, doi: 10.1016/j.ymben.2013.08.004 (2013).
14. Moss, M. O. & Ryall, C. Distribution of Chromobacteriaceae in a Lowland River. Microbiol Ecol 7, 139–149, doi: 10.1111/j.1365-2946 (1981).
15. Hoshino, T. Violacin and related tryptophan metabolites produced by Chromobacterium violaceum: biosynthetic mechanism and pathway for construction of violacin core. Appl Microbiol Biotech 91, 1463–1475, doi: 10.1007/s00253-011-3468-z (2011).
16. McCarthy, S. A., Johnson, R. M., Kakimoto, D. & Sakata, T. Effects of Various Agents on the Pigment (Violacin) and Antibiotic Production of Alteromonas-Luteoviolaceae. B Jpn Soc Sci Fish 51, 1115–1121 (1985).
17. Zhang, X. & Enomoto, K. Characterization of a gene cluster and its putative promoter region for violacin biosynthesis in Pseudovolatromonas sp 520P1. Appl Microbiol Biotech 90, 1963–1971, doi: 10.1007/s00253-011-3203-9 (2011).
18. Lu, Y. et al. Production of violet pigment by a newly isolated psychrotrophic bacterium from a glacier in Xinjiang, China. Biochem Eng J 43, 135–141, doi: 10.1016/j.biee.2008.09.009 (2009).
19. Pantanella, F. et al. Violacin and biofilm production in Janthinobacterium lividum. J Microbiol 102, 992–999, doi: 10.1111/j.1365-2946.2008.01515.x (2007).
20. Wang, H. et al. Optimization of culture conditions for violacin production by a new strain of Duganella sp B2. Biochem Eng J 44, 119–124, doi: 10.1016/j.biee.2008.11.008 (2009).
21. Avgustin, J. A., Bertok, D. Z., Kostanjsek, R. & Avgustin, G. Isolation and characterization of a novel violacin-like pigment producing psychrotrophic bacterial species Janthinobacterium sballardensis sp nov. Anton Leeuw Int J G 103, 763–769, doi: 10.1007/s00482-012-9858-0 (2013).
22. Kim, S. J. et al. Genome Sequence of Janthinobacterium sp Strain PAMC 25724, Isolated from Alpine Glacier Cryoconite. J Bacteriol 194, 2096–2096, doi: 10.1128/JB.00096-12 (2012).
23. Moss, M. O., Ryall, C. & Logan, N. A. Classification and Characterization of Chromobacteria from a Lowland River. J Gen Microbiol 105, 11–21 (1978).
24. Li, W. J. et al. Duganella violaeinigra sp. nov., a novel mesophilic bacterium isolated from forest soil. Int J Syst Evol Microbiol 54, 1811–1814, doi: 10.1099/ijs.0.63141-0 (2004).
25. Aranda, S., Montes-Borrego, M. & Landa, B. B. Purple-Pigmented Violacin-Producing Duganella sp. inhabit the Rhizosphere of Wild and Cultivated Olives in Southern Spain. Microbial Ecol 62, 446–459, doi: 10.1007/s00248-011-9840-9 (2011).
26. Hakvag, S. et al. Violaenic-producing Colimonas sp. from the sea surface microlayer of coastal waters in Trondelag, Norway. Mar Drugs 7, 576–588, doi: 10.3390/md7040576 (2009).
27. Kämmer, P., Wellner, S., Lohse, K., Martin, K. & Lodders, N. Duganella phyllosphaerae sp. nov., isolated from the leaf surface of Trifolium repens and proposal to reclassify Duganella violaeinigra into a novel genus as Pseudoduganella violaeinigra gen. nov., comb. nov. (vol 35, pg 19, 2012). Syst Appl Microbiol 35, 278–278, doi: 10.1016/j.syapm.2012.02.001 (2012).
28. Hahn, M. W. & Holle, M. G. Flagellate predation on a bacterial model community: interplay of size-selective grazing, specific bacterial cell size, and bacterial community composition. Appl Environ Microbiol 65, 4863–4872 (1999).
29. Vandamme, D., Fouvert, I. & Muylaert, K. Flocculation as a low-cost method for harvesting microalgae for bulk biomass production. Trends Biotechnol 31, 233–239, doi: 10.1016/j.tibtech.2012.12.005 (2013).
30. Witte, W. Antibiotic resistance in gram-positive bacteria: epidemiological aspects. J Antimicrob Chemother 44, Suppl A, 1–9 (1999).
31. Archer, G. L. Staphylococcus aureus: a well-armed pathogen. Clin Infect Dis 26, 1179–1181 (1998).
32. Chambers, H. F. & DeLeo, F. R. Waves of resistance: Staphylococcus aureus in the antibiotic era. Nat Rev Microbiol 7, 629–641, doi: 10.1038/nrmicro2200 (2009).
33. Niazi, J. H., Kim, B. C., Ahn, J. M. & Gu, M. B. A novel bioluminescent bacterial biosensor using the highly specific oxidative stress-inducible pgi gene. Biosens Bioelectron 24, 670–675, doi: 10.1016/j.bios.2008.06.026 (2008).
34. Wang, H. S. et al. Biosynthesis and characterization of violacein, deoxyviolacein and oxyviolacein in heterologous host, and their antimicrobial activities. Biochem Eng J 67, 148–155, doi: 10.1016/j.bej.2012.06.005 (2012).
35. Ahmad, W. A., Yusof, N. Z., Nordin, N., Zakaria, Z. A. & Rezali, M. F. Production and characterization of violacein by locally isolated Chromobacterium violaceum grown in agricultural wastes. Appl Biochem Biotechnol 167, 1220–1234, doi: 10.1007/s12010-012-9553-7 (2012).
36. Mavrodi, D. V. et al. Diversity and evolution of the phenazine biosynthesis pathway. Appl Environ Microbiol 76, 866–879, doi: 10.1128/AEM.02009-09 (2010).
37. Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30, 2725–2729, doi: 10.1093/molbev/mst197 (2013).
38. Adler, C. et al. Catecholate Siderophores Protect Bacteria from Pyochelin Toxicity. PLoS One 7, e46754, doi: 10.1371/journal.pone.0046754 (2012).
39. Sarker, S. D., Nahar, L. & Kumarasamy, Y. Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals. Methods 42, 321–324, doi: 10.1016/j.ymeth.2007.01.006 (2007).

Acknowledgements
This work was supported by grants from the Korea Health Industry Development Institute (KHIDI) (Grant #HI13C13550000), the National Research Foundation of Korea (Grant No. 2013R1A1A2008590), and the Korea Institute for Advancement of Technology (KIAT) through the EUREKA program (Grant #N019800009). SBK also acknowledges support from the 2014 CNU Research Fund (Grant #2014-0784-01). We are all thankful for the support. We would also like to thank Dr. Sujin Lee, MD, at the Pusan National University Hospital-Yang San for the clinical isolate of Staphylococcus aureus isolate used in this study.

Author Contributions
S.Y.C., S.L. and R.J.M. designed the study; S.Y.C., S.K. and S.L. performed the experiments; S.Y.C. and S.B.K. performed the phylogenetic analyses; S.Y.C., S.K. and R.J.M. wrote the paper. All authors reviewed the manuscript.

Additional Information
Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Choi, S.Y. et al. High-level production of violacein by the newly isolated Duganella violaceinigra str. NI28 and its impact on Staphylococcus aureus. Sci. Rep. 5, 15598; doi: 10.1038/srep15598 (2015).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/