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METHOD PAPER

Tolerance of plastic-encapsulated *Pseudomonas putida* KT2440 to chemical stress

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Abstract *Pseudomonas putida* dried in the presence of hydroxyectoine or trehalose can withstand exposure to organic solvents and therefore can be encapsulated inside plastics such as polystyrene. Here we show that *P. putida* in a plastic-encapsulated dried tablet exhibits remarkable tolerance to chemical stress, comparable to that of spores of *Bacillus subtilis*.

Keywords Anhydrobiosis · *Pseudomonas putida* · Chemical stress · Hydroxyectoine · Trehalose · *Bacillus subtilis*

Bacterial spores are one of the hardiest forms of life, exhibiting a high degree of resistance to various physical and chemical insults, including heat (Bond et al. 1973; Bloomfield and Arthur 1994; Fox and Eder 1969; Gerhardt and Marquis 1989), irradiation (Gould 1983; Setlow and Setlow 1988), extreme desiccation (Gould 1983), pressure (Russell 1982), freeze-drying and resistance to chemical agents (Bloomfield and Arthur 1994; McDonnell and Russell 1999; reviewed in reference Nicholson et al. 2000). Four important factors have been identified in spore resistance including low core water content, presence of spore coats, the non-permeability of the spore core to hydrophilic chemicals and mechanisms of DNA reparation and protection (Bloomfield and Arthur 1994; Loshon et al. 1999; Russell 1990; Setlow 1999). From a biotechnological point of view, it would be highly desirable to be able to confer similar biostability on beneficial non-sporulating microorganisms.

Previously, we described methods for stabilising Gram-negative bacteria in the dry state using xeroprotectants such as trehalose and hydroxyectoine (Garcia de Castro et al. 2000; Manzanera et al. 2002; Manzanera et al. 2004a). We have termed this process anhydrobiotic engineering, since it was inspired by the extreme desiccation tolerance of anhydrobiotic organisms (Crowe et al. 1992). When stabilised in this manner, bacteria show increased resistance to a number of stresses, including exposure to organic solvents such as chloroform and acetone. This allowed us to suspend dried, powdered *P. putida* in a solution of polystyrene in organic solvent. The suspension can be applied to surfaces and the solvent allowed to evaporate, after which the dried bacteria are encapsulated in plastic. As an example of the utility of this method, we partially coated maize seeds with dried *P. putida* in polystyrene and, on germination, which broke the plastic coat, the bacteria populated the plant root system (Manzanera et al. 2004b). This arrangement, of dry, dormant micro-organisms embedded in an impermeable protective coat, is reminiscent of naturally occurring spores and we therefore postulated that bacteria should show increased resistance to

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chemical attack in aqueous solution, perhaps comparable to that of spores themselves. We have therefore compared the chemical resistance of plastic-encapsulated *P. putida* with that of spores of *B. subtilis*.

The encapsulation method used previously (Manzanera et al. 2004b) was slightly modified to allow quantitation of bacterial survival after chemical treatment. Tablets were prepared from a powdered mixture of dried cells and trehalose: 500 mg of trehalose containing 10^4 dried *P. putida*, prepared as described (Manzanera et al. 2004b), were tableted in a KBr press at 10 atms of pressure for 1–5 min. The resulting tablets were 3 mm in height and 14 mm in diameter (Fig. 1). To rule out any possible deleterious effect of pressure on the cells, 500 mg of the same trehalose/dried cell mixture were dissolved in 25 ml of sterile phosphate buffer before and after moulding. The cfu of the suspensions in three different experiments showed similar results both before and after compression, indicating that the pressure applied at this level has no detrimental effect on the survival of dried cells (data not shown). Tablets containing dried *P. putida* were painted with a solution of 200 mg of polystyrene dissolved in 1 ml of a mixture of chloroform and acetone 1:1 (vol:vol). During solvent evaporation, a thin plastic layer was formed around the tablet. Laminated tablets were allowed to dry for 3 h (although touch dry within 15 min) before experiments were conducted.

To determine whether the plastic layer applied was waterproof, coated tablets were incubated with agitation in a rotary shaker at 100 rpm at ambient temperature for 1 h in 25 ml of phosphate buffer. The tablets appeared completely intact after this incubation, but to check that no cells were released, replicate 100 µl drops of the buffer solution were plated on LB agar. No colonies were found on the plates the following day. In contrast, if laminated tablets were disrupted and the dried cells allowed to dissolve in 25 ml phosphate buffer, almost full recovery (i.e. $\sim 10^4$ cells) of *P. putida* was obtained. This result also showed that lamination of tablets using organic solvent and polystyrene does not affect the survival rate of stabilised, dried cells.

Coating dried cells with polystyrene provides a waterproof layer that should protect the cells from the surrounding environment. This situation resembles the strategy that natural spores adopt when environmental conditions are unfavourable. Since natural spores are more resistant to anti-microbial agents than vegetative cells, we decided to test the tolerance of the encapsulated tablets of dried *P. putida* to a variety of chemical insults. Therefore, both laminated and non-laminated tablets were incubated in 25 ml of either 70% ethanol, 4% formaldehyde, 0.1 M K_2MnO_4, 1% bleach, 1/80 solution of seldine (a commercial iodine-based disinfectant), 10 mM NaOH (pH 12), or 10 mM HCl (pH 2), solutions well-known as biocides (McDonnell and Russell 1999); 25 ml of phosphate buffer was used as a control. After 30 min incubation at 70 rpm (ambient temperature), serial dilutions from each solution were made and plated on sterile LB-agar plates for colony counting. Intact laminated tablets were withdrawn from the solutions and washed twice with sterile milli-Q water. After disruption of the plastic layer the tablets were incubated in sterile phosphate buffer for 30 min and replicate 100 µl drops were plated for colony counting. No colonies were found in any of the solutions in which non-laminated tablets were used, except for the phosphate buffer control, where $\sim 10^4$ cells were recovered. However, the survival rates of the laminated cells ranged from 10–100% of control values (Table 1). In the case of the laminated tablets incubated in the seldine solution the plastic around the tablet was disrupted, possibly due to the oxidant effect of the iodine solution, which would explain the lack of survival of *P. putida*.

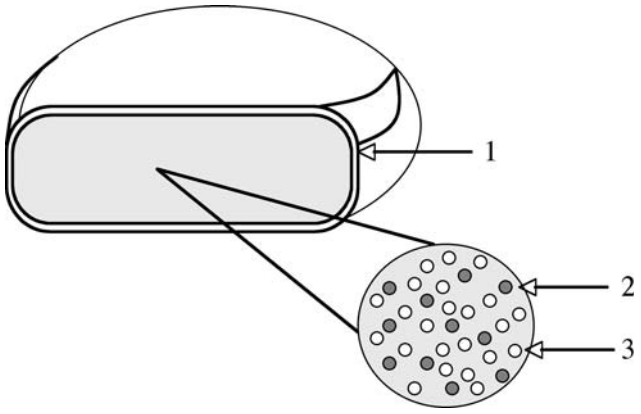

Fig. 1 Schematic drawing of laminated tablet. *1* represents the polystyrene coat, *2* the trehalose granules containing *P. putida,* and *3* the trehalose granules forming the bulk of the tablet

Table 1 Survival of unprotected *P. putida* (non-laminated tablets), encapsulated *P. putida* (laminated tablets) and *B. subtilis* spores after treatment with different disinfectants

	Survival of *P. putida* in non-laminated tablets	Survival of encapsulated *P. putida*	Survival of *B. subtilis* spores
M9	0.8×10^4	1×10^4	51×10^4
Ethanol (70%)	ND	1.5×10^4	36×10^4
Formaldehyde (4%)	ND	1.8×10^4	39×10^4
0.1 M K_2MnO_4	ND	0.3×10^4	28×10^4
Bleach (1%)	ND	1.3×10^4	44×10^4
1/80 Seldine	ND	ND	13×10^4
10 mM NaOH (pH 12)	ND	0.1×10^4	45×10^4
10 mM HCl (pH 2)	ND	0.9×10^4	49×10^4

ND no survivors detected

For comparison, an identical experiment was performed using *B. subtilis* spores. *Bacillus subtilis* was grown in M9 with 0.5% (*v/v*) glycerol as sole C-source. To obtain spores, 10^7–10^9 cells were collected by centrifugation and the pellets washed with sterile distilled water and incubated at 72°C for 30 min. Around 30% of spores per vegetative cell were obtained. A high degree of spore survival was obtained—in the range 25–100%—for all chemical treatments (Table 1). These results support previous findings that spores are generally significantly more resistant than vegetative cells to a wide variety of toxic chemicals and physical insults (Bloomfield and Arthur 1994; McDonnell and Russell 1999; Russell 1990). The observation that plastic-encapsulated, dried *P. putida* exhibit similar chemical resistance demonstrates the potential of this novel biotechnology for stabilising non-sporulating organisms. Storage of culture collections and libraries could be simplified using a plastic encapsulation procedure, for example, since there is no requirement for freezing or storage under vacuum or in an inert atmosphere.

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