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Improving the Catabolic Functions of Desiccation-Tolerant Soil Bacteria

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Abstract

Bacterial strains were selected from a desiccated polluted soil for their drought tolerance and their ability to grow on diesel oil in view of incorporating them in a bioaugmentation product. These products are useful in case of recalcitrant xenobiotic pollution, where there is no intrinsic biodegradation activity in the soil. These strains grow on the easily degradable components of diesel oil. Introduction of new catabolic genes into these desiccation-tolerant bacteria in order to improve their catabolic functions was considered.

Plasmid-borne catabolic genes coding for enzymes involved in the degradation of more recalcitrant compounds (Isopropylbenzene, trichloroethene, 3-chlorobenzoate, 4-chlorobiphenyl, biphenyl) were successfully introduced in some of the desiccation-tolerant strains by means of natural conjugation. Strains exhibiting good tolerance to desiccation and able to grow on the new carbon sources were obtained. The frequencies of integration of the plasmids ranged from 2×10^{-8} to $9.2\ 10^{-2}$ transconjugants/acceptor.

Drought-tolerance is indeed important for bioaugmentation because of its intrinsic ecological significance and because a bioaugmentation starter has to be conditioned in a desiccated form to ensure good shelf-life. The conservation of the properties during storage was evaluated by accelerated storage tests.

Index Entries: Bioaugmentation; drought tolerance; conjugation; plasmids; preservation.

Introduction

Aromatic hydrocarbons are important sources of pollution in many soils of industrial sites *(1)* as well as domestic subsurface. In case of pollution

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with recalcitrant hydrocarbons, it is sometimes useful to use bioaugmentation technique with starter cultures of microorganisms that are adapted to the pollutant *(2,3)* to achieve the biodegradation of the pollutant in a reasonable time period. In case of use of starter culture, it is of prime importance to have a product with good shelf-life in a ready-to-use form. The desiccated form fulfills these criteria. However, most of biodegrading bacterial strains do not tolerate desiccation *(2)*.

In previous papers *(2,4)*, we reported the selection of xenobiotic degrading bacteria with a marked tolerance to desiccation. These bacterial strains could be used in dried bioaugmentation products, but the range of xenobiotic compounds on which they were able to grow was restricted to the aliphatic fraction of diesel oil. The introduction of plasmid-borne genes into a selected strain with selected properties (such as drought-tolerance) has been used to improve the performances of such strain. Marconi et al. *(5)*, for example, have recently enlarged the degradation properties of a solvent-tolerant *Pseudomonas putida* and Ramos et al. *(6)* have expanded the catabolic potential of another *Pseudomonas putida* able to resist higher concentration of aromatic hydrocarbons. In this article, we report the improvement of the catabolic functions of strains selected for their drought-tolerance by the introduction of plasmid-borne catabolic genes responsible for the degradation pathway of isopropylbenzene (IPB), trichoroethene (TCE) or biphenyl (BP).

Although the genus *Rhodococcus* has been shown to comprise a group of Gram-positive soil bacteria known for their ability to degrade various xenobiotic compounds, including phenols *(7)*, insecticides *(8)*, herbicides *(9)*, acrylamides *(10)*, or halogenated alkanes *(11)*, more plasmids are known in the Gram-negative strains. Many *Pseudomonas* species are known to grow on monoalkylbenzenes such as isopropylbenzene, ethylbenzene, and toluene *(12)*, but they belong to Gram-negative species, whereas most of the drought-tolerant strains are Gram-positive *(13)*. It is therefore difficult to introduce and express these plasmids into the Gram-positive desiccation-tolerant strains.

We compared the drought-tolerance of the constructed strains with the donors and other reference strains *(14)*. We analyzed the stability of the new phenotype in the improved strains. The survival after drying and the stability in time of the surviving fraction are necessary, but not sufficient conditions for a starter culture to be competitive. In addition, the degradation properties must be maintained in the surviving cells. Actually, ensuring phenotypic stability after drying and during preservation is a problem because the viability of the cells after preservation may not necessarily correlate with the full maintenance of all properties. Plasmid-encoded degradation activities may be lost at high frequencies during drying of a culture, although little loss of viability occurs *(15)*. Changes in various properties have been reported, especially during inadequate lyophilization *(16,17)*. Although Lang and Malik *(15)* found a loss of certain proper-

Table 1
Identification of Four Dried Soil Isolates
and the Main Carbon Sources They Metabolize

Strain	Genera and species (Gram coloration)	Metabolized carbon sources
T902	*Rhodococcus erythropolis* (+)	Alkanes (C_{10}–C_{15}), branched alkanes, diesel oil
TFI	*Acinetobacterjohnsonii* (–)	Branched alkanes
TF7	*Micrococcus luteus* (+)	Benzene, phenol, m-xylene, diesel oil
TFLI	*Methylobacterium extorquens* (+)	Diesel oil

ties in their strains, they could not detect any plasmid loss. An accelerated storage test *(18,19)*, based on the Arrhenius activation energy principle, may be applied to predict the stability of the dry product during long-term preservation. With this test, the half-life of the dry product can be determined, for any storage temperature, in relatively short experimental time. An adaptation of the technique allows the evaluation of the activity maintenance of the dry product.

Material and Methods

Bacterial Strains and Growth Conditions

The drought-tolerant strains (*see* Table 1) were isolated from desiccated soil samples as described elsewhere *(2)*.

The plasmid bearer strains come from Dr. L. Diels's VITO (Mol, Belgium) strain collection. The *Rhodococcus erythropolis*, BD2, strain was kindly provided by Dr. Beate Haverhoff from the Institut für Mikrobiologie, Georg-August-Universität, Göttingen, Germany. The *Rhodococcus erythropolis*, LMG5359, strain, and the *Deinococcus radiodurans* ATCC 13939 were purchased from the Belgian Type Culture Collection from The Laboratorium voor Mikrobiologïe van Gent (Gent, Belgium).

All cultures were incubated at 30°C either in rich 869 medium (peptone from casein 10 g/L; yeast extract 5 g/L; NaCl 5 g/L; glucose 1 g/L and $CaCl_2$ 0.345 g/L; pH = 7.0) or in 284 minimal mineral medium (50 m*M* Tris-HCl; 80 m*M* NaCl; 20 m*M* KCl; 20 m*M* NH_4Cl; 3 m*M* Na_2SO_4; 1 m*M* $MgCl_2 \cdot 6H_2O$; 0.2 m*M* $CaCl_2$; 0.2 m*M* $Na_2HPO_4 \cdot 2H_2O$; 4.8 mg/L Fe (III) NH_4 citrate; 1 mL/L trace elements solution; pH 7.0). The trace element solution had the following composition: 10 m*M* HCl; 0.5 m*M* $ZnSO_4 \cdot 7H_2O$; 0.5 m*M* $MnCl_2 \cdot 4H_2O$; 1 m*M* H_3BO_3; 0.8 m*M* $CoCl_2 \cdot 6H_2O$; 0.1 m*M* $CuCl_2 \cdot 2H_2O$; 0.1 m*M* $NiCl_2 \cdot 6H_2O$; 0.15 m*M* $Na_2MoO_4 \cdot 2H_2O$. For solid media, agar was added to a concentration of 1.2% (w/v). When growing at the expense of volatile aromatic compounds such as IPB, the substrate was provided via the gas phase. One-mL portion of the substrate was added in a dish inside a tightly-closed jar wherein the plates were incubated.

Selection of Rifampicin-Resistant Mutants of R. erythropolis *T902*

In order to have a phenotype to differentiate T902 from BD2, mutant colonies of T902 were isolated on 869 medium containing 150 mg/L of rifampicin. The isolated colonies were streaked again on 869 medium with the same concentration of the antibiotic to obtain a whole population of rifampicin-resistant mutants. The mutant was named $T902_{rif}$.

Plasmids and Conjugation

Plasmids (*see* Table 2) were chosen with genes responsible for degradation pathways of recalcitrant compounds. Plasmid transfers were attempted by plate mating. The donor and receptor strains were pre-grown overnight in 5 mL 869 liquid medium with selective antibiotic marker when relevant. The cells were harvested and washed in 2 vol of magnesium sulfate (10^{-2} *M*) and resuspended in 1 vol of $MgSO_4$. The mating occurred during 24 h on 869 agar plates by depositing 20 µL of each parental strain on the same spot and 20 µL apart of each other to check that each parental strain had grown. The appropriate dilutions of the mating colony were plated out on a selective medium allowing growth of the receptor strain and a selective medium allowing only growth of the transconjugants combining the properties of both parental strains. The mating frequency was evaluated by the ratio of these two colony-forming units (CFU) counts and expressed as the number of transconjugant CFU/acceptor CFU. The supposed-transconjugant colonies were checked for the presence of both phenotypes from the donor and recipient strains. Several verifications were operated on the mated strains. The absence of growth of both recipient and donor strains was checked on the transconjugants-selective-medium. When selection was made according to one phenotype, the other phenotypes from the donor-strain plasmid were tested on the supposed-transconjugants. Eventually, the presence of the plasmid in the recipient strain was confirmed by gel electrophoresis analysis and comparison with the donor strain.

Checking the Phenotype of the Transconjugant Colonies

When colonies were found to grow on the transconjugants-selective-medium, 10 of these colonies were collected. Replications of these colonies were deposited on different media containing each of the markers that are likely to be expressed in the transconjugants.

Screening for Indole Oxidation and Meta-Cleavage Activity

IPB is oxidized to 3-isopropylcathechol by the isopropyl dioxygenase. The opening of the benzene cycle occurs then with 3-isopropylcathechol 2,3-dioxygenase to give the meta-cleavage product, the 2-hydroxy-6-oxo-7-methylocta-2,4-dienoate. Enzymes were detected by activity staining on agar plates. Therefore, 10 µL of 2 *M* indole dissolved in methanol was placed near a single colony, and after 30 min of incubation at room temperature, the colony turned blue owing to the formation of indigo *(15)*.

Table 2
Description of the Plasmids Used[a]

Gene	Plasmid	Incompatibility group	Marker	Strain	Genera and species	Phenotype
	pRK2013	Inc Q	Km^+	CM404[b]	*E. coli*	Helper, aux
bph (JHR22)	pLAFR3::bph	Inc P1	Tc^+	CM1470	*E. coli*	BP^+, aux
bph (A5)	pLAFR3::bph	Inc P1	Tc^+	CM2014	*E. coli*	BP^+, $4CBP^+$, aux
bph (JHR22)	pLAFR3::bph	Inc P1	Tc^+	CM2422	*E. coli*	BP^+, aux
bph (A5)	RP4::Tn4371[c]	Inc P1	Tc^+, Amp^+, Km^+	CM844	*E. coli*	BP^+, $4CBP^+$, aux
nah	pNAH7	Inc P9	–	PpG7[d]	*P. putida*	$Naphtalene^+$
	pBD2[e]		–	BD2	*R. erythropolis*	IPB^+, TCE^+, rif^-

[a]D. Springael, personal communication.
[b]*See* ref. *22*.
[c]*See* ref. *21*.
[d]*See* ref. *13*.
[e]*See* ref. *20*.

Km^+, resistant to 50 mg/L of kanamycin; Tc^+, resistant to 20 mg/L of tetracycline; Amp^+, resistant to 50 mg/L of ampicillin; rif^-, sensitive to 150 mg/L of rifampicin; BP^+, able to grow on biphenyl; naphtalene$^+$, able to grow on naphtalene; $4CBP^+$, able to grow on 4-chlorobiphenyl; IPB^+, able to grow on isopropyl-benzene; TCE^+, able to grow on trichloroethene; aux, unable to grow on minimal medium (auxotroph).

Preparation of DNA for Detection of Linear Megaplasmid

Strains with megaplasmids were cultivated overnight in 5 mL 869 medium and DNA samples embedded in agarose plugs were prepared according to the method described by Dabrock et al. *(20)*.

Pulsed-Field Gel Electrophoresis

Pulsed field gel electrophoresis (PFGE) was used to isolate pBD2 plasmid from the transconjugant strain and from BD2 strain. A CHEFE I system obtained from Bio-Rad (Bio-Rad S. A. Belgium, Nazateth, Belgium) was used in the conditions described by Dabrock et al. *(20)*.

Drying Methods

Before drying, cells were grown overnight in 869 rich medium. They were harvested and washed with 2 vol of magnesium sulfate (10^{-2} M).

Fluidized Bed Drying

Fluidized bed drying was achieved in an Aeromatic system (Niro-Aeromatic AG, Budendorf, Switzerland), with 500 g silica as a solid substratum on which the 500 mL $MgSO_4$ cell suspension was sprayed with 10% (w/v) maltodextrine (Roquette, Lestrem, France) as protective agent. The inlet air temperature was set at 45°C and the outlet air temperature ranged from 21°C to 28°C according to the advancement of the drying process. The final dry matter of the product reached 98%.

Spray Drying

Spray drying was achieved in a Mobile Minor Niro spray drier (Niro, Copenhagen, Denmark). The composition of the cell suspension medium was the same as for fluidized bed drying. The air inlet temperature was set at 110°C and the outlet air temperature was maintained at 55°C by adapting the liquid inlet flow rate. The final dry matter of the product reached 92%.

Lyophilization

After the washing step, the samples were resuspended in 1 vol of $MgSO_4$ (10^{-2} *M*) either with 0.5% (w/v) trehalose or without protector. The lyophilization was made after freezing at –60°C and was carried out for 24 h in a Leybold-Heraeus Lyovac GT2 (Leybold-Heraeus, Köln, Germany) at a pressure of 10^{-5} bar and with a trap temperature of –40°C.

Rehydration Procedure

All dried samples were rehydrated in $MgSO_4$ (10^{-2} M) at 30°C under shaking for 30 min.

Determination of the Maintenance of the Biodegradation Properties After Drying and During Long-Term Preservation

Accelerated Storage Tests

Before and after drying, cells were plated out on 869 agar and simultaneously on 284 mineral medium with IPB as sole source of carbon. The

survival ratio was determined by the ratio of the rich medium count before and after treatment. The still-active fraction was measured by the ratio between the mineral medium count and rich medium count, both done at the same time after drying.

Dried samples were stored at different temperatures (55, 37, 25, and 4°C) in air-tight closed aluminized bags under vacuum and in the dark. Colony counts and biodegradation properties were measured at regular time intervals. All samples were done in duplicates and the colony counts values are averages of 10 counts/sample. At each temperature, the $\log_{10}$ of the number of CFU on rich medium was plotted vs time. The $\log_{10}$ of the colony count on the minimum selective medium reflecting the maintenance of the biodegradation activity of the cells was plotted on the same graph. The $\log_{10}$ of the slope of the different lines obtained by linear regression on these data were collected and plotted vs 1/T in a so-called Arrhenius plot, where T is the absolute temperature. On this graph, the half-life of the product can be determined for any storage temperature.

Results and Discussion

Introduction of Catabolic Genes into Drought-Tolerant Strains

Mating Experiments

Several mating experiments (*see* Table 3) were attempted with a drought-tolerant strain as the recipient strain in order to improve its catabolic functions. The goal of these experiments was to obtain drought-tolerant strains with high metabolic activity towards recalcitrant xenobiotics. These new strains obtained by natural conjugation would be good candidates for a desiccated bioaugmentation product to introduce in the environment.

In two mating experiments, a rifampicin-resistant mutant of T902 was used to differentiate the recipient strain from the donor. In the same conditions of isolation of T902 rifampicin-resistant mutant, no mutant of BD2 could be isolated. BD2 is rifampicin-sensitive and the *P. putida* PpG7 was also found to be sensitive to rifampicin. When mating a strain with an *E. coli* plasmid bearer (CM strains), there is no need for a specific recipient strain marker, because the *E. coli* strains are unable to grow on minimum selective media.

Mating between the Gram-positive strain, *R. erythropolis* T902 and the *R. erythropolis* BD2, harboring the pBD2 plasmid responsible for the degradation pathway of isopropylbenzene was first attempted and gave transconjugants (named T902.1) with a high frequency (9.2×10^{-2} transconjugants/acceptor). These two strains belong to the same species and are phylogenitically close to each other, which enables the high frequency of conjugation. However, few genes are known to be borne on this type of plasmid. pBD2 is a linear megaplasmid originally isolated from the Gram-positive *R. erythropolis* BD2 strain *(20)*.

In a second phase, transfer of plasmids harbored in Gram-negative strains into the Gram-positive *Rhodococcus* were attempted. These matings

Table 3
Mating Experiments Attempted between Drought-Tolerant Strain T902 and Strains Harboring Plasmids Encoding for Genes Responsible for Recalcitrant Xenobiotic Compounds Degradation

Recipient[a]	Donor	Helper	Selective media[b]	Frequency
T902 (rif$^+$)	BD2	–	284+IPB+rif$_{150}$	$9.2\ 10^{-2}$
T902	CM844	–	284+BP+TC$_{20}$	$<3\ 10^{-10}$
T902	CM1470	–	284+BP+TC$_{20}$	$2\ 10^{-8}$
T902 (rif$^+$)	PpG7	–	284+naphtalene+rif$_{150}$	$<1\ 10^{-9}$
T902	CM2014	CM404	284+BP+TC$_{20}$	$<1\ 10^{-9}$
T902	CM2422	CM404	284+BP	$<1\ 10^{-9}$

[a]Rif$^+$, resistant to 150 mg/L rifampicin.
[b]Tc20, culture medium containing 20 mg/L of tetracycline; rif$_{150}$, culture medium containing 150 mg/L of rifampicin; BP, biphenyl; IPB, isopropylbenzene.

Table 4
Verification of the Phenotype of the Transconjugant Colonies Obtained by the Mating of the *Rhodococci erythropolis* T902 and BD2

	869rif	284	284+IPB	284+rif+IPB
T902	+	–	–	–
BD2	–	–	+	–
T902.1	+	–	+	+

+, growth; –, no growth detected.

would open a wider range of possible genes to introduce into the drought-tolerant Gram-positive strains. However, only one mating of the Gram-positive strain *R. erythropolis* T902 with Gram-negative strains gave CFU's exhibiting the phenotype of transconjugants. The *E. coli* strain CM1470, harboring a broad host range plasmid from the Inc P incompatibility group afforded a frequency of conjugation of 2×10^{-8} transconjugants/acceptor (named T902.2) when mated with T902. Although this frequency was low, it is an important opportunity to introduce other genes from Gram-negative strains into the *Rhodococcus* ones by means of the pLAFR3 which could potentially be used as a vector.

Checking the Conjugation Products

In order to verify whether the collected transconjugants are really bearing the plasmid and that they really derive from the recipient strain, some tests were undertaken. The donor strain, the recipient strain and 10 transconjugant colonies were screened for the properties of the parental strains. Table 4 gives the result of these verifications for the first mating.

All the markers of the parental strains were expressed in all 10 tested transconjugant colonies. There was no residual growth on the minimal medium without carbon source. The recipient strain (T902) did not grow on

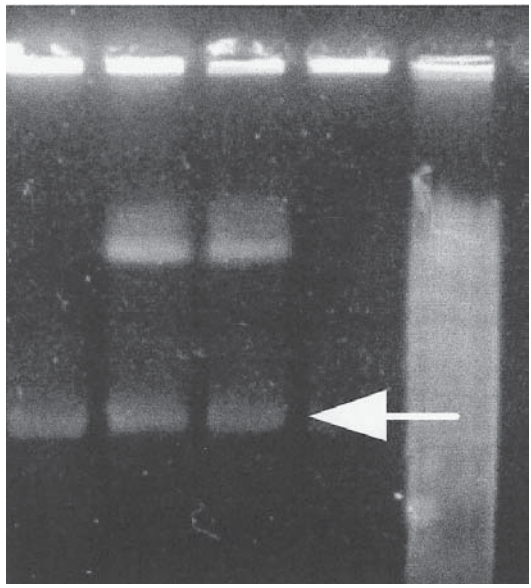

Fig. 1. Pulsed field gel electrophoresis of strains BD2 and T902.1. Lane 1, BD2; lanes 2 and 3, T902.1; lane 5, λ-ladder size standard of 48.5 kb. The arrow indicates the pBD2 plasmid of an approximate size of 212 kb.

the hydrocarbons for the degradation of which the plasmid codes. The donor strain (BD2) was sensitive to the selection marker (rifampicin) of the recipient one. All the verifications indicated that the T902.1 colonies were the product of the introduction of the pBD2 plasmid into the strain T902. This conjugation improved the catabolic functions of the drought-tolerant T902 strain.

As the final proof of the presence of the linear pBD2 megaplasmid in the drought-tolerant strain, T902.1, plasmid extraction and separation with PFGE technique was conducted (*see* Fig. 1).

The pBD2 plasmid was present in the T902.1 strain. Also seen on this figure was another plasmid of an approximate size of 400 kb present in T902.1. This plasmid comes from the T902 recipient strain in which it is also present (result not shown).

The second conjugation experiment giving transconjugants occurred between the Gram-positive T902 strain and the Gram-negative CM1470 strain. The same verifications were operated (*see* Table 5). All verifications indicated that the T902.2 colonies were the product of the introduction of the pLAFR3::bph plasmid into the strain T902. The introduction of Gram-negative plasmids into Gram-positive bacteria by natural conjugation is not common. Only one attempt gave rise to transconjugant colonies in this work with a very low frequency. Most of the drought-tolerant strains being Gram-positive, and most known plasmids involved in the degradation of xenobiotics being found in Gram-negative bacteria, this result is

Table 5
Verification of the Phenotype of the Transconjugant Colonies Obtained by the Mating of the *Rodococcus erythropolis* T902 Strain and the *E. coli* CM1470 Strain, Harboring the Plasmid pLAFR3::bph

	869+Tc_{20}	869+Amp_{50}	869+Km_{50}	284	284+BP	284+Tc_{20}+BP
T902	–	–	+[a]	–	–	–
CM1470	+	–	–	–	–	–
T902.2	+	–	+[a]	–	+	+

[a]T902 was found to be naturally resistant to 50 mg/L of kanamycin.
+, growth; –, no growth detected.

promising. However, the stability of this plasmid in the Gram-positive strain has not yet been studied.

Expression of New Properties in Drought-Tolerant Strain T902.1 and Comparison with Donor Strains

The transconjugant strain, named T902.1, resulting from the conjugation of BD2 with T902 were characterized. The expression of the genes borne on the plasmid were measured in T902.1.

Growth on Hydrocarbons

The optical density (OD) of the liquid minimal medium cultures of T902, BD2 and the transconjugant strain T902.1 was measured (*see* Fig. 2) with 1000 ppm of IPB as sole source of carbon. No growth of T902 on this source of carbon could be detected. The transconjugant strain grows well on this carbon source. The genes encoding for the pathway of IPB degradation borne on the pBD2 plasmid are expressed in the T902.1 strain.

The meta-cleavage activity (3-isopropylcatechol 2,3-dioxygenase activity) could be detected by indigo staining in T902.1 and BD2 colonies grown on minimal medium with IPB. In colonies grown on complex medium (869), no activity was detected after incubation with indole for 1 h. The activity could be induced in these colonies by adding IPB to the culture and measuring the activity after 30 min.

Tolerance to Mercury Chloride

The pBD2 plasmid encodes for the resistance to mercury. The level of tolerance to mercury chloride was measured in the parental strains and in the transconjugant one (*see* Fig. 3).

Another *R. erythropolis*, LMG 5359, from a type culture collection was used as a reference without *a priori* mercury resistance. The concentrations tested ranged from 0.5 to 15 µ*M* in mercury (0.5, 1, 2.5, 5, 10, 15).

The BD2 donor strain does not grow on a concentration of mercury chloride above 2.5 µ*M*. The recipient strain T902 is already resistant to a concentration up to 10 µ*M* without the presence of the pBD2 plasmid. Another plasmid is present in the T902 strain and its T902.1 derivative

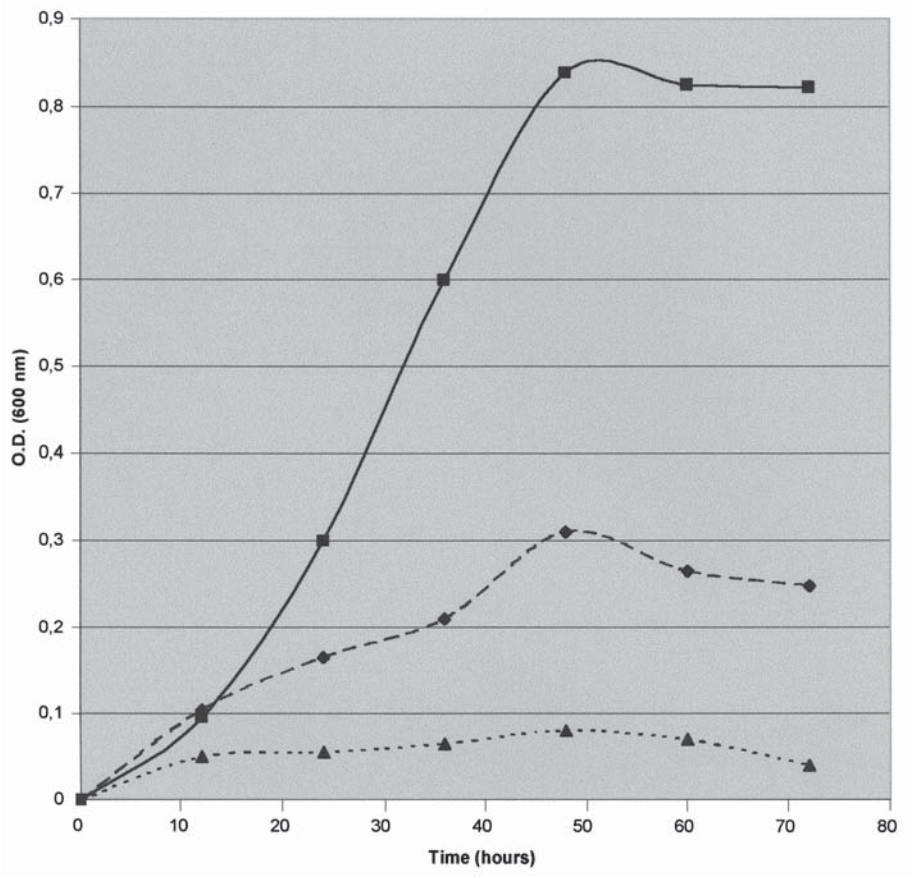

Fig. 2. Optical density of the 284 liquid cultures of T902 (- -▲- -), BD2 (– –●– –), and T902.1 (—■—) on 1000 ppm IPB.

(*see* Fig. 1). This other plasmid might be responsible for this high value. The introduction of the pBD2 plasmid into T902 (strain T902.1) does not give rise to a higher level of tolerance toward mercury.

Drought Tolerance

Since the original property of the recipient strain was its drought-tolerance, the drought-tolerance of the transconjugant cells was determined under different drying conditions (*see* Table 6). The T902 strain was selected for its tolerance to desiccation, LMG5359 was chosen as a reference of the same genera and species without *a priori* desiccation-tolerance properties and BD2 was the plasmid bearer. The *D. radiodurans* is known to be desiccation tolerant *(14)* in some conditions and was chosen as a positive reference.

The drought tolerance of all four *R. erythropolis* strains is of the same order of magnitude with all techniques. The drought-tolerant strain T902 does not exhibit a better behavior when dried. This could indicate that the

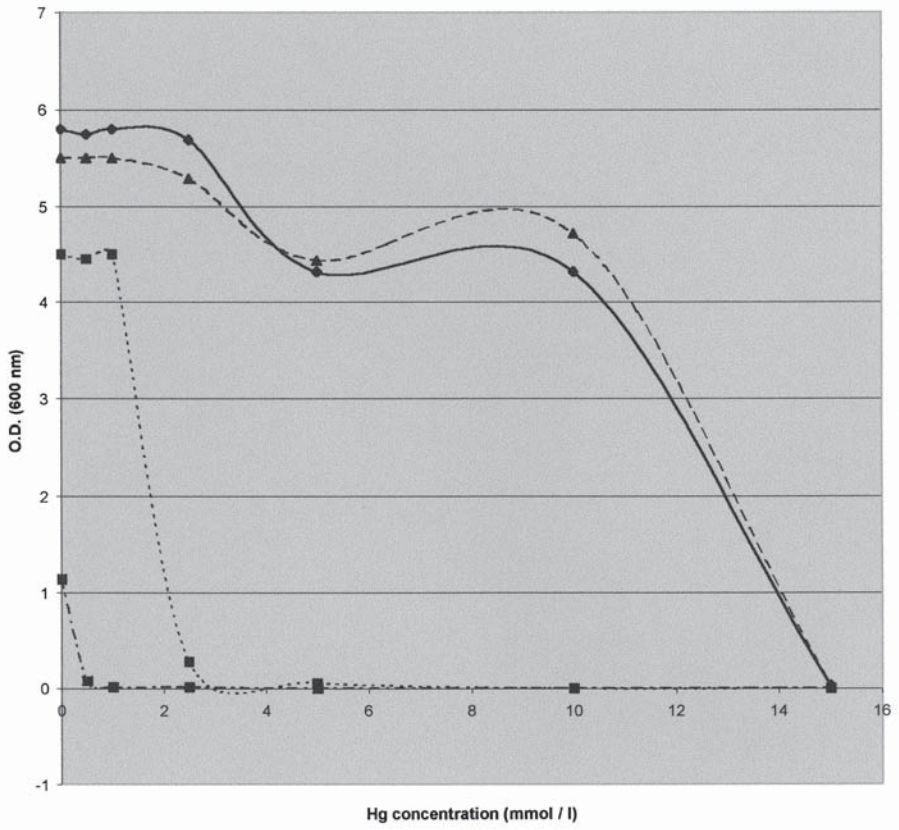

Fig. 3. Optical density of 869 liquid cultures of T902 (––▲––), BD2 (- -■- -), LMG5359 (– –■– –), and T902.1 (—■—) in presence of various concentrations of mercury chloride (from 0 to 15 µmol/L).

Table 6
Comparison of the Surviving Ratio of Four *Rhodococcus erythropolis* Strains After Lyophilization (with 0.5% trehalose), Fluidized Bed Drying, and Spray Drying, Respectively, Comparison with the Positive Reference Strain *Deinococcus radiodurans*

Strain	Lyophilization	Fluidized bed drying	Spray drying
T902	25%	26%	38%
BD2	25%	15%	18%
T902.1	26%	21%	31%
LMG5359	15%	ND[a]	ND[a]
ATCC13939	16%	4.1%	ND[a]

[a]ND, not determined.

R. erythropolis constitutes a desiccation tolerant species just as does the positive reference *D. radiodurans*. The drought-tolerance of the *Rhodococcus* strains is indeed comparable or higher than that of *D. radiodurans*.

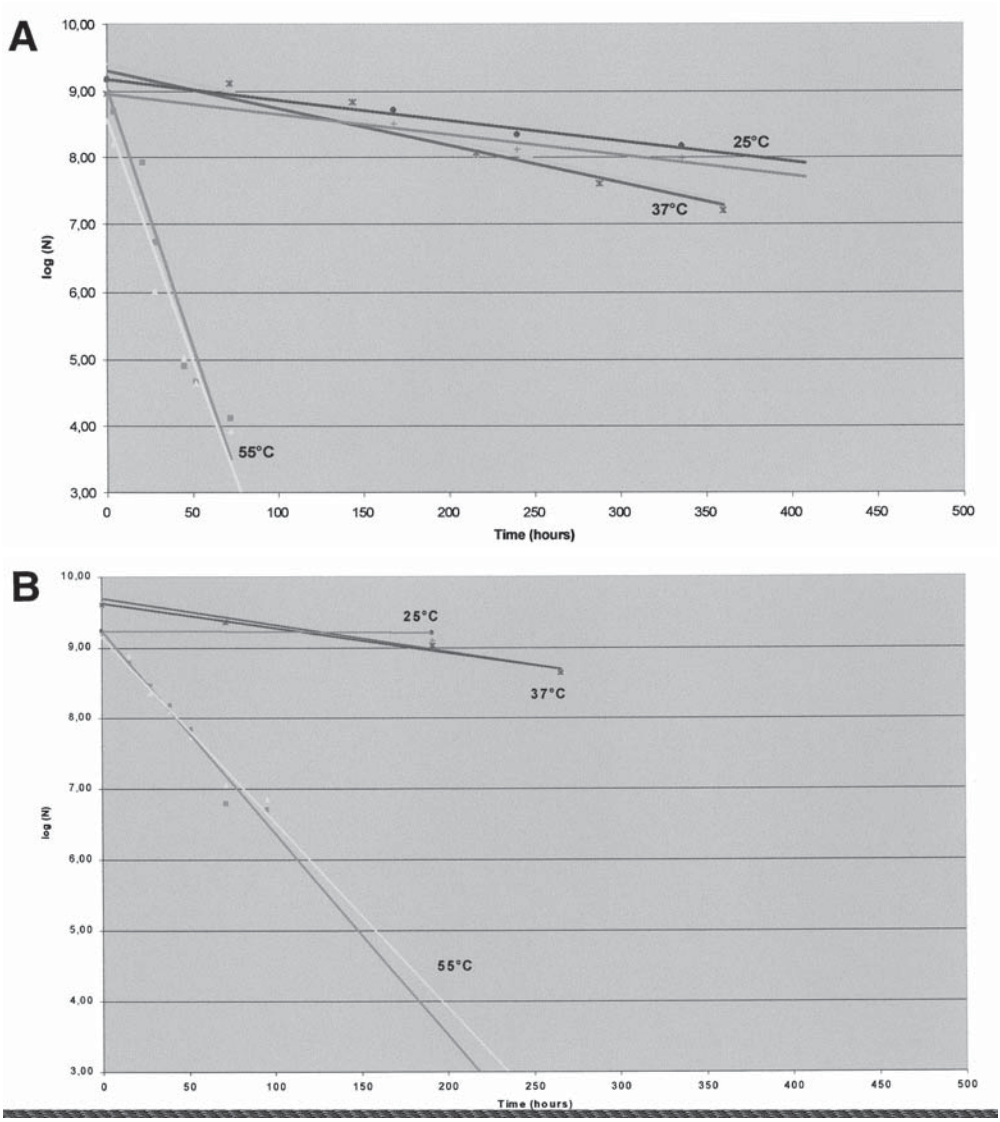

Fig. 4. Evolution during storage at 55, 37, and 25°C of the colony count after fluidized bed drying of strain **(A)** T902.1 and **(B)** BD2 on complex (869) medium and minimal medium (284) with IPB.

Evaluation of Survival and Maintenance of Biodegradation Properties During Long-Term Preservation

The survival after drying is not the only parameter to monitor. It is important to control the evolution in time, during long-term storage of the surviving fraction of the population. Even more important is the monitoring of the maintenance of biodegradation activity during storage. The evolution in time of the surviving fraction of T902.1 after fluidized bed drying (*see* Fig. 4A) and of BD2 (*see* Fig. 4B) was followed, as well as the CFU count on 284 minimal medium with IPB as sole source of carbon. These evolutions

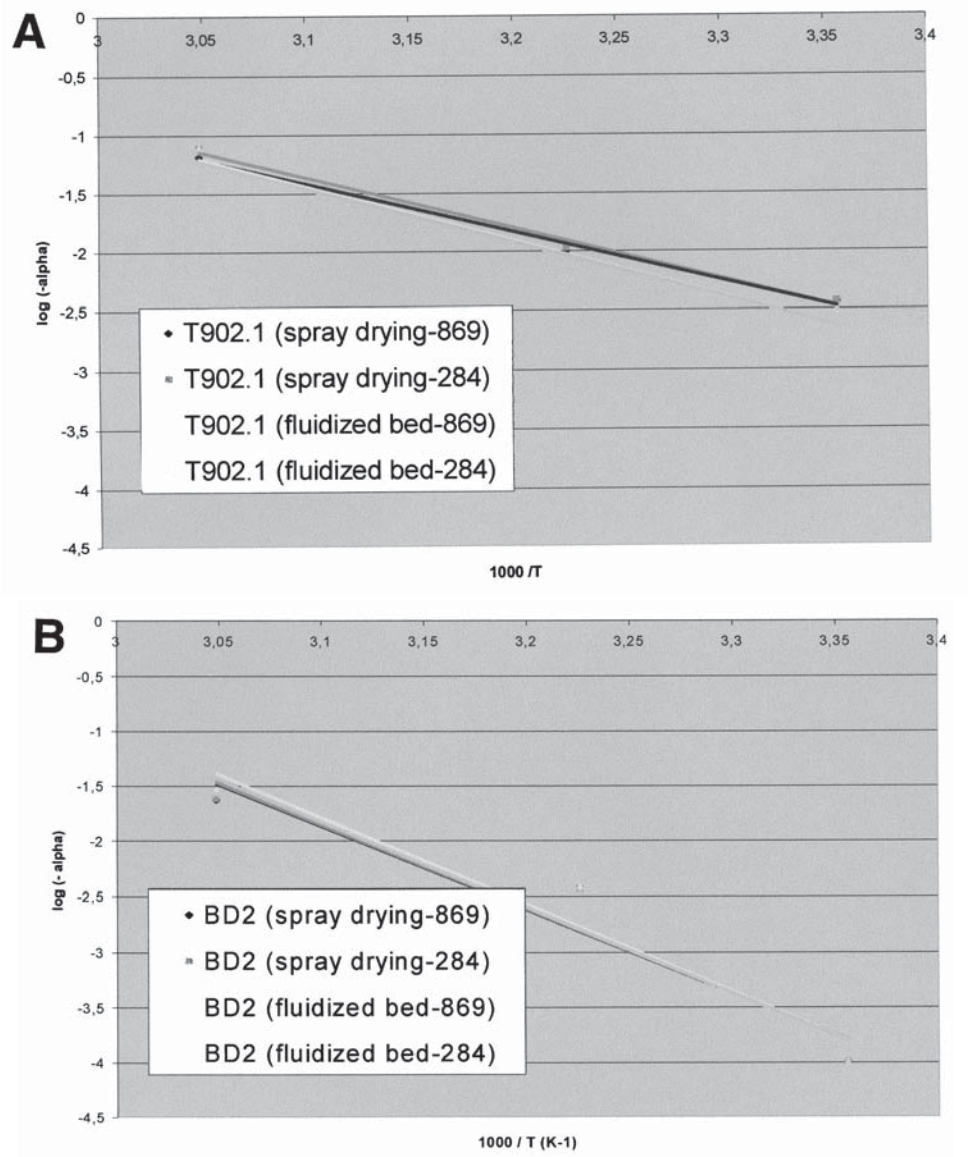

Fig. 5. Arrhenius plots of surviving fractions and active fractions after storage of spray dried and fluidized powders of **(A)** T902.1 and **(B)** BD2.

were monitored at storage temperatures of 55, 37, and 25°C. From the slopes of the linear least square fits of these data, the Arrhenius plots can be derived (*see* Fig. 5).

In Fig. 4, no difference is seen between the maintenance of the surviving fraction and maintenance of the IPB biodegradation activity. The rate of loss of survival is equivalent to the rate of loss of activity at the same temperature. No spontaneous IPB– mutants were isolated. Although the IPB degradation activity is borne on a plasmid, the storage at tempera-

Table 7
Half-Lives Estimated at 0, 4, and 25°C from the Arrhenius Plots[a]
for T902.1 and BD2 After the Two Drying Processes
and Expressed in Days (D) or Months (Mo)

Strain	Drying technique	Half-life at 0°C		Half-life at 4°C		Half-life at 25°C	
		(D)	(Mo)	(D)	(Mo)	(D)	(Mo)
T902.1	Fluidized bed	145	4,8	82	2,7	5	0,2
	Spray drying	70	2,3	42	1,4	4	0,1
BD2	Fluidized bed	22008	733	7615	253	77	2,6
	Spray drying	12249	408	4859	162	59	2

[a]*See* Fig. 6.

tures as high as 55°C did not induce plasmid loss. From 10 colonies tested for the presence of plasmid by gel electrophoresis (result not shown) after storage at high temperature, none had lost its plasmid. The pBD2 plasmid seems to be stable in the conditions of storage that were tested. In Fig. 4, the slopes for the strain T902.1 are steeper than those of the strain BD2. The *R. erythropolis* BD2 is significantly more stable during storage than T902.1.

The smaller the angular coefficient of the lines of Fig. 4 are, the more stable the product are. From the Arrhenius plots (plots of log α vs $1/T$ as in Fig. 5), one can calculate the half-life of a product at any temperature. The half-lives at a room temperature of 25, 4, and at 0°C were calculated (*see* Table 7). When stored at high temperatures (25°C), the products have comparable half-lives, but when the storage temperature is lowered, the differences between the strains and between the drying techniques get more significant. Evidently, the samples stored at colder temperatures are more stable. From Table 7, one can see that the fluidized product of T902.1 is more stable than the spray-dried one. The dry matter content of the fluidized product is 98% whereas that of the spray-dried product is only of 92%. Generally, a drier product is more stable.

When stored at 0°C, the T902.1 strain has a half-life of a few months, compatible with the commercialization requirements. The BD2 product has that half-life even when stored at 25°C.

Conclusions

The metabolic activity of the drought-tolerant strain T902 could be improved by plasmid transfer by conjugation. The demonstration of the applicability of the strategy is made. It is possible to select drought-tolerant strains with some biodegradation properties and afterward, in a second phase, to expend its properties by "doping" it with plasmids bearing genes responsible for the synthesis of enzymes responsible for the degradation of more recalcitrant xenobiotic compounds. We also demonstrated the feasibility of the introduction of plasmids from Gram-negative bacteria into the Gram-positive desiccation-tolerant ones. This broadens the possibility of

doping for these strains. A drought-tolerant strain with an enhanced recalcitrant xenobiotic metabolic activity was constructed.

Interestingly, it seems that all *R. erythropolis* strains express desiccation-tolerance phenotype. This could open the way to the characterization of the drought-tolerance phenotype of this species.

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