Curr Microbiol (2014) 69:412–416
DOI 10.1007/s00284-014-0604-x

The Effects of *Paenibacillus polymyxa* E681 on Antifungal and Crack Remediation of Cement Paste

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Received: 17 February 2014 / Accepted: 13 April 2014 / Published online: 14 May 2014

Abstract This study investigated the antifungal effects of cement paste containing *Paenibacillus polymyxa* E681 against *Aspergillus niger,* a deleterious fungus commonly found in cement buildings and structures. To test the antifungal effects, cement paste containing *P. polymyxa* E681 was neutralized by CO_2 gas, and the fungal growth inhibition was examined according to the clear zone around the cement specimen. In addition to the antifungal effects of the cement paste added with bacteria, calcium crystal precipitation of *P. polymyxa* E681 was examined by qualitative and quantitative analyses. The cement paste containing *P. polymyxa* E681 showed strong antifungal effects but *fusA* mutant (deficient in fusaricidin synthesis) showed no antifungal activity. Crack sealing of the cement paste treated with *P. polymyxa* E681 was captured by light microscope showed fungal growth inhibition and crack repairing in cement paste.

Introduction

Cement is one of the most commonly used building materials in the world due to its durability and relatively inexpensive price. However, mature cement structures are neutralized by bicarbonate in the air, resulting in the colonization of bacteria and fungi onto or within the building material as it ages. Fungal growth is a major biodeterioration factor affecting cement materials and therefore is particularly important to prevent [6]. Acidic metabolites of fungi can lead to neutralization of contaminated cement surfaces and reduced compressive strength [12, 13]. Further, the hyphae of fungi can penetrate into the cement structure, leading to physical disruption [10].

The current study confirmed that an organic–inorganic antifungal agent was effective against *Aspergillus niger*, common in the interiors and exteriors of building [5, 6, 9, 10, 12]. According to Do et al. 2005, isothiazoline/carbamate prevents the growth of *Aspergillus niger* with cement mortar containing 5 % isothiazoline/carbamate produced a clear zone with no fungal growth. On the other hand, zinc oxide and ammonium bromide have been used as algaecide agents for the prevention of algal growth which is a major contaminant of concrete surfaces in humid environments. Enhanced algaecide properties have been demonstrated on concrete surfaces through the addition of these chemicals [4, 6, 9, 11]. Furthermore, various organo-metallic compounds such as zeolite, zeocarbon, silver, copper, and nickel sulfate have been used for fungal growth inhibition [5, 6, 9, 10]. These inorganic treatments are effective for fungal growth inhibition but often have harmful effects on humans and the surrounding environment [14]. We hypothesized that the strong antifungal effects of bacteria could be applied to the antifungal cement paste, thus we investigated the potential of antifungal effects of *Paenibacillus polymyxa* E681 in cement paste.

The addition of microorganisms to cement paste for the stimulation of organic–inorganic biomineralization has been applied as a novel approach in cement technology research [8, 11, 14]. This biomaterial is a progressive concept and environmentally harmless in comparison with currently used synthetic polymers in the cement technology field [8, 14]. This concept has been applied in order to coat

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concrete surfaces [1], enhance the durability of cement–sand mortars [3, 9], and repair cracks in cement structures. In addition, carbonate precipitation by bacteria could be used as a self-healing agent for improving concrete durability through biomineralization controlled by bacteria on cementitious materials [8].

This study investigated the potential use of *P. polymyxa* E681 in the development of novel, multifunctional cement formulations showing antifungal effects and calcium carbonate precipitation. In our results, E681 induced calcium carbonate crystals on calcium-rich medium, and artificial cracks in cement paste were sealed by calcium carbonate crystals produced by E681. In addition, this E681 induced coating of calcium aggregates onto the cement paste surface, and the compressive strength of cement–sand mortar containing *P. polymyxa* E681 was increased. Above all, the growth of *A. niger*, a well-known fungal strain with deleterious effects on cement surfaces, was severely inhibited by the antifungal properties of *P. polymyxa* E681.

Materials and Methods

Bacterial Strains and Culture Media

The *P. polymyxa* E681 which was obtained from the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea) was used in this study. The deleterious fungal strain *A. niger* KCTC6906 was purchased from the Korean Biological Resource Center (Daejeon, Korea). *P. polymyxa* E681 demonstrates strong antifungal activity against *A. niger*, whereas the *fusA* mutant strain shows no antifungal activity [2]. These bacteria were precultured in tryptic soy broth (TSB, Becton Dickson, USA) media, followed by incubation at 30 °C for 24 h with 160 rpm shaking. Potato dextrose broth (PDA, Becton Dickson, USA) was used for inoculum preparation of the fungal strain. B4 liquid and solid medium (0.4 % yeast extract, 0.5 % dextrose, and 0.25 % calcium acetate) were used for the determination of microbiologic calcium carbonate precipitation (MCCP). *Escherichia coli* K12, non-calcium carbonate-forming bacteria with no antifungal activity, was used as a negative control.

Preparation of the Fungal Spore Suspension

To examine the antifungal activity of *P. polymyxa* E681, a spore suspension of *A. niger* was prepared as described below [12, 13]. PDA media inoculated with the fungal strain was incubated at 25 °C for 1–2 weeks in order to generate spores. After culturing, 5 ml of 0.1 % (w/w) Tween 80 was added, and the fungal colonies were scrubbed using a sterile plastic loop. The suspension was then centrifuged at 12,000×*g* and 4 °C for 15 min. After centrifugation, the sample was washed twice with sterile distilled water (SDW), and the concentration of fungal spores was adjusted to 10^6 spores/ml using a hemocytometer (Marienfeld Co., Germany) [12].

Compressive Strength Test of Cement Mortar

These test strains were inoculated in 300 ml of TSB and then cultured at 30 °C for 24 h with 160 rpm shaking. Cell pellets were harvested by centrifugation at 8,000 rpm and washed twice with SDW for the elimination of TSB constitutions. The same concentrations of bacterial samples were then adjusted to an O.D. of 0.8 at 600 nm, where O.D. value means 10^9 CFU/ml. A mixture consisting of 240 g of cement, 660 g of sand, and 116.4 ml of SDW was used to cast three cement mortars with dimensions of 50.8 mm × 50.8 mm × 50.8 mm [11]. Six cement mortars containing bacteria were then immersed separately in 1 l of B4 liquid medium containing each treatment, followed by incubation at 30 °C under aerobic conditions for 28 days. After incubation, specimens were removed from the B4 liquid medium and dried completely at 25 °C, after which the compressive strengths of all specimens were measured using a compressive strength machine (Universal Test Machine, Shimadzu Corporation, Kyoto, Japan) [11].

Artificial Crack Remediation

Bacterial strains were tested for their ability to remediate cracks in cement paste. Cement pastes were made by mixing aliquots of SDW with cement at a 0.4 water/cement weight ratio and then drying at 60 °C for 24 h, after which cracks were artificially made by applying physical force. Crack sizes were measured by stereo microscopy (10×). Bacterial samples suspended in 100 µl of liquid B4 medium were applied to cracks in the cement pastes, after which the samples were incubated at 30 °C for 24 h. This process was repeated three times within 3 days at 24-hour intervals. After 5 days, digital images of the pastes were captured using a Zentech Digicam.

Preparation and Neutralization of Cement Paste Containing Microorganism

Paenibacillus polymyxa E681 and *fusA* mutant strain which did not show antifungal effects were inoculated into 5 ml of TSB medium and then cultured at 30 °C with 160 rpm shaking for 24 h. After incubation, 5 ml of each sample was inoculated into 500 ml of TSB and then cultured at 30 °C with 160 rpm shaking for 24 h. Cell pellets were collected by centrifugation at 7,000 rpm, followed by resuspension in 20 ml of SDW. The concentration of each

strain was adjusted to 10^9 CFU/ml by the dilution method [11]. A mixture containing 50 g of cement and 20 ml of bacterial suspension was prepared, after which 10.0 g (±0.5 g) of the mixture was poured into 3.5-cm-diameter plastic dish. After 1 day, cement mortar samples were separated from the plastic dish, transferred into a plastic chamber (30 × 20 × 10 cm), and incubated at room temperature with CO_2 gas in order to induce fungal growth. The neutralized cement paste containing *P. polymyxa* E681 and *fusA* mutant was placed on PDA media containing 10^6 spores/ml of *A. niger*. The PDA plate was incubated at 25 °C for 5 days, after which images of the resulting fungal growth were captured using a digital camera (D5100, Nikon Corp. Japan).

Antifungal Effects on the Surface of Cement Paste

To examine the antifungal effects on the surface of cement paste, *P. polymyxa* E681 and *fusA* mutant strain were cultured by the above cell inoculation method, and 10 ml of bacteria solution containing *A. niger* was treated on the center line of the neutralized cement paste. In the surface treatment, these concentrations of E681 and *E. coli* K12 were adjusted to 10^9 CFU/ml by the dilution method [11–13]. The fungal growths on the surface of cement pastes were captured using a Zentech Digicam.

Results and Discussion

Figure 1 displayed the precipitation of calcium carbonate crystals induced by *P. polymyxa* E681 grown on B4 calcium-rich medium. Morphologies of these crystals were regular or irregular circular and transparent yellow, and the size ranging from 50 to 300 μm as shown in Fig. 1a. As shown in Fig. 1b, the diffraction peaks in the XRD analysis of the calcium crystals were determined to be vaterite. The specificity of crystals is mainly due to differences among bacterial genera, and bacteria-specific calcium crystal precipitation can have different effects on the durability of cement materials [7, 13]. Thus, various MCCP bacteria have been reported and characterized to improve the durability of cement materials.

To examine the effects of *P. polymyxa* E681 crystal formation on strength improvement of cement, cement mortar was immersed in a *P. polymyxa* E681 solution with a cell concentration of 10^9 CFU/ml. *P. polymyxa* E681 treatment induced the increase in compressive strength (15.1 %) compared with other non-calcium carbonate-forming bacteria (*E. coli* K12) treatment (7.2 %) (Table 1). This result suggests that *P. polymyxa* E681 calcium crystal formation can be induced in the surface of cement paste, and the biomaterial has no negative effect on the durability of cement mortar.

Further, the artificial crack of around 0.3 mm of cement paste was sealed by the treatment of E681 as seen in Fig. 2c. In the result, the crack treated with E681 was gradually repaired during 3–5 days, whereas treatment with *E. coli* K12 (Fig. 2b) and only B4 calcium-rich medium treatment (Fig. 2a) did not show sealing effect. These results supported that the biodeposition of *P. polymyxa* E681, which mixed with calcium aggregates and biomolecules, could be applied on microcrack of cement paste.

Table 1 Compressive strength test of cement pastes added bacteria in 28 days after

	Compressive strength ± SD[a]	Strength increase (%)
Control[b]	51.8 ± 3.9	0
E. coli K12	55.8 ± 0.24	7.2
E681	61.0 ± 3.5	15.1

[a] Average and standard deviation values obtained from six samples

[b] Only B4 medium

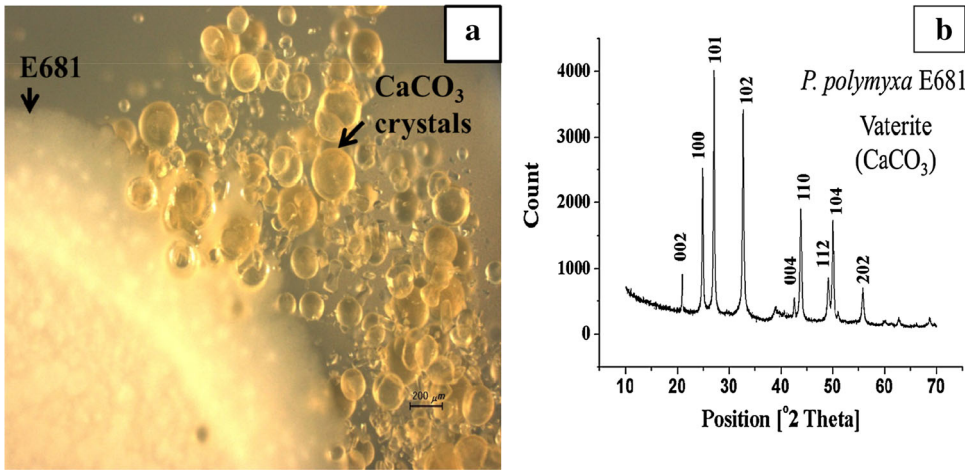

Fig. 1 Light microscopic image and X-ray diffraction patterns of calcium aggregates induced by *P. polymyxa* E681. **a** Calcium carbonate crystals produced by *P. polymyxa* E681. **b** XRD patterns of calcium crystals produced by *P. polymyxa* E681 (Color figure online)

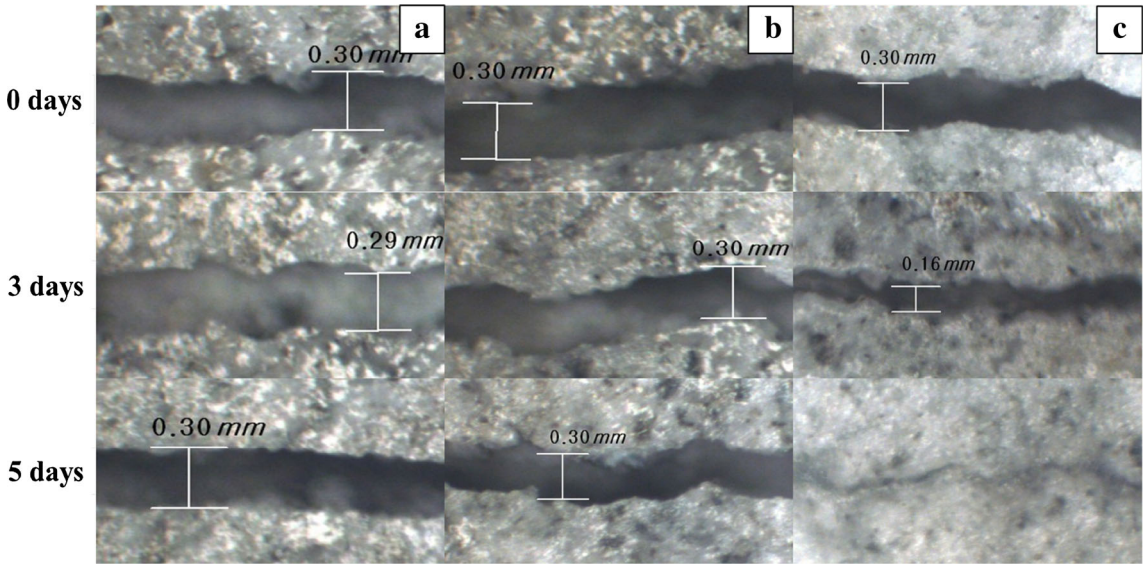

Fig. 2 Artificial crack remediation by *P. polymyxa* E681. **a** Crack of cement paste treated with only B4 medium. **b** Non-calcium carbonate-forming *E. coli* K12. **c** *P. polymyxa* E681 treatment (Color figure online)

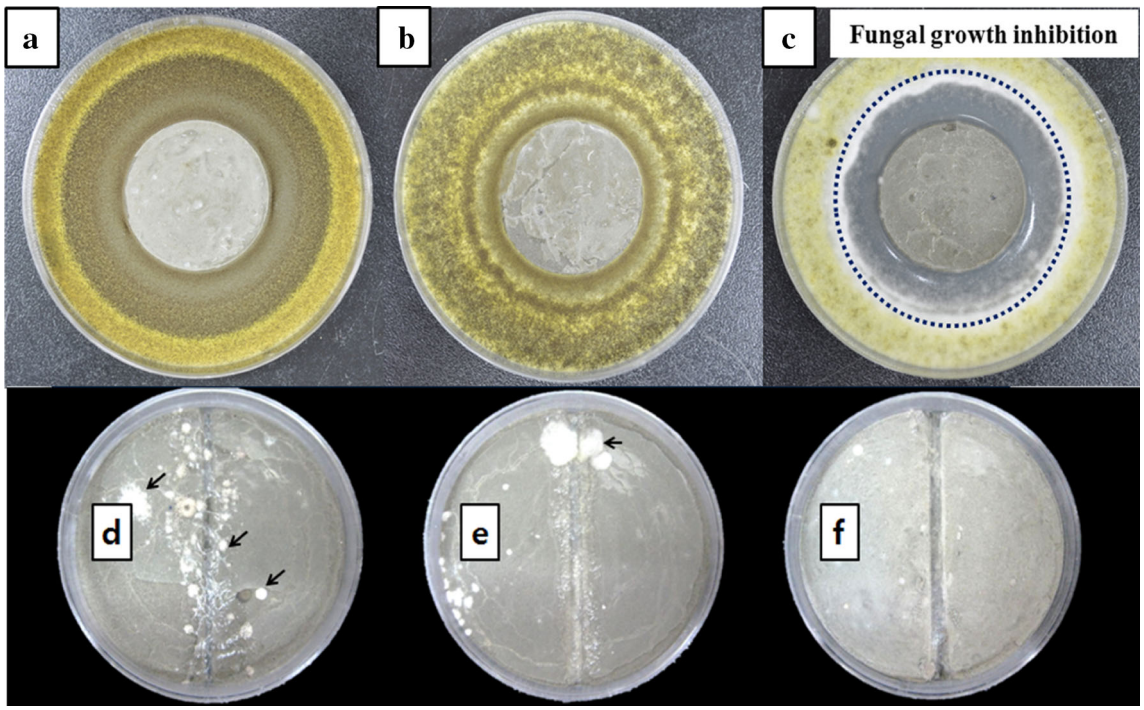

Fig. 3 Fungal growth inhibition of cement paste containing *P. polymyxa* E681 and *FusA* mutant strains. **a** Only SDW treatment, **b** *FusA* mutant strain as negative control, **c** *P. polymyxa* E681 treatment, **d** surface treatment of distilled water, **e** *FusA* mutant strain, and **f** *P. polymyxa* E681 on cement paste. *Black arrows* indicate fungal growth (**d**, **e**) (Color figure online)

Inhibition of the fungal strain surrounding the cement paste added with E681 is shown in Fig. 3c, f. In our results, cement paste containing *P. polymyxa* E681 resulted in the inhibition of *A. niger* spores (10^6 spores/ml) (Fig. 3c). However, *E. coli* K12 and SDW treatment did not show fungal growth inhibition (Fig. 3a, b). In addition to the antifungal effects of the cement paste added with *P. polymyxa* E681, the E681 was treated on the surface of cement paste. As shown in Fig. 3f, the cement paste treated with *P. polymyxa* E681 has no fungal growth, but the surface of cement paste treated with *E. coli* K12, which has no antifungal activity, and only SDW treatment were observed to

have broad fungal growth (Fig. 3d, e). These results suggest that the *P. polymyxa* E681 has strong antifungal effects and could be applied to enhance hygienic conditions in cement material by both adding and surface treatment methods.

In conclusion, *P. polymyxa* E681 has two physiological activities, strong antifungal activity against by *A. niger* which is a major bio-deteriorating agent and calcium carbonate precipitation. When *P. polymyxa* E681 was cultured in calcium-rich medium, much calcium crystals were formed and the crystal structure was determined as vaterite. The calcium precipitation of *P. polymyxa* E681 induced strength increase and crack repair. The cement paste containing *P. polymyxa* E681 showed antifungal effects, and the fungal growth was not detected on the surface of cement paste treated with the *P. polymyxa* E681. All these results support that the antifungal activity and crystal formation of bacteria could be used as multifunctional biomaterial on the development of multifunctional cement mortar.

Nucleotide Sequence

The genome sequence of *P. polymyxa* E681 is available in Genbank under the accession number CP000154 and in the Genome Encyclopedia of Microbes (GEM; http://www.gem.re.kr/).

Acknowledgments This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology of Korea (No. 2010-0024780).

References

1. Adolphe JP, Hourimèche A, Loubière JF, Paradas J, Soleilhavoup F (1989) Les formations carbonates- d'origine bactérienne, formations continentales d'Afrique du Nord. Bull Soc Geol Fr 8:55–62
2. Choi SK, Park SY, Kim R, Lee CH, Kim JH, Park SH (2008) Identification and functional analysis of the fusaricidin biosynthetic gene of *Paenibacillus polymyxa* E681. Biochem Biophys Res Commun 365:89–95
3. De Muynck W, De Belie N, Verstraete W (2010) Antimicrobial mortar surfaces for the improvement of hygienic conditions. J App Microbiol 108:62–72
4. Do JY, So HS, Soh YS (2002) Antifungal activities of isothiazoline/cabamate based organic antifungal agent activated-cement mortars. (AACM) KCI Concr J 14:171–177
5. Do JY, Song H, So HS, Soh YS (2005) Antifungal effects of cement mortars with two types of organic antifungal agents. Cem Concr Res 35:371–376
6. Gaylarde C, Ribas SM, Warscheid TH (2003) Microbial impact on building materials: an overview. Mater Struct 36:342–352
7. Ghosh P, Mandal S, Chattopadhyay BD, Pal S (2005) Use of microorganism to improve the strength of cement mortar. Cem Concr Res 35:1980–1983
8. Jonkers HM, Thijssen A, Muyzer G, Copuroglu O, Schlangen E (2010) Application of bacteria as self-healing agent for the development of sustainable concrete. Ecol Eng 26:230–235
9. Okabe S, Odagiri M, Ito T, Satoh H (2007) Succession of sulfur-oxidizing bacteria in the microbial community on corroding concrete in sewer systems. Appl Environ Microbiol 73: 971–980
10. Park SK, Kim Jay JH, Nam JW, Phan HD, Kim JK (2009) Development of antifungal mortar and concrete using zeolite and zeocarbon microcapsules. Cem Concr Compos 31:447–453
11. Park SJ, Park YM, Chun WY, Kim WJ, Ghim SY (2010) Calcite-forming bacteria for compressive strength improvement in mortar. J Microbiol Biotechnol 20:782–788
12. Park SJ, Park JM, Kim WJ, Ghim SY (2012) Application of *Bacillus subtilis* 168 as a multifunctional agent for improvement of the durability of cement mortar. J Microbiol Biotechnol 22:1568–1574
13. Park JM, Park SJ, Kim WJ, Ghim SY (2012) Application of antifungal CFB to increase the durability of cement mortar. J Microbiol Biotechnol 22:1015–1020
14. Rodriguez-Navarro C, Rodriguez-Gallego M, Ben Chekroun K, Gonzalez-Munoz MT (2003) Conservation of ornamental stone by *Myxococcus xanthus*-induced carbonate biomineralization. Appl Environ Microbiol 69:2182–2193