Full Length Research Paper

Chryseobacterium indologenes improves survival of the *Chromobacterium violaceum* and violacein production

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Chromobacterium violaceum* is a human opportunistic pathogen which appears in soil and water environments. It produces a purple-colored bactericide named violacein. The aim of this study was to investigate the correlation between violacein production and the competitive ability of *C. violaceum* in co-culture with other soil bacteria. The *C. violaceum* (3), *Chryseobacterium indologenes* (1), *Serratia marcescens* (2), *Pantoea agglomerans* (4 and 5), *Enterobacter asburiae* (6) and *Bacillus megaterium* (7), were isolated together from a nest of *Crematogaster biroi*. In the duel species competition experiments, *C. violaceum* (3) inhibited the growth of *C. indologenes* (1), while being dramatically inhibited by *S. marcescens* (2). The growth of *C. violaceum* (3) was unaffected by *P. agglomerans* (4 and 5) and *E. asburiae* (6), but it was slightly reduced by *B. megaterium* (7) (after 48 h of incubation). In the multiple competition experiments, the survival of *C. violaceum* (3) was increased in the presence of *C. indologenes* (1). The production of violacein was reduced in co-culture with all the examined bacterial strains, except *C. indologenes* (1). The violacein degradation assay demonstrated that, *S. marcescens* (2), *P. agglomerans* (4 and 5) and *E. asburiae* (6) were capable of decolorizing violacein, while *C. indologenes* (1) and *B. megaterium* (7) had no effect on violacein stability in 24 h of incubation. Taken together, these results might be useful for industrial violacein production.

Key words: *Chromobacterium violaceum*, *Chryseobacterium indologenes*, growth competition, violacein.

INTRODUCTION

Chromobacterium violaceum is a Gram-negative bacterium found in the soil and aquatic environments of tropical and subtropical regions. It is also an opportunistic human pathogen that invades its hosts through traumatic wounds or ingestion of contamination water (Midani and Rathore, 1998). Infection by *C. violaceum* is rare; it causes sepsis and visceral abscesses in the liver, lungs, kidneys and spleen, which without appropriate treatment result in death (Lee et al., 1999; Moore et al., 2001). Clinically, *C. violaceum* can be quickly identified by its tendency to produce a purple pigment named violacein, a polyphenolic compound composed of two modified tryptophan molecules. In *C. violaceum*, violacein synthesis is catalyzed by a group of enzymes (VioA, VioB, VioC, VioD and VioE) (Balibar and Walsh, 2006; Hirano et al., 2008; Ryan et al., 2008; Sanchez et al., 2006). Expression of VioA-E is regulated by the quorum sensing molecule N-hexanoyl-L-homoserin lactone (HHL). HHL can induce the expression of HHL synthase regulator (CviR), a transcriptional activator that increases both the expression of VioA-E and violacein production (Martinelli et al., 2004; McClean et al., 1997). Because of this colored product, *C. violaceum* is widely used as an indicator in the study of quorum sensing mechanisms (Blosser and Gray, 2000).

Violacein has many biological activities such as antibacterial (Lichstein, 1946), antioxidant (Konzen et al., 2006) and anti-parasite (Leon et al., 2001; Matz et al., 2004) activities. These properties could enhance the survival capacity of *C. violaceum* against environment stresses (Duran et al., 2007). Searching for color bacteria,

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Table 1. Characteristics of the seven soil bacterial isolates.

Isolate	Colony pigmentation	Cell morphology	Closest relative	Identity (%)
1	Yellow	Rods	*C. indologenes*	99
2	Red	Cocci	*S. marcescens*	100
3	Purple	Rods	*C. violaceum*	99
4	White	Cocci	*P. agglomerans*	99
5	White	Cocci	*P. agglomerans*	99
6	White	Cocci	*E. asburiae*	99
7	White	Large rods	*B. megaterium*	100

we isolated a purple bacterium together with other six soil bacteria from a nest of *Crematogaster biroi*. This violet bacterium was identified as *C. violaceum* based on the homology of 16S rDNA sequence (Table 1). On the original plate, we found that the *C. violaceum* colonies were outnumbered by other bacterial colonies. Since these bacteria were directly isolated from the soil samples without further enrichment processes, we concluded that, this *C. violaceum* strain was relatively scarce in the soil sample. The object of this study was to investigate the ability of *C. violaceum* to compete against six other bacteria and to determine whether violacein levels and its decomposition would affect survival of *C. violaceum* in competition experiments

MATERIALS AND METHODS

Collection of soil samples

The soil samples used in this study were collected from the nests of *C. biroi* at Lily Lake, Taichung, Taiwan, between June and August 2007.

Bacteria identification

The soil samples were dissolved in sterile water, diluted 10-fold serially to 10^{-7} and 200 µl of the 10^{-5}, 10^{-6} and 10^{-7} dilutions were spread out on Luria-Bertani (LB) agar plates. The plates were incubated at room temperature for two days. On one plate violet color bacteria were observed. This violet bacterium and several other colonies from the same plate were selected and inoculated in LB broth for DNA extraction. All bacterial DNA was prepared using a Gene-Spin[TM]-V2 Genomic DNA isolation kit (Bio-Protech, Taiwan) according to the manufacturer's instructions and the purified DNA at a concentration of 20 ng/ml was stored at -20°C. To clone 16S rDNA, PCR was performed using a BD Advantage[TM] 2 PCR enzyme system (BD Biosciencs, U.S.A.). Total bacterial DNA was used as a template and the forward primer (AGAGTTTGATCC TGGCTCAG) and reverse primer (GGCTACCTTGTTACGACTT) were designed based on the genome sequence of *E. coli* 16S rDNA (Edwards, 1989; Lane, 1991). The PCR condition was as follows: 95°C for 1 min; 95°C for 30 s; 55°C for 1 min: 68°C for 3 min (35 cycles); and 70°C for 10 min. Amplification products were sequenced by the ABI 3730XL DNA Analyzer (Applied Biosystems, U.S.A.). The sequences were subjected to GenBank homology searches against a bacterial DNA database using the BLAST program.

Growth competition

A single bacterial colony was inoculated into a 50 ml Falcon polypropylene tube containing 5 ml LB medium at 25°C for 12 h. After incubation, cells were diluted to an appropriate density with the fresh LB medium. In the competition experiments, bacterial strains with a density of OD_{600} 0.1 were mixed and incubated at 25°C. For analysis of survival, 1 ml of cell suspensions was taken at 0, 4, 8, 16, 24, 48, 72 and 96 h, diluted serially and diluted 10-fold serially to 10^{-7} and 200 µl of the 10^{-6} and 10^{-7} dilutions were spread out on three Luria-Bertani (LB) agar plates. After incubation at 25°C, the numbers of colonies of different bacteria were counted based on their colors. All experiments were performed thrice.

Extraction of violacein from the *C. violaceum* (3)

The cultures containing *C. violaceum* (3) cells alone or co-cultured with other soil bacteria were taken at 0, 4, 8, 16, 24, 48, 72 and 96 h post-incubation. To extract violacein, an equal amount of ethyl acetate (Sigma Aldrich, U.S.A.) was added and incubated at room temperature for 12 h. After incubation, the ethyl acetate solution containing violacein was separated from the medium by centrifugation at 8000 rcf for 10 min. The relative concentrations of violacein were measured by a spectrophotometer at 562 nm.

Degradation of violacein by soil bacteria

To avoid the organic solvent effect, we isolated violacein directly from the culture medium by filtration without using organic solvents. Briefly, *C. violaceum* (3) was grown in LB broth at room temperature for 48 h. After incubation, bacteria were removed from the medium by centrifugation at 8000 rcf for 10 min. The supernatants were passed through a 0.2 µm filter (Sartorius, Germany). After filtration, the medium containing violacein was incubated with the soil bacteria at 25°C for 24 h. Violacein in the culture medium was extracted by ethyl acetate and measured by a spectrophotometer at 562 nm. All the experiments were performed in triplicates and the Duncan's test was used for statistical comparisons.

RESULTS AND DISCUSSION

Identification and characterization of soil bacteria

Seven bacteria strains (1 to 7) including a purple bacterium were isolated from a soil sample in Taiwan. In LB broth, the growth rates of the soil bacteria were similar, except that no. 7 grew slower in the first 8 h of incubation.
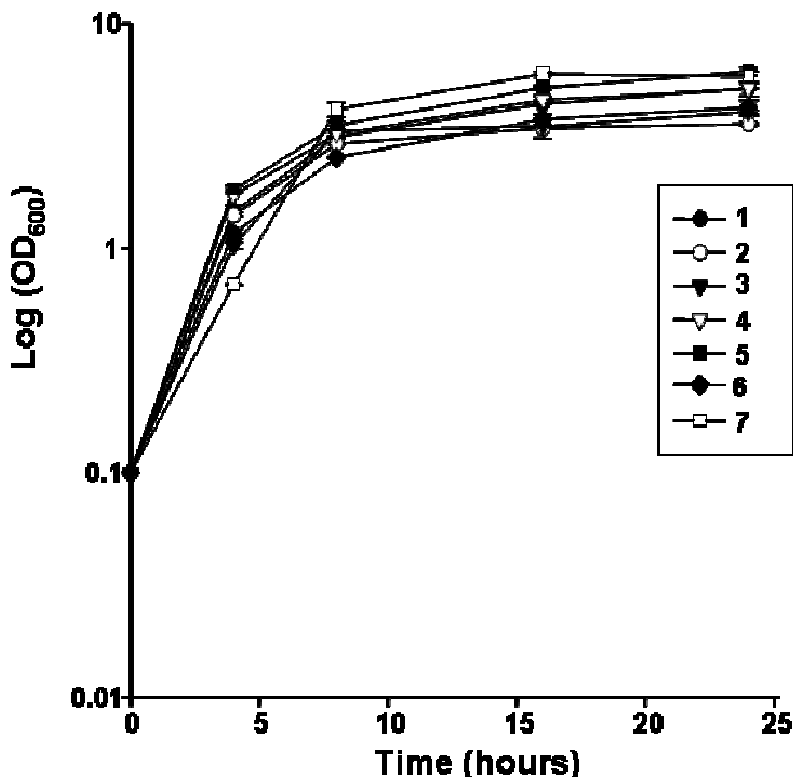
Figure 1. Growth rate of the soil bacteria in this study. Seven soil bacterial strains (1-7) were inoculated in LB broth at 25°C. The cell densities were measured by a spectrophotometer at 562 nm after incubation for 4, 8, 16 and 24 h. The bacterial name abbreviations are shown in Table 1.

After incubation for 24 h, the cell densities of all seven bacterial strains were similar (Figure 1). These strains were further identified based on their morphology and 16S rDNA sequences. The morphology of 1 and 3 was of a short rod and that of 7 was of a large rod, while that of 2, 4, 5 and 6 was round under a light microscope (data not shown). The color of the colonies grown on LB plates were yellow (1), red (2), purple (3) and white (4 to 6). Sequence alignments showed that, these soil bacteria closely aligned with *Chryseobacterium indologenes* (1), *Serratia marcescens* (2), *C. violaceum* (3), *Pantoea agglomerans* (4 and 5), *Enterobacter asburiae* (6) and *Bacillus megaterium* (7) (Table 1).

Survival of the *C. violaceum* and violacein production in the pair species competition experiments

The cell densities of the *C. violaceum* (3) in the co-culture with the other soil bacterial strains are shown in Figure 2. In the presence of *C. indologenes* (1), the growth of the *C. violaceum* strain (3) cells was not affected (Figure 2a) and their number was relatively constant at the end of the experiment. In contrast, the number of *C. indologenes* (1) cells decreased during the incubation time. In the presence of *S. marcescens* (2), a slight increase in the *C.*

violaceum (3) growth was observed before 4 h post-coincubation. After that, the number of *C. violaceum* (3) cells declined rapidly. After 96 h of incubation, only 10^3cfu/ml *C. violaceum* (3) remained (Figure 2b). Co-culture with *P. agglomerans* (4 and 5) or *E. asburiae* (6) had slightly decreased the number of *C. violaceum* strain (3) (Figure 2c to e). In the presence of *B. megaterium* (7), the growth of *C. violaceum* (3) was also not affected until 48 h post-incubation. After that, it decreased slightly (Figure 2f).

The violacein production of *C. violaceum* (3) in the co-culture with the other soil bacteria was also investigated (Figure 2). Compared with *C. violaceum* (3) cultured alone, the production of violacein increased in the co-culture with *C. indologenes* (1) and decreased in the co-culture with *S. marcescens* (2), *P. agglomerans* (4 and 5) and *E. asburiae* (6) after incubation for 18 h. Co-culture with *B. megaterium* (7) also increased the production of violacein before 48 h, after which it decreased.

It has been demonstrated that, increased violacein production occurs after cells enter the stationary phase (Riveros et al., 1989). At that time, a high concentration of HHL that accumulated in the medium could diffuse back to the cells and result in both an increase in the expression of VioA-E and in violacein production (Martinelli et al., 2004; McClean et al., 1997). In spite of different violacein levels, similar expression profiles of violacein
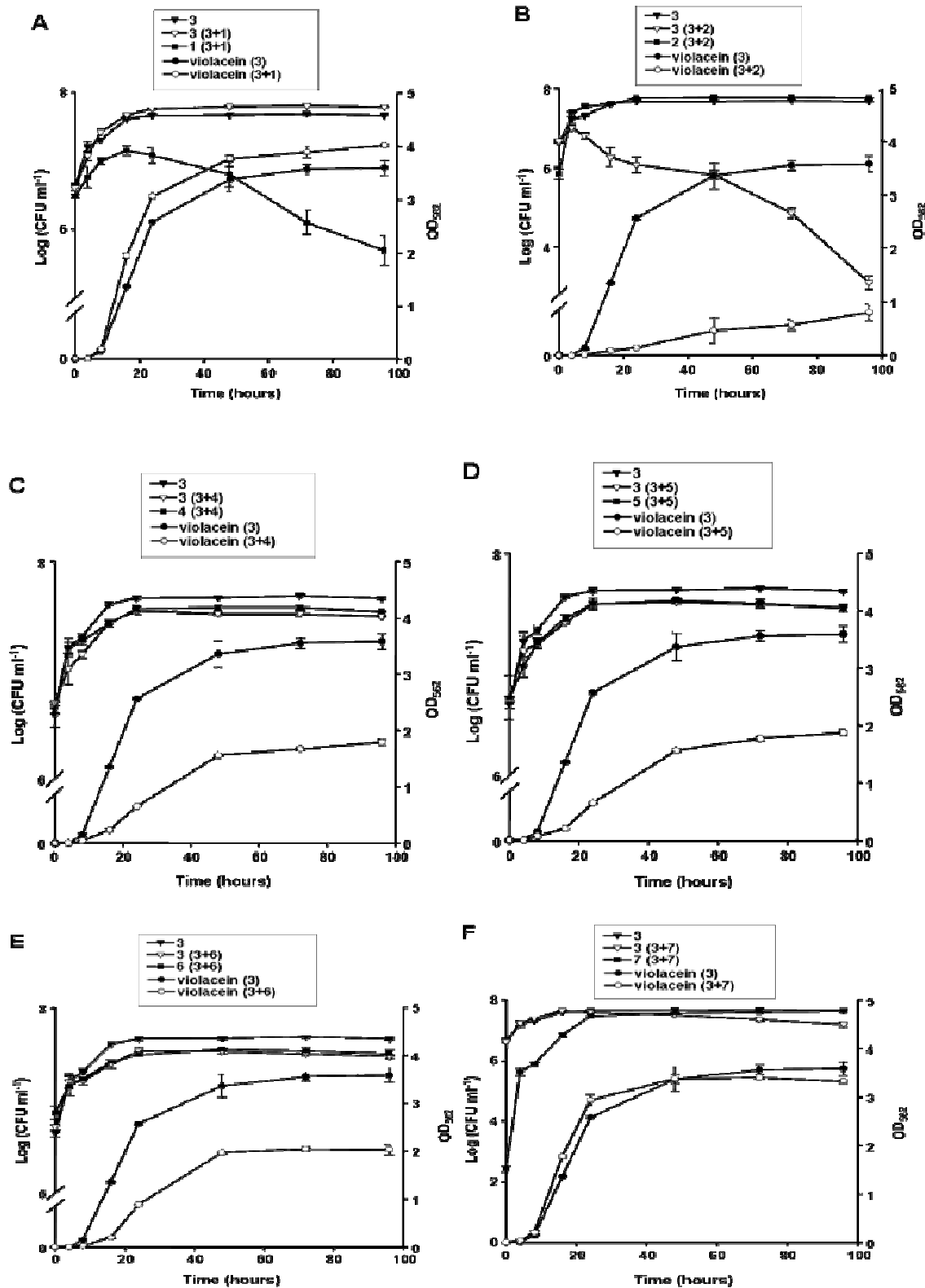
Figure 2. Growth and violacein levels of *C. violaceum* (3) in the competition experiments. The black triangles represent the growth of *C. violaceum* (3) alone in the culture medium. The white triangles and black squares represent the growth of *C. violaceum* (3) and of the other bacterial strain (e.g. *C. indologenes* (1), *S. marcescens* (2), *P. agglomerans* (4 and 5), *E. asburiae* (6) or *B. megaterium* (7)) in the co-culture, respectively. The black circles and white circles represent the amount of violacein in the media without or with the other bacterial strain. Error bars indicate standard deviations.
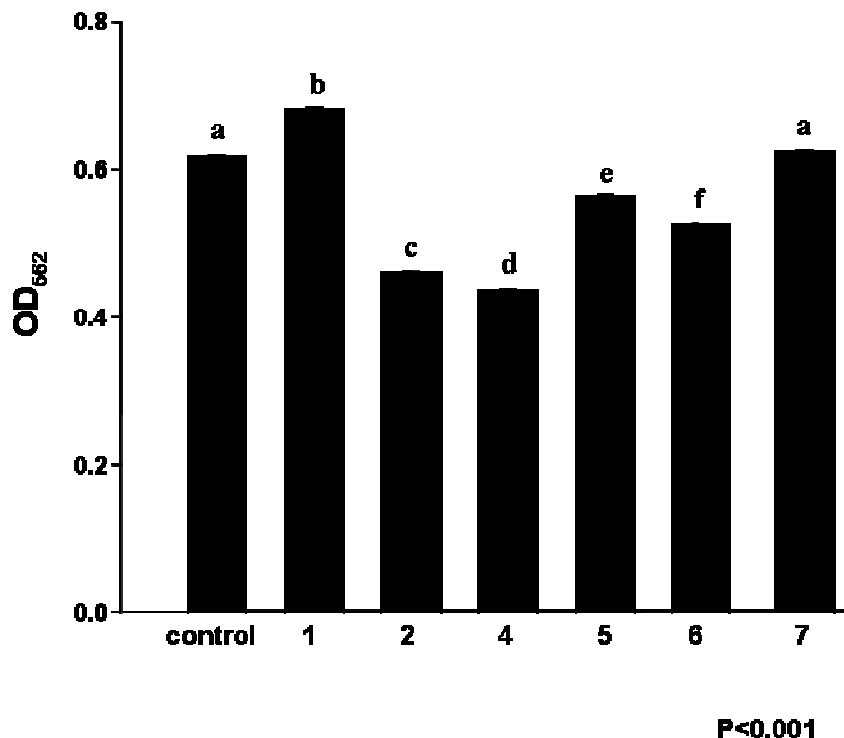
Figure 3. Degradation of violacein by the diverse bacteria. The culture media containing violacein were inoculated with the soil bacteria at 25°C for 24 h. After incubation, the remaining violacein was extracted, and its relative amounts were measured by a spectrophotometer at 562 nm. Values presented by different letters represent significant differences (p < 0.001).

production were observed in the co-culture with all these bacteria, except for *S. marcescens* (2). However, we also noticed that the number of *C. violaceum* (3) cells in culture without or with *P. agglomerans* (4 and 5) and *E. asburiae* (6) were similar, but the violacein production was lower in the co-culture experiments.

Violacein degradation assay

Since several microorganisms have been shown to digest violacein (Bromberg and Duran, 2001), we tested whether the lower violacein production in dual competition experiments may have resulted from degradation of violacein by these soil bacteria. By incubating violacein with these strains individually, we found that *S. marcescens* (2), *P. agglomerans* (4 and 5) and *E. asburiae* (6) were capable of digesting violacein and the amount of violacein remaining in the co-culture medium was 75, 71, 92 and 85%, respectively. By contrast, both *C. indologenes* (1) and *B. megaterium* (7) were unable to digest violacein in 24 h (Figure 3). This suggested that low violacein production in the duel competition experiments might at least in part, be due to degradation of violacein by the bacterial strains present in the culture medium.

Survival of *C. violaceum* strain (3) in the multiple species competition experiments

The results shown in Figure 2 indicate that, *S. marcescens* (2) was the major threat to the survival of *C. violaceum* (3). To investigate which bacterial strain could protect *C. violaceum* (3) against *S. marcescens* (2), we performed competition experiments containing *S. marcescens* (2) and each of the other soil bacteria. As demonstrated in Figure 4a, in the presence of *C. indologenes* strain (1), *S. marcescens* (2) was unable to reduced the amount of *C. violaceum* (3) throughout the experiment (144 h of incubation), even though *C. indologenes* (1) was undetectable after 72 h of incubation. However, in the presence of *B. megaterium* (7), the survival of *C. violaceum* (3) was constantly monitored during 72 h, but undetectable after 144 h.

To better define the roles of *C. indologenes* (1) and *B. megaterium* (7) in the competition experiments, we investigated whether *S. marcescens* (2) could inhibit their growth. In the presence of *S. marcescens* (2), the *B. megaterium* (7) was undetectable after growth for 48 h, while the amount of *C. indologenes* (1) remained constant throughout the experiment (data not shown). We also found that, the numbers of *P. agglomerans* (4 and 5) and *E. asburiae* (6) were decreased in co-culture with *S.*

B
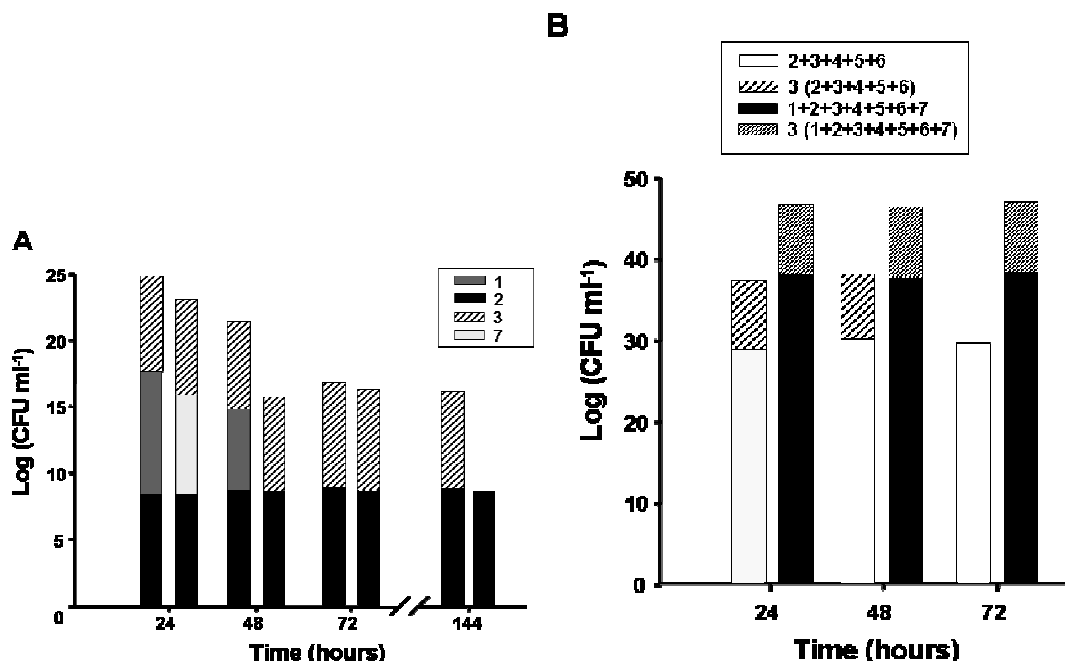
Figure 4. Growth of *C. violaceum* (3) in the multiple species competition experiments. (A) *C. violaceum* (3) and *S. marcescens* (2) were co-incubated with *C. indologenes* (1) or *B. megaterium* (7). The growth (number of cells) of each bacterial strain in the co-cultures is represented by a special bar: *C. violaceum* (3) - a coarse striation, *S. marcescens* (2) - black bars, *C. indologenes* (1)- gray bars and *B. megaterium* (7)- white bars; (B) The growth of *C. violaceum* (3) cells without or with *C. indologenes* (1) and *B. megaterium* (7) are indicated by white bars with a coarse or medium striation, respectively and the growth of the other bacterial cells are represented by white or black bars.

marcescens (2) (data not shown). *S. marcescens* (2) managed to survive in all experiments.

To confirm that the presence of *C. indologenes* (1) and *B. megaterium* (7) were important for the survival of *C. violaceum* (3), we performed multiple species competition experiments in the presence or absence of these two strains (Figure 4b). Not surprisingly, the survival of *C. violaceum* (3) was only observed in the medium containing *C. indologenes* (1) and *B. megaterium* (7). The presence of *P. agglomerans* (4 and 5) and *E. asburiae* (6) had no effect on the survival of *C. violaceum* (3) against *S. marcescens* (2).

In conclusion, we demonstrated that *C. violaceum* (3) could kill *C. indologenes* (1) and live with *P. agglomerans* (4 and 5) and *E. asburiae* (6), but be eliminated by *S. marcescens* (2) and *B. megaterium* (7) (after 48 h). We also found that, *C. indologenes* (1) could increase viola-cein production and improved survival of the *C. violaceum* (3) in the competition experiments. Future works will focus on identification of the materials from *C. indologenes* (1), which can increase violacein production in *C. violaceum* (3).

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