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NOTE

Stabilities of Artificially Transconjugated Plasmids for the Bioremediation of Cocontaminated Sites

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Here, we attempted to evaluate the activity of artificially transconjugated multiple plasmids in "designer biocatalysts" for the bioremediation of cocontaminated sites under nonselective conditions. We observed profound losses in the percent survivals of artificially transconjugated plasmid activity (66 - 78% loss immediately after freeze-drying, 99.45 - 99.88% loss by the end of 6 months storage) in reconstituted *Pseudomonas* sp. KM12TC. Such unpredictable high losses of this particular plasmid appeared to clearly be a deleterious effect. However, even after 6 months of storage, the cells remained able to degrade 95% of phenol within 9 days, and the full efflux of ^{73}As, as compared to that of the non-freeze-dried cells, was successfully achieved 4 to 9 days later. These results indicate that "stable designer biocatalysts" can remain viable, even after freeze-drying and 6 months of storage.

Key words: *Pseudomonas* sp. KM12TC, artificially transconjugated plasmid activity, cocontaminated site

Heavy metals often occur as cocontaminants, and have been reported to exert detrimental effects on biodegradation (Agency for Toxic Substances and Disease Registry, 1994, 1997, 1999, 2000; Falih, 1997; Leighton *et al.*, 1998; Amor *et al.*, 2001; Groudeva *et al.*, 2001). Therefore, the successful bioremediation of cocontaminated sites requires the stable activity of microorganisms that are both resistant to multiple metals, and capable of xenobiotic degradation. In the preparation of such microorganisms, it has been generally accepted that horizontal gene transfer (i.e. conjugation) may provide the potential for both toxic heavy metal resistance and novel biodegradation pathways in bioremediation systems (Top *et al.*, 2002).

During the last decade, only a few reports have been published with regard to the horizontal transfer of catabolic (xenobiotic-degradative) plasmids among bacteria (Top *et al.*, 2002), and there have been only a few reports regarding the horizontal transfer of heavy metal-resistant genes among bacteria, although a significant study on this topic has recently been published (Yoon, 2003).

The degree to which bioaugmentation involving artificially transconjugated microorganisms can be considered successful will depend principally on the maintenance of the artificially transconjugated plasmids, even under nonselective conditions. Long term freeze-dried storage conditions may constitute one such nonselective condition, as naturally residing plasmid-containing microorganisms are frequently stored in freeze-dried form, thus retaining viability between culture, and subsequent application for the purposes of bioaugmentation. With regard to the stability of naturally residing plasmid-phenotypes, only a few studies have been submitted. The immediate loss of plasmid-encoded catechol 2,3-oxygenase phenotype was shown to exceed 99% after freeze-drying, with additional loss occurring during storage, in *Alcaligenes eutrophus* and *Pseudomonas putida* (Lange and Weber. 1995). Nierman and Feldblyum (1984) also reported plasmid loss occurring in freeze-dried *B. subtilis* and *S. cerevisiae* cultures, after a storage period of 9 months. Gaiek *et al.* (1994) reported a greater than 99.99% viability loss after 6 months of storage of a freeze-dried phenol-degrading mixed culture. Such high loss rates of naturally residing plasmids clearly contraindicate these techniques with regard to transconjugation strategies for the bioremediation of cocontaminated sites.

However, little or no published data is currently available regarding the loss of artificially transconjugated multiple species of plasmids present in a single cell, for environmental application to cocontaminated sites. A possible reason for this paucity of research might be that the

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issue of horizontally transferred bacteria, engineered to be both multiple heavy metal-resistant and capable of xenobiotic degradation, has only recently arisen in response to the growing importance of cocontaminated site bioremediation techniques.

In order to determine the degree of artificially transconjugated plasmid activity, naturally residing plasmid activity, and cell viability occurring subsequent to freeze-drying and one week of storage, we have carried out the following experiments. We used *Pseudomonas* sp. KM10, which harbors pKM10 plasmids (Yoon, 1998), *Pseudomonas* sp. KM20, which harbors pKM20 plasmids (Yoon, 2002), and transconjugant *Pseudomonas* sp. KM12TC (Yoon, 2003), which harbors both the pKM10 plasmid and the transconjugated pKM20 plasmid, obtained by solid surface artificial transconjugation between *Pseudomonas* sp. KM10 and *Pseudomonas* sp. KM20. In addition, we used *P. aeruginosa* TC (Yoon, 2003) as a negative control. Gram-positive *Staphylococcus aureus,* which harbors the pI258 plasmid, was used in order to compare the maintenance, expression, and stability of the plasmids with Gram-negative *Pseudomonas* sp., as pI258 has already been well characterized (Yoon *et al.*, 1991).

Plasmid pKM10's presence in the cells was determined predicated on its ability to utilize phenol as a sole carbon and energy source, and plasmid pKM20's presence was detected according to its arsenical resistance. Plasmid pI258's presence was determined by virtue of its cadmium resistance. The presence or absence of each plasmid in each experiment was confirmed by standard plasmid extractions and 0.8% agarose gel electrophoresis, as has been described elsewhere (Yoon *et al.* 1991). One non-selective medium and four selective media were also employed in this research: (i) Non-selective Luria-Bertani (LB) medium containing 10 g of Bacto Peptone, 5 g of Bacto yeast extract, and 10 g of NaCl in 1 liter of distilled water at pH7.5; (ii) pKM10-selective (phenol degradation-selective) phenol/basal medium (basal medium supplemented with phenol as the sole carbon and energy source at a concentration of 1 g/l). The basal medium has been thoroughly described elsewhere (Yoon, 2002). (iii) pKM20-selective (arsenical resistance-selective) As/LB medium (LB supplemented with 5 mM $Na_2HAsO_4 \cdot 7H_2O$); (iv) both pKM10 and pKM20-selective phenol · As/basal medium (basal medium supplemented with both phenol and arsenate as specified above); and (v) pI258-selective (cadmium resistance-selective) Cd/LB medium (LB supplemented with 1 mM $CdCl_2$).

For the freeze-drying and storage, overnight cultures of transconjugant *Pseudomonas* sp. KM12TC in phenol · As/basal medium, *Pseudomonas* sp. KM10 in phenol/basal medium, *Pseudomonas* sp. KM20 in As/LB medium, and *S. aureus* pI258 in Cd/LB medium were prepared. In order to prepare the cryoprotective cells, half of the cell pellet was resuspended in phosphate buffer (10 mM, pH 7.0) supplemented with 15% skim milk (Hunter-Cevera and Belt, 1996), yielding the same cell concentration as the non-cryoprotective (no skim milk added) cells. Both the non-cryoprotected and cryoprotected cell suspensions were then separated into 0.2 ml aliquots in 1 ml tubular-type ampoules (Wheaton Scientific, USA), and the standard freezing-drying method was employed in order to prepare all of the freeze-dried samples, using a Heto Freeze Dryer FD3000.

The estimation of artificially transconjugated plasmid activity, naturally residing plasmid activity, and cell viability were carried out as follows. In order to reconstitute the freeze-dried samples and count the colonies, ampoules of each sample were opened, the contents were reconstituted with 0.5 mM phosphate buffer (pH 7.2), yielding 10^2, 10^4, or 10^6 fold dilutions, and then 0.1 ml of the diluted cells were spread on nonselective agar LB plates and incubated, for two days at 28°C for *Pseudomonas* sp., or for 2 days at 37°C for *S. aureus.* Phenol-degradation activity (pKM10) was quantified via replica-plating on phenol/basal agar plates; arsenical-resistant activity (pKM20) on As/LB agar plates; dual activity (both phenol-degradation and arsenical-resistant phenotypes in a single cell) on phenol · As/basal agar plates; and cadmium-resistant activity on Cd/LB agar plates. Plasmid activity was expressed as percent survival (CFU on selective agar plates after freeze-drying and storage, divided by CFU on the same kind of agar plates before freeze-drying). Cell viability tests were carried out using identical reconstitution methods, and expressed as percent survival (CFU on LB agar plates after freeze-drying and storage divided by CFU on the same kind of agar plate before freeze-drying). These reconstitution, plating, and counting steps were repeated three times using triplicate vials at each time point, and the percent survivals are shown as the mean of three independent experimental results, including the error bars, with ±1 standard deviation.

Fig. 1 shows the percent survivals of artificially transconjugated plasmid activity of *Pseudomonas* sp. KM12TC, and the naturally-residing plasmid activities of *Pseudomonas* sp. KM10, *Pseudomonas* sp. KM20, and *S. aureus* pI258, during the first seven days of freeze-drying and storage. Their cell viabilities on LB agar plates during the same period are also shown. The percent survival immediately before and after freeze-drying are shown at -0.5 and 0 days, respectively, followed by percent survival after 1, 3, and 7 days.

The profound losses observed in the percent survivals of artificially transconjugated plasmid activity (22 - 34% in Fig. 1C) and naturally residing plasmid activity (23 - 32% in Fig. 1A; 22 - 34% in Fig. 1B; 57 - 72% in Fig. 1D) were seen immediately after freeze-drying. Then, the linear profiles were discernable after 1 day of storage. In fact, the losses in the percent survival of plasmid activities were found to be more severe than the losses of cell via-

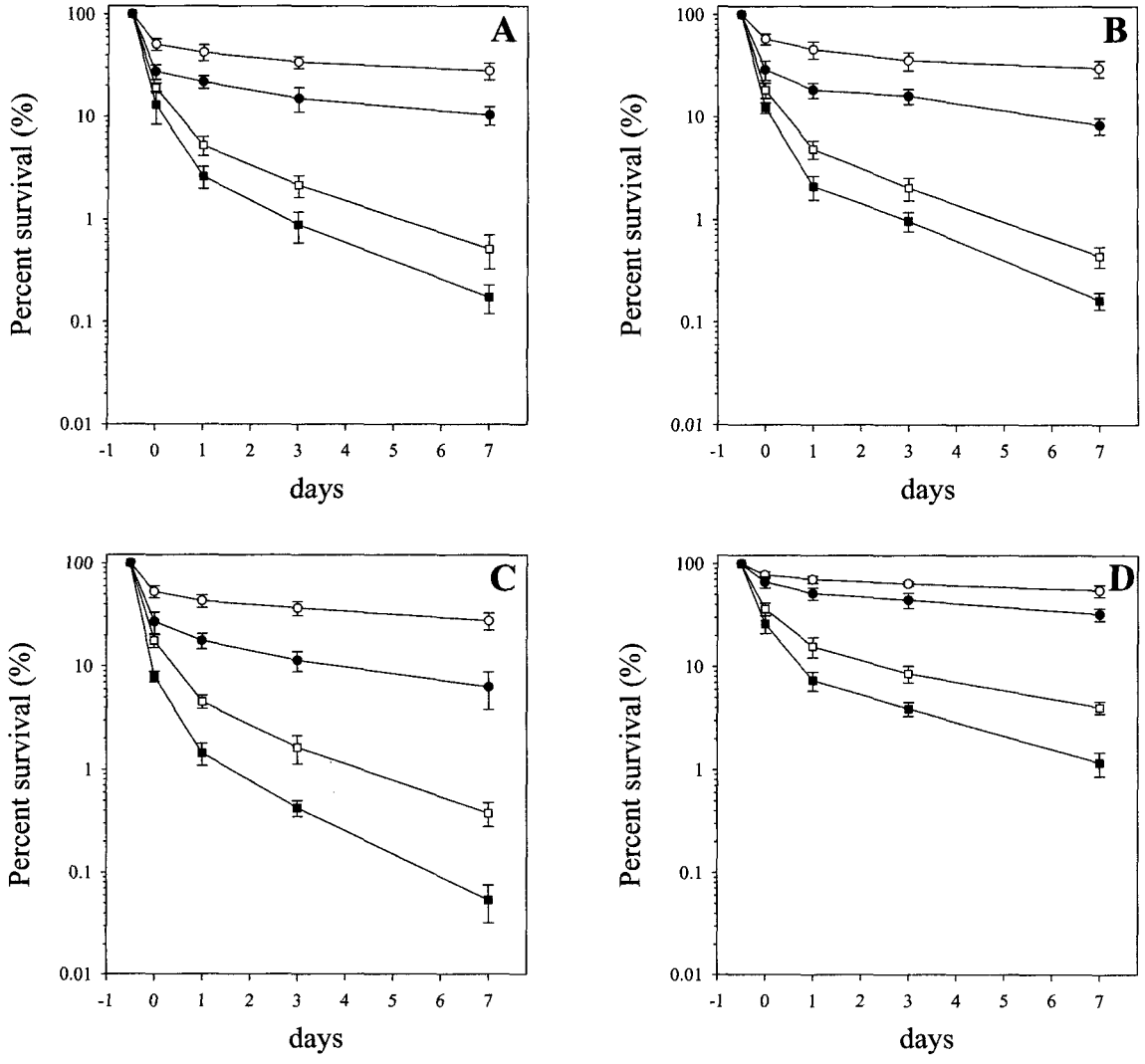

Fig. 1. Estimation of artificially transconjugated and naturally residing plasmid activities and their host cells, both before and after freeze-drying and storage. The plasmid activity and cell viability were expressed as percent survival values, as described. Data collected immediately before freeze-drying were shown at -0.5 days, and considered to be an initial 100% survival point. In all figures, data points represent the mean of three independent trials, and error bars represent standard deviation (±1SD). When error bars are invisible, they are hidden behind the symbols. Panels: A, *Pseudomonas* sp. KM10; B, *Pseudomonas* sp. KM20; C, *Pseudomonas* sp. KM12TC; D, *S. aureus* pI258. Symbols: ○, cell viability of cryoprotected cells; ●, activity of artificially transconjugated or naturally residing plasmids in cryoprotected cells; □, cell viability of non-cryoprotected cells; ■, activity of artificially transconjugated or naturally residing plasmids in non-cryoprotected cells.

bility in all four bacteria (44 - 57% in Fig. 1A; 51 - 65% in Fig. 1B; 46 - 60% in Fig. 1C; 72 - 83% in Fig. 1D). Lange and Weber (1995) evaluated only naturally-residing plasmid activity, reporting that the plasmid activity (plasmid-encoded phenotype) of catechol 2,3-oxygenase was reduced by an order of magnitude, as compared to the viability of its host, *A. eutrophus*, during freeze-drying. Such differences might be attributable to a variety of freeze-drying and storage conditions, including initial freezing temperatures, freezing time, freeze-drying temperatures, storage temperatures, and rehydration conditions (Hunter-Cevera and Belt, 1996).

The observed differences between the percent survival of artificially transconjugated plasmid activity (in *Pseudomonas* sp. KM12TC) and those of naturally residing plasmid activities (in *Pseudomonas* sp. KM10 and *Pseudomonas* sp. KM20) initially appeared to be insignificant, but these differences exhibited a tendency to increase with increasing storage time. The artificially transconjugated plasmids appeared less stable than the naturally-residing plasmids. This tendency to increase (faster decreases in percent survival of the artificially transconjugated plasmid activity) might be detrimental to the transconjugation strategy for the bioremediation of cocontaminated sites.

The percent survivals of cell viability of the non-cryo-

protected *Pseudomonas* sp. immediately after freeze-drying were in the range of 15 to 21% (Fig. 1A, B, and C). In the case of the cryoprotected (freeze-dried in 15% skim milk) *Pseudomonas* sp., percent survivals ranged between 44 and 61% (Fig. 1A, B, and C). The majority of viability loss occurring during freeze-drying is the result of injury to the cytoplasmic membrane and cell wall during drying, and the possible loss of activity upon rehydration (Allison *et al.*, 1998; Pieters *et al.*, 2002). However, these undesirable effects were determined to be less pronounced when a cryoprotectant, such as 15% skim milk, was used, as was expected. Miyamoto-Shinohara *et. al.* (2000) used both 10% skim milk and 1% sodium glutamate as cryoprotectants, reporting that the survival rate of gram-negative bacteria was around 50%, which is similar to the 44-61% percent survivals seen with the cryoprotected *Pseudomonas* sp. (Fig. 1A, B, and C). In addition, Fig. 1D illustrates that the percent survival of the non-cryoprotected *S. aureus* was 21 - 31% immediately after freeze-drying. In the case of the cryoprotected *S. aureus,* this value was 72 - 83%, similar to the approximately 80% cell viability of the gram-positive bacteria after freeze-drying (Miyamoto-Shinohara *et. al.*, 2000). The gram-positive bacteria exhibit a greater resistance to drying than do the gram-negative bacteria, and this difference might be the result of the different structure of the cell surface (Pembrey *et al.*, 1999). Cryoprotectants, such as 15% skim milk, did indeed increase the percent survival rates of plasmid activity and cell viability.

There have been only a few reports regarding plasmid loss during freeze-drying and storage (Nierman and Feldblyum, 1984; Gaiek *et al.*, 1994; Lange and Weber, 1995). However, none of these focused on the loss of artificially transconjugated multispecies of plasmids in a single cell. *Pseudomonas* sp. KM12TC harbors two distinct plasmid species (Yoon, 2003). One of them is pKM10, which contains genes necessary for phenol biodegradation (Yoon, 1998). The other is pKM20, which contains an arsenic-resistant determinant (Yoon, 2002). The pKM20 plasmid of *Pseudomonas* sp. KM20 was horizontally transferred to *Pseudomonas* sp. KM10 via conjugation, in the hopes of generating multiple heavy metal-resistant and phenol-degrading Pseudomonads (Yoon, 2003).

The percent survival of cell viability seen in association with *Pseudomonas* sp. KM12TC (Fig. 1C) was similar to those determined for both *Pseudomonas* sp. KM10 (Fig. 1A) and *Pseudomonas* sp. KM20 (Fig. 1B). However, the percent survival of the dual plasmid activities (phenol-degrading and arsenic-resistant) of *Pseudomonas* sp. KM12TC (Fig. 1C) was found to be significantly lower than that seen for both *Pseudomonas* sp. KM10 (Fig. 1A) and *Pseudomonas* sp. KM20 (Fig. 1B). The lower percent survival of dual plasmids activity in Fig. 1C indicates that the collaboration of both phenol-degrading and arsenic-resistant plasmid activities in a single cell was somehow stunted, possibly due to the loss of either the phenol-degrading plasmid or arsenic-resistant plasmid, or even of both plasmids.

A study has been conducted in order to identify the species of each plasmid which might be present in the reconstituted *Pseudomonas* sp. KM12TC, which failed to grow on phenol · As/basal agar medium. The identification of plasmid species in the cryoprotected *Pseudomonas sp.* KM12TC was performed as follows. Three different ampoules of cryoprotected *Pseudomonas* sp. KM12TC were randomly chosen, at storage days of 0, 1, 3, and 7. The reconstituted cells were then spread onto LB plates, as previously described. Growing colonies on the LB plates were then replica-plated to phenol/basal, As/LB, and phenol · As/basal agar plates. Then, we carried out direct colony counts. The CFU on a phenol/basal agar plate reflected the sum of the number of bacteria that harbored both pKM10 and pKM20 in a single cell, as well as the number of bacteria that harbored only pKM10. The CFU on an As/LB agar plate reflected the sum of the number of bacteria that harbored both pKM10 and pKM20 in a single cell, as well as the number of bacteria that harbored only pKM20. The CFU on a phenol · As/basal agar plate represents the number of bacteria that harbored both pKM10 and pKM20 in a single cell. The number of bacteria harboring only pKM10 was calculated by the subtraction of the CFU on the phenol · As/basal agar plate from the CFU on the phenol/basal agar plate. Similarly, the number of bacteria that harbored only pKM20 was obtained the subtraction of the CFU on the phenol · As/basal agar plate from the CFU on the As/LB agar plate. Also, the number of bacteria that harbored none of these plasmids was derived by the subtraction of the sum of the number of bacteria that harbored pKM10 and the number of bacteria harboring pKM20, and the number of bacteria that harbored both pKM10 and pKM20, from the CFU on the LB agar plate. The presence of either an individual plasmid or none was expressed as a percentage (CFU of specific bacteria harboring a specific plasmid or none divided by the sum of the number of bacteria bearing pKM10 and that of bacteria harboring pKM20 and that of bacteria harboring none of plasmids).

Fig. 2 illustrates the comparisons of the percentages of bacteria that harbored either pKM10 or pKM20, or none, as the result of direct counting on selective agar plates, as described above. We determined there to be no significant correlation between the survival percentages of pKM10 and those of pKM20 (Fig. 2). Our results suggested that a particular plasmid had been selected and lost randomly during freeze-drying and storage. This is the first report of the random loss of multiple plasmids resulting from artificial transconjugation in a single cell designed for the bioremediation of cocontaminated sites. Based on these results, the plasmids appeared to be lost randomly due to the physical cellular damage associated with the freeze-

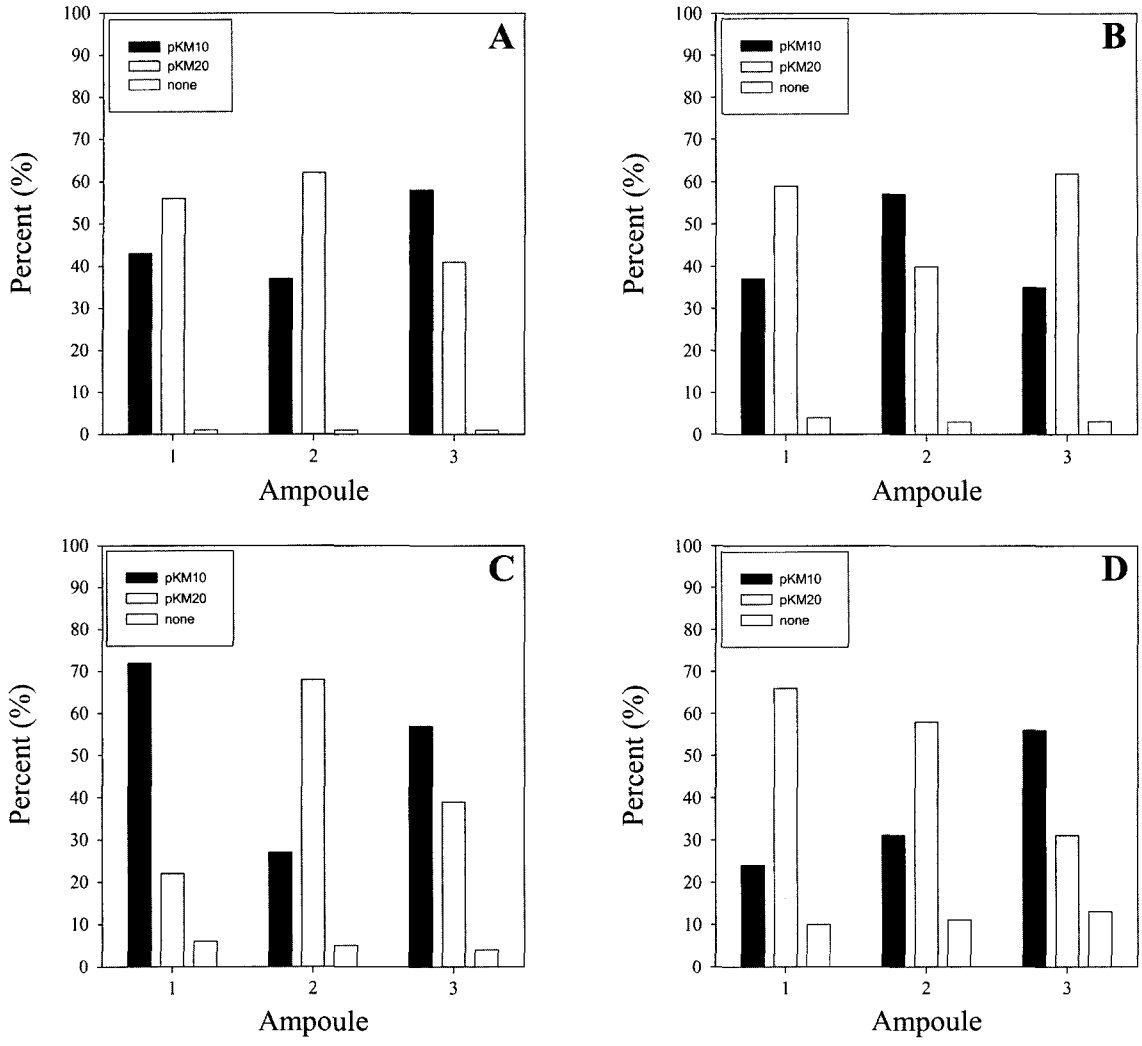

Fig. 2. Identification of plasmid species in the cryoprotected *Pseudomonas* sp. KM12TC. Three different ampoules were chosen. Then, reconstitution and replica plating were conducted as described. Panels: A, 0 day storage; B, 1 day storage; C, 3 days storage; D, 7 days storage.

drying and storage. This random loss could prove greatly detrimental to the development of transconjugation strategies for the bioremediation of cocontaminated sites, as this strategy employed the transconjugation of a specific plasmid with desirable activity, and the loss of this transconjugated plasmid occurred in an unpredictable way.

A long-term analysis of artificially transconjugated plasmid activity and strain viability was therefore conducted, for 6 months. Fig. 3 illustrates that the percent survival of the artificially transconjugated plasmid activity of cryoprotected *Pseudomonas* sp. KM12TC exhibited a relatively rapid decrease, to 1 - 2% during the first month of storage, and a gradual decrease, to 0.12 - 0.55% by the end of 6 months of storage. The percent survival of the cell viability of the same cells decreased less rapidly, to 11 - 19% over the first month, and then more gradually, to 2 - 5% by the end of 6 months of storage. As expected, the overall percent survivals of the cryoprotected cells

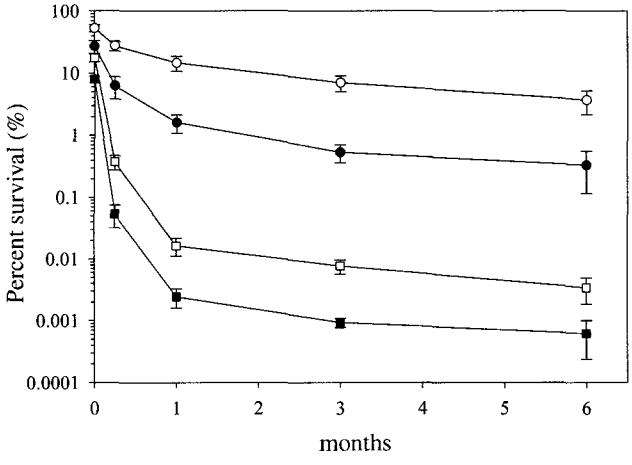

Fig. 3. Activities of artificially transconjugated plasmids and their host cells, *Pseudomonas* sp. KM12TC, during long-term storage. Symbols: ○, cell viability of cryoprotected cells; ●, artificially transconjugated plasmid activity in cryoprotected cells; □, cell viability of non-cryoprotected cells; ■, artificially transconjugated plasmid activity in non-cryoprotected cells.

were, indeed, significantly higher than those of the non-cryoprotected cells. More than a 99% loss of naturally residing plasmid-encoded catechol 2,3-oxygenase phenotype was observed after freeze-drying, with additional loss during storage, in *Alcaligenes eutrophus* and *Pseudomonas putida* (Lange and Weber, 1995), and a more than 99.99% loss of viability was observed after 6 months of storage of a freeze-dried phenol-degrading mixed culture (Gaiek *et al.*, 1994). A similar rapid loss was observed with the artificially transconjugated plasmid activity during the first month of storage, but the loss rate appeared to stabilize remarkably after that (Fig. 3). Such a great loss, though would still be disastrous in an actual bioaugmentation program, especially when the desired plasmid-encode phenotype expression is important to the success of the bioaugmentation.

Therefore, another experiment was conducted, in order to determine whether pKM10-mediated phenol degradation could be achieved after freeze-drying and subsequent storage in the presence of 5 mM arsenate ($Na_2HAsO_4 \cdot 7H_2O$). Estimation of the phenol degradation efficiency has been previously described elsewhere (Yoon, 1998). The values of phenol degradation efficiency for the cryoprotected *Pseudomonas* sp. KM12TC after specified storage times are shown in Fig. 4. Non-freeze-dried heavy metal-resistant *Pseudomonas* sp. KM12TC proved able to degrade phenol within 2 days (a positive control), but the heavy metal-sensitive *P. aeruginosa* TC failed to degrade phenol, even after 5 days of incubation (a negative control), as shown in Fig. 4. This was the expected result. A lag associated with low phenol degradation efficiency was also observed, and this lag grew more pronounced with increased storage times, as is shown in Fig. 4. It took about 4 days for the cells at 0 days of storage to degrade about 95% of the phenol. However, after 1 week of storage, it took 5 days to achieve a similar efficiency. After 1 month of storage, the cells could degrade about 95% of the phenol in about 7 days. Even after 6 months of storage, these cells retained the ability to degrade 95% of the phenol within 9 days, as shown in Fig. 4. The cells that were noncryoprotectively freeze-dried and stored for 6 months failed to degrade phenol, even after only 10 days (data not shown). These results strongly suggest that cryoprotected *Pseudomonas* sp. KM12TC retains phenol-degradation ability after up to 6 months of storage, albeit with prolonged degradation time and a significant loss of plasmid activity over long storage times.

Active efflux of metal lowers intracellular heavy metal concentration levels, and is a method which is frequently employed in order to induce resistance (Yoon, 2002). However, as the reconstituted *Pseudomonas* sp. KM12TC exhibited a rapid and random loss of plasmids, we decided to examine the pKM20-mediated arsenic efflux activity of *Pseudomonas* sp. KM12TC. Arsenic efflux was measured according to a method previously described elsewhere (Yoon, 2002). In Fig. 5, the non-freeze-dried *Pseudomonas* sp. KM12TC cells were confirmed to have successfully removed the preloaded ^{73}As, resulting from the intact efflux activity of pKM20. The non-freeze-dried arsenate-sensitive *P. aeruginosa* TC (without pKM20) failed to lower arsenate levels, even after 4 days of incubation. This was, of course, the expected result. The cells at 0, 1, and 6 months of storage required 4, 6.5, and 9 days, respectively, to achieve arsenate levels comparable to those evidenced by the non-freeze-dried *Pseudomonas* sp. KM12TC. These data were consistent with the phenol-degradation performance data shown in Fig. 4. Even

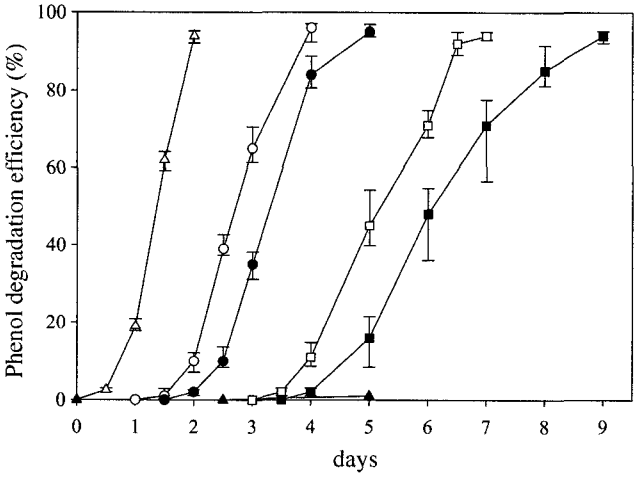

Fig. 4. Phenol degradation performance of cryoprotected *Pseudomonas* sp. KM12TC after different storage times. The preparation of cells and the determination of phenol degradation efficiencies were conducted as described. Symbols: ○, 0 day storage; ●, 1 week storage; □, 1 month storage; ■, 6 months storage; △, non-freeze-dried *Pseudomonas* sp. KM12TC; ▲, non-freeze-dried *P. aeruginosa* TC.

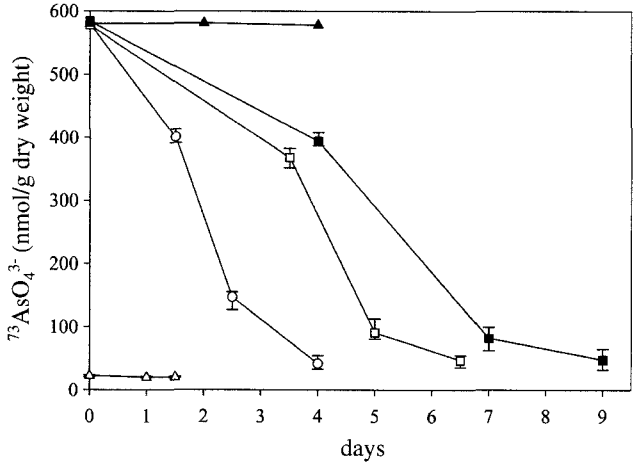

Fig. 5. Efflux of ^{73}As by cryoprotected *Pseudomonas* sp. KM12TC after different storage times. Arsenic efflux was measured after preloading the cells with 50 μM $^{73}AsO_4^{3-}$ as described. Symbols: ○, 0 day storage; □, 1 month storage; ■, 6 months storage; △, non-freeze-dried *Pseudomonas* sp. KM12TC; ▲, non-freeze-dried *P. aeruginosa* TC.

though the freeze-dried and stored cells had rapidly lost their plasmids, the full efflux of ^{73}As was successfully achieved 4 to 9 days later, at a level comparable to that evidenced by the non-freeze-dried cells.

In conclusion, little remains known regarding the stability and performance of artificially transconjugated plasmids when they are present in multi-species for the bioremediation of cocontaminated sites. This is because transconjugation strategies for the bioremediation of cocontaminated sites have only recently emerged as a focus of study. Although the artificially transconjugated plasmids suffered extensive losses immediately after the inception of freeze-drying, the loss rate appeared to stabilize significantly after the first month of storage. During the freeze-drying and storage, plasmids appeared to be chosen and lost at random, regardless of the plasmid species. This is ostensibly due to the damage incurred by the cell membranes of the cells during freeze-drying and storage. Initially, such great losses appeared to contraindicate this bioaugmentation program strategy, especially when the desired plasmid-encode phenotype expression was important to the success of the bioaugmentation of the cocontaminated sites.

However, the 0.12 - 0.55% survival of the artificially transconjugated dual plasmid activities of the cryoprotected *Pseudomonas* sp. KM12TC after six months of storage may remain sufficient for bioaugmentation. The freeze-dried cells achieved a phenol degradation efficiency and an ^{73}As efflux efficiency comparable to that of the non-freeze-dried cells, within 9 days of the incubation. Our results indicate that when it is necessary to ensure a high degree of multiple plasmid activity in freeze-dried bacteria, particularly with regard to the bioremediation of cocontaminated sites dependent on multiple plasmid-encoded activities, it is also necessary to carry out pre-culturing with selective force. The transconjugation strategy for the preparation of "stable designer biocatalysts" harboring artificially transconjugated multiple plasmids with desirable activities in a single cell might constitute a feasible strategy for the bioremediation of cocontaminated sites.

Additional experiments involving the activity of artificially transconjugated plasmids under other nonselective conditions should be carried out, in order to thoroughly delineate the activities of these plasmid, and then, pilot-scale field tests are recommended for the evaluation of the feasibility of the application of artificially transconjugated plasmids for the bioremediation of cocontaminated sites.

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