RESEARCH ARTICLE

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Rapid spectrofluorometric screening of poly-hydroxyalkanoate-producing bacteria from microbial mats

Summary. Microbial mat ecosystems are characterized by both seasonal and diel fluctuations in several physicochemical variables, so that resident microorganisms must frequently adapt to the changing conditions of their environment. It has been pointed out that, under stress conditions, bacterial cells with higher contents of poly-hydroxyalkanoates (PHA) survive longer than those with lower PHA content. In the present study, PHA-producing strains from Ebro Delta microbial mats were selected using the Nile red dying technique and the relative accumulation of PHA was monitored during further laboratory cultivation. The number of heterotrophic isolates in trypticase soy agar (TSA) was ca. 10^7 colony-forming units/g microbial mat. Of these, 100 randomly chosen colonies were replicated on mineral salt agar limited in nitrogen, and Nile red was added to the medium to detect PHA. Orange fluorescence, produced upon binding of the dye to polymer granules in the cell, was detected in approximately 10% of the replicated heterotrophic isolates. The kinetics of PHA accumulation in *Pseudomonas putida*, and *P. oleovorans* were compared with those of several of the environmental isolates spectrofluorometry. PHA accumulation, measured as relative fluorescence intensity, resulted in a steady-state concentration after 48 h of incubation in all strains assayed. At 72 h, the maximum fluorescence intensity of each strain incubated with glucose and fructose was usually similar. MAT-28 strain accumulated more PHA than the other isolates. The results show that data obtained from environmental isolates can highly improve studies based on modeling-simulation programs, and that microbial mats constitute an excellent source for the isolation of PHA-producing strains with industrial applications. [**Int Microbiol** 2006; 9(2):95-102]

Key words: *Pseudomonas putida* · *P. oleovorans* · poly-hydroxyalkanoates · Ebro Delta microbial mats · Nile red · spectrofluorometry · screening
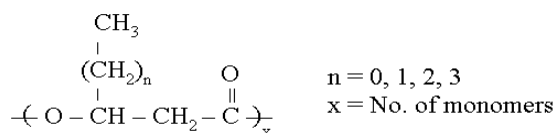
Introduction

Microbial mats are stratified microbial ecosystems characterized by cyclic seasonal fluctuations of flooding and desiccation, and by diel fluctuations in the concentrations of oxygen, sulfide, and other chemical nutrients. Microorganisms rapidly respond to changes in various physicochemical gradients, and locate themselves according to the most favorable environmental conditions. This behavior is likely to govern the vertical species stratification that results from the active migration of motile cells in response to the shifting gradients of electron donors and/or acceptors observed within microbial mats [4,6,8,22]. Prokaryotes have evolved numerous

mechanisms of resistance to stress conditions. For example, many microorganisms have an inherent ability to form resting stages (e.g., cysts and spores), which allows them to survive in desiccated environments [16]; others, such as the spirochete *Spirosymplokos deltaiberi,* become swollen and form refractile resistant bodies on exposure to air [17]. Alternatively, other bacteria exhibit a metabolic versatility in order to cope with fluctuations in the chemical conditions of their environment.

The accumulation of intracellular storage polymers is another bacterial strategy that increases survival in a changing environment [19]. Poly-hydroxyalkanoates (PHA) serve as an endogenous source of carbon and energy during starvation [13]. In Ebro Delta mats, anoxygenic photosynthesis represents about 26% of total organic carbon production [18] and PHA distribution may contribute about 2% of the organic carbon of the photosynthetic layers [25]. The content of PHA in the microbial mat usually increases over the day, reaching its maximum at 18:00 h, and decreases overnight, with a minimum at 6:00 h [20]. Thus, dusk and dawn are periods of adaptation or accommodation to new physicochemical environmental conditions within microbial mats. Consequently, the necessity for organisms to adapt to extreme environmental fluctuations by alternating between the production and utilization of storage compounds may explain the diel pattern of in situ PHA accumulation observed in such environments [20]. More detailed, in situ analysis of PHA and their repeating-unit composition in stratified photosynthetic microbial mats from the Ebro Delta showed the higher prevalence of hydroxyvalerate (HV) repeating units over hydroxybutyrate (HB) repeating units at approximately 1 HB:2 HV [25].

PHA are members of a family of polyesters that includes a wide range of different D-hydroxyalkanoids acids characterized by the general chemical structure:

$$
\underset{\displaystyle \overset{\textstyle O-CH-CH_2-C}{\big(}}{}
$$

$$
\begin{array}{c}
CH_3 \\
| \\
(CH_2)_n \\
| \\
-\!\!\leftarrow O - CH - CH_2 - \overset{\displaystyle O}{\overset{\|}{C}} \rightarrow_x
\end{array}
\qquad
\begin{array}{l}
n = 0, 1, 2, 3 \\
x = No.\ of\ monomers
\end{array}
$$

Since poly-β-hydroxybutyrate (PHB), one of the most abundant PHA, was first described in *Bacillus megaterium* by Lemoigne in 1926, several studies have demonstrated the production of PHA by a wide variety of prokaryotes and also by several plants and animals. However, only prokaryotes accumulate high-molecular-weight PHA in cytoplasmic granules [9,27]. The granules are coated with a monolayer of phospholipids and proteins. These granule-associated proteins play a major role in the synthesis and degradation of PHA and in granule formation [23]. In *Bacteria*, PHA constitute a major carbon and energy storage material, which is accumulates when a carbon source is provided in excess and another nutrient (such as nitrogen, sulfur, phosphate, iron, magnesium, potassium, or oxygen) is limiting. The polymerization of soluble intermediates into insoluble molecules does not change the osmotic state of the cell, thereby avoiding leakage of these nutrient-rich compounds out of the cell. In addition, PHA-producing bacteria have the advantage of nutrient storage at a relatively low maintenance cost and with a secured return of energy [15].

The analytical method first used to determine PHB accumulation in bacteria was based on the degradation of PHB with sulfuric acid to crotonic acid, and measurement of the reaction at 235 nm by spectroscopy [14]. Later, other techniques were developed to measure PHA: gas chromatography [3], mass spectrometry [2], Fourier-transform infrared spectroscopy [10], staining with the lipid coloring agent Nile red followed by spectrofluorometry [5,7], and flow cytometry [30]. Of these methods, Nile red fluorescence offers an easy, rapid screening technique that can be used to isolate potential PHA-producing strains from environmental samples. In the present study, we used Nile red to select such strains from Ebro Delta microbial mats and to monitor the relative amounts of product (measured as relative fluorescence intensity) that accumulated during laboratory cultivation.

Material and methods

Collection microorganisms. Two collection strains were used as controls in the screening of PHA-producing strains from Ebro Delta microbial mats and in monitoring the accumulation of PHA by spectrofluorometry: *Pseudomonas putida* CECT 324 and *P. oleovorans* CECT 4079 as PHA-producing bacteria; and *Escherichia coli* ATCC 10536 as a non-PHA-producing bacterium.

Isolation of PHA-accumulating heterotrophic bacteria from microbial mats. One gram of mat sample from Ebro Delta microbial mats was homogenized in 10 ml of Ringer ¼. A dilution series was made in Ringer ¼ to obtain 30–300 colony-forming units (cfu) per plate. Aliquots of 0.1 ml were plated onto Luria-Bertani (LB) agar (Scharlau, Barcelona, Spain) containing 1.0 % NaCl and on trypticase soy agar (TSA) (Scharlau). The plates were incubated for 24–48 h at 30ºC. Colonies that developed on agar were differentiated by color, elevation, form, and edge appearance. Isolates of axenic colonies were randomly picked and cultured in solid mineral salt medium (MSM) containing (in g/l): agar 15, $Na_2HPO_4 \cdot 7H_2O$ 6.7, NaCl 10, KH_2PO_4 1.5, NH_4Cl 0.1, $MgSO_4 \cdot 7H_2O$ 0.2, $CaCl_2$ 0.01, ferrous ammonium citrate 0.06, and 1 ml trace elements [24]. MSM was supplemented with 5 g glucose/l and 0.5 mg Nile red (Sigma, St. Louis, MO, USA) (dissolved in dimethylsulfoxide)/ml [29]. Petri dishes were incubated for 4–5 days at 30ºC. The amount of orange fluorescence observed in PHA-positive colonies, particularly those of *P. putida*, was much higher than the barely visible fluorescence of PHA-negative colonies, such as *E. coli*. A sample of each of the PHA$^+$ isolates was smeared on a glass slide, heat-fixed, and stained with Nile blue A [21] to detect the presence of intracellular PHA

granules. Briefly, the isolates were stained with a 1% aqueous solution of Nile blue A for 10 min, washed with water and 8% aqueous acetic acid for 1 min to remove excess stain, dried with filter paper, and covered with a glass cover slip (to protect the stained cells from immersion oil). The preparation was examined using an Olympus BX-40 epifluorescence microscope and an excitation wavelength of approximately 460 nm. PHA granules stained with Nile blue A fluoresced bright orange, with individual granules often visible within a cell.

General biochemical and physiological characteristics of PHA-accumulating strains isolated from Ebro Delta microbial mats. PHA[+] isolates were studied using several general biochemical and physiological probes, such as Gram stain, pigmentation on TSA, and temperature, pH, and salinity ranges. Oxidase and growth and fermentation on glucose, fructose, lactose, xylitol, arabinose, cellobiose, trehalose, melibiose, rhamnose, galactose, xylose, sucrose, mannose were also analyzed (Table 1S, ONLINE).

Spectrofluorometric monitoring of PHA accumulation. PHA was monitored spectrofluorometrically with Nile red as a fluorochrome following a modification of the procedure of Delegau et al. [5]. The microorganisms used in the assay were: *E. coli*, *P. oleovorans*, *P. putida*, and five PHA[+] isolates (MAT-07, MAT-13, MAT-16, MAT-17 and MAT-28) obtained from the Ebro Delta microbial mats. These strains were grown overnight in TSB broth (3% NaCl) at 30ºC. Aliquots (1/100) from each culture were transferred into 100-ml flasks containing nitrogen-limited MSM, glucose (5 g/l), and 0.5 µg Nile red dye (dissolved in dimethylsulfoxide)/ml. Liquid cultures were incubated in an orbital shaker (100 rpm) at 30ºC for several days. At 24, 48 and 72 h, a 1-ml sample was removed and then centrifuged in a microcentrifuge at 10,000 rpm at room temperature. Pellets were washed in 1 ml of PBS (pH 7.0), suspended in 1 ml of 0.1 M glycine-HCl (pH 3.0), and incubated at room temperature in the dark for at least 2 h. The relative amount of PHA within the cells, as indicated by the intensity of Nile-red orange fluorescence, was measured using an SLM Aminco 8100 spectrofluorometer. The fluorescence excitation and emission wavelengths of the stained cells in 0.1 M glycine-HCl (pH 3) were 543 nm and 598 nm, respectively. Slits of excitation and emission were set to 10 nm at 900 V.

PHA accumulation after 72 h of incubation with glucose and fructose was compared between the control strains and the isolates. The conditions used in this assay were as explained above.

Transmission electron microscopy. One-milliliter aliquots of 48 and 72 h cultures of MAT-28 in MSM with glucose were collected for TEM analysis. They were centrifuged in a microcentrifuge at 10,000 rpm at room temperature. Pellets were washed in 1 ml of PBS (pH 7.0), and fixed with 2% of glutaraldehyde-PBS and then stained with osmium tetroxide and uranyl acetate. Samples were viewed in a Leica transmission electron microscope.

Results

Several different heterotrophic bacteria were isolated from microbial mats. Approximately 2.3×10^7 cfu/g mat were obtained in TSA and ca. 10^4 cfu/g mat in LB agar with 1% salt. One hundred randomly chosen colonies were replicated on agar/nitrogen-limited MSM containing glucose (5 g/l) and Nile red. The dye produces orange fluorescence on binding to polymer granules in the cell [29]. Approximately 10% of replicated heterotrophic isolates showed orange fluorescence, while Nile blue A staining confirmed the presence of intracellular lipidic granules (Fig. 1). The colonies exhibited a diverse range of morphologies, although there were only minimal differences in the results of the biochemical analysis (Table 1 and Table 1S ONLINE), which may reflect the fact that these microorganisms shared the same habitat. Most of the isolated strains were gram-negative and motile, able to grow from pH 6 to pH 9, from 4 to 37ºC, and on 0.5–7% NaCl. The isolates were oxidase-positive and showed no pigmentation on TSA. All of the isolates utilized the substrates assayed, some of them fermentatively (see Table 1S ONLINE).

Five of the PHA[+] isolates, MAT-07, MAT-13, MAT-16, MAT-17 and MAT-28, were randomly selected for further study. The relative fluorescence intensity of strains cultured in MSM-glucose medium was tested in three conditions: (i) after more than 2 h of incubation in PBS buffer-Nile red (at room
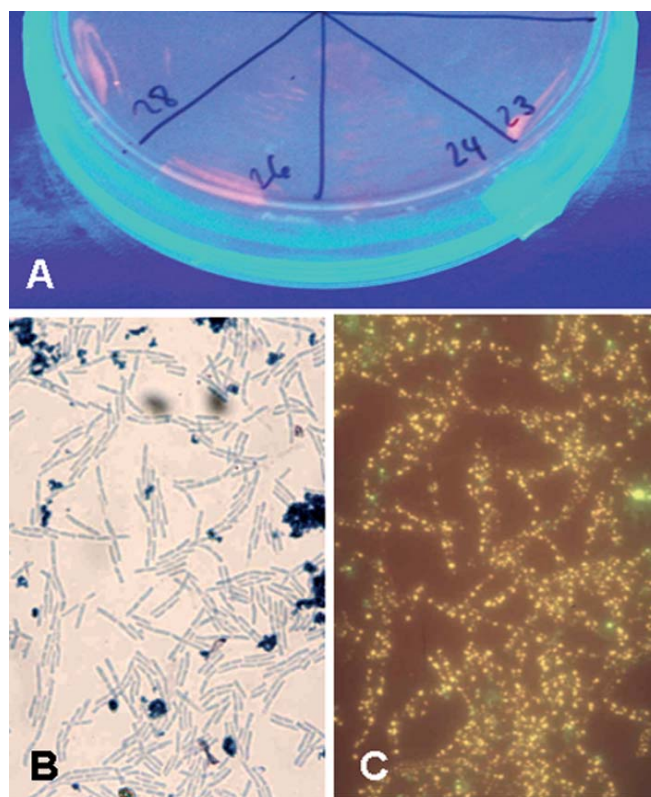

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Fig. 1. (A) Fluorescent Nile red staining of strains from Ebro Delta microbial mats. The amount of orange fluorescence in poly-hydroxyalkanoate (PHA)-positive colonies was much higher than that of PHA-negative colonies. **(B)** An isolate from Ebro Delta microbial mats observed by optical microscopy. **(C)** The same microorganisms, stained with Nile blue dye, observed by epifluorescesce microscopy. PHA granules fluoresce bright orange within the cells. (Photograhs made by A. Navarrete.)

Table 1. Distinctive physiological characteristics of several PHA-producing strains isolated from Ebro Delta microbial mats (see Table 1S ONLINE)

	MAT-07	MAT-13	MAT-16	MAT-17	MAT-28
Gram stain	–	–	–	–	–
Round, slimy colonies	+	+	+	+	+
Oxidase	+	+	+	+	+
Growth at 45ºC	–	–	+	–	–
Growth at 7% NaCl	–	–	–	–	+
Arabinose fermentation[a]	–	+	+	–	–
Melibiose fermentation[a]	–	+	+	+	+
Galactose fermentation[a]	–	+	+	+	+
Xylose fermentation[a]	+	+	+	+	+
Mannose fermentation[a]	+	+	+	–	–
PHA production[b]	+	+	+	++	+++
Depolymerase (hydrolysis of PHA)	+	–	+	+	+

[a]Fermentantion after 7 days of incubation at 30ºC.
[b]Production of PHA after growth in MSM-glucose at 30ºC, at 48–72 h.

temperature, in the dark); (ii) after more than 2 h of incubation in 0.1 M glycine-HCl-Nile red (at room temperature, in the dark); and (iii) immediate sonication in 0.1 M glycine-HCl-Nile red. The relative fluorescence intensity was lower in cells stained with PBS-Nile red (condition i) than in those stained with glycine-HCl-Nile red (condition ii). However, the relative fluorescence intensity in bacteria incubacted in glycine-HCl-Nile red (condition ii), was similar to that in
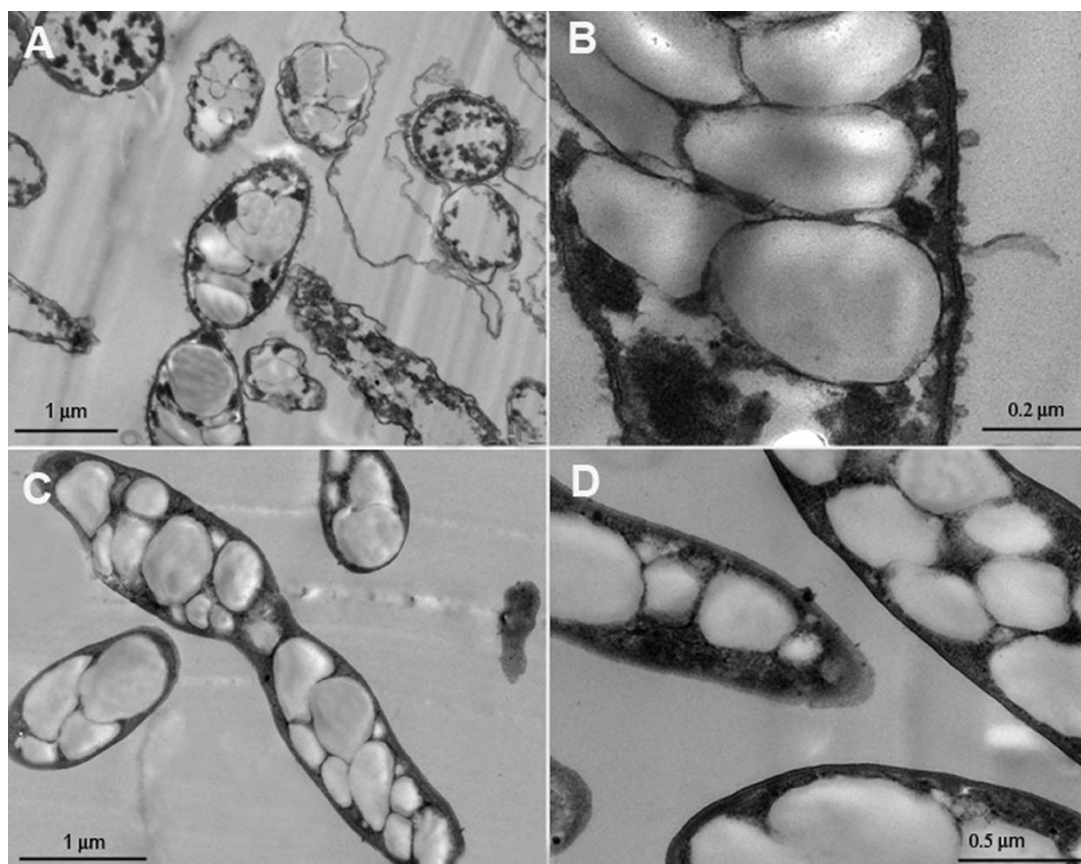
Fig. 2. Transmision electron micrographs of strain MAT-28 grown on MSM medium with glucose for 48 h, and incubated for 2 h in glycine-HCl-Nile red (**A,B**) and PBS-Nile red (**C,D**).
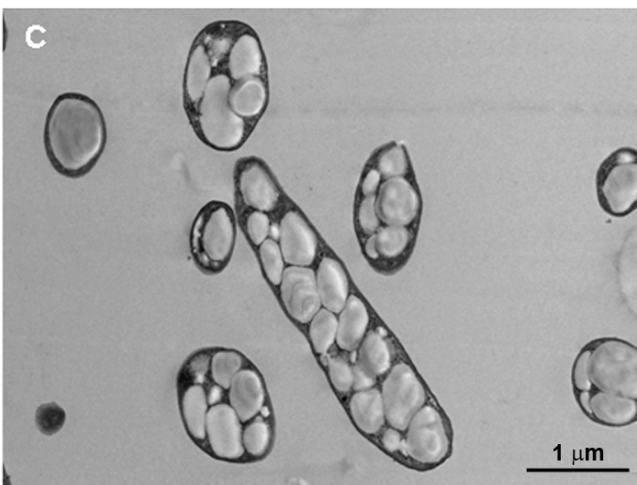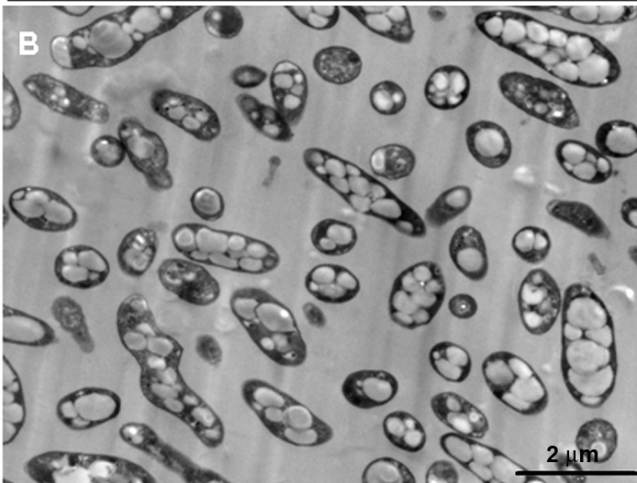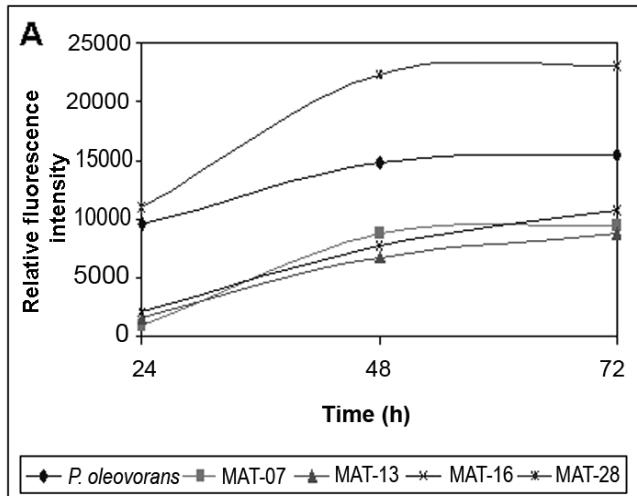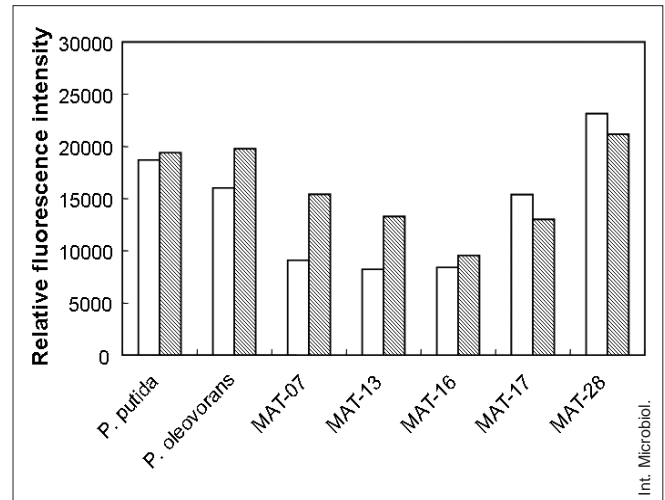
Fig. 4. PHA accumulation in *Pseudomonas putida*, *P. oleovorans*, and the mat isolates MAT-07, MAT-13, MAT-16, MAT-17 and MAT-28 as measured spectrofluorometrically at 72 h of incubation with glucose (white bars) or fructose (gray bars). The final concentration of Nile red used in the assay was 0.5 µg/ml. The data shown are the average of the results of three independent experiments. In each case, the coefficient of variation (i.e., the standard deviation/mean × 100) was less than 17%.

Fig. 3. (**A**) PHA accumulation in *Pseudomonas oleovorans* and the mat isolates MAT-07, MAT-13, MAT-16 and MAT-28 as measured spectrofluorometrically. The final concentration of Nile red used in the assay was 0.5 µg/ml. The data shown are the average of the results of three independent experiments. In each case, the coefficient of variation (i.e., the standard deviation/mean × 100) was less than 22%. (**B**) Transmision electron micrograph of MAT-28 growing on MSM medium with glucose at 48 h. (**C**) Transmision electron micrograph of MAT-28 at 72 h.

cells immediately sonicated (condition iii). This may be a consequence of the disruption of the cell envelope when they were incubated with glycine-HCl-Nile red (Fig. 2).

PHA accumulation, measured as relative fluorescence intensity, was determined spectrofluorometrically in cells grown under the same conditions as stated above. The kinetics of PHA accumulation in *P. oleovorans* and *E. coli* were compared with those of four of the environmental isolates (Fig. 3). The experiments were done three times independently, and the variation coefficient of each data point was less than 22%. The lipid concentration in non-PHA-producing *E. coli* was lower than the level that could be detected spectrofluorometrically. In all PHA-producing strains, accumulation of the polymer led to a steady-state concentration after approximately 48 h. When grown on glucose and fructose, the steady-state concentration of PHA accumulated at 72 h, measured as the relative intensity of Nile red fluorescence, was similar in all strains assayed, except for MAT-07 and MAT-13, which accumulated more PHA when grown on fructose. Of all the isolates, MAT-28 accumulated the most PHA (Fig. 4).

Discussion

Among the different organisms that make up most of the mat biomass, there are heterotrophic microorganisms (e.g., fermentative bacteria, sulfur oxidizers) that do not form stable

layers; instead, their position in the mat depends on the availability of organic matter and on other environmental variables. Aerobic heterotrophic bacteria inhabiting microbial mats may play an important role in carbon cycling, yet information about their identity is scarce. Molecular phylogenetic studies are currently underway to examine the microbial diversity in naturally occurring mats. Preliminary findings of 16S rRNA sequences in Guerrero Negro microbial mats showed that heterotrophs were abundant, including α-, γ-, δ-, and ε-proteobacteria, firmicutes, spirochetes, and representatives of the *Cytophaga/Flexibacter/Bacteroides* (CFB) group. The variability in numbers of different kinds of organisms at different times and at different depths in the mats is extensive, reflecting the enormous complexity within both the mats and individual strata [28]. Jonkers and Abed [12] were able to phylogenetically identify the apparent dominant populations of aerobic heterotrophs in a hypersaline microbial mat from Eilat by combining cultivation and molecular techniques. The isolated populations were related to the genera *Rhodobacter* and *Roseobacter* (α-proteobacteria) and to *Marinobacter* and *Halomonas* (γ-proteobacteria).

We have used a cultivation step to obtain heterotrophs from Ebro Delta microbial mats, with the aim of isolating potential PHA-producing strains. The lipophilic dye Nile red can be directly added to the medium to stain colonies that store PHA, and at concentrations that do not inhibit cell growth. Nile red staining thus allows both the detection of PHA-producing strain from environmental samples and quantification of the polymer in liquid culture, resulting in a significant reduction of analysis time and manpower. However, Nile red cannot be used to identify the monomer composition of the accumulated PHA [7]. The fluorescence intensity of stained cells depends on the PHA concentration. Bacteria that do not produce PHA are only lightly fluorescent because they contain almost no lipids [26] (see Figs. 1 and 2). In the PHA-producing strains isolated from Ebro Delta microbial mats, the kinetics of PHA accumulation were similar to those of *P. putida*, assayed under the same conditions.

In bacteria, PHA accumulation probably serves to increase survival and stress tolerance in changing environments and competitive settings, i.e., when carbon and energy sources are limited, such as occurs in stratified microbial mats. Bacterial cells containing higher amounts of PHA, and thus larger nutrient stores, may survive longer than those with a lower PHA content. Alternatively, the polymer may protect against additional adverse factors [1,11]. Microbial mats (a complex biofilm) have a highly structured spatial distribution of biotic and abiotic variables. This distribution plays a significant role in determining the composition and activity of the microbial populations that form the mat. Our results could be applied to modeling studies aimed at understanding the intricate relationship between the structure and the activity of the microbial community, as they allow the spatial distribution of the different populations in the mat matrix to be monitored according to substrate availability [8,18,31]. We propose that microbial mats constitute a potential source for the isolation of new PHA-producing strains, although culture conditions must be optimized to obtain polymer concentrations that are high enough to be industrially and commercially exploited.

References

1. Aneja P, Zachertowska A, Charles TC (2005) Comparison of the symbiotic and competition phenotypes of *Sinorhizobium meliloti* PHB synthesis and degradation pathway mutants. J Can Microbiol 51:599-604
2. Ballistreri A, Garozzo D, Giuffrida M, Impallomeni G, Montaudo G (1989) Sequencing bacterial poly(β-hydroxybutyrate-*co*-hydroxyvalerate) by partial methanolysis, high-performance liquid chromatography fraction, and fast atom bombardment mass spectrometry analysis. Macromolecules 22:2107-2111
3. Braunegg G, Sonnleitner B, Lafferty RM (1978) A rapid gas chromatographic method for the determination of poly-β-hydroxybutyric acid in microbial biomass. Eur J Appl Microbiol Biotechnol 6:29-37
4. Brune A, Frenzel P, Cypionka H (2000) Life at the oxic-anoxic interface: microbial activities and adaptations. FEMS Microbiol Rev 24:691-710
5. Degelau A, Scheper T, Bailey JE, Guske C (1995) Fluorometric measurement of poly-β-hydroxybutyrate in *Alcaligenes eutrophus* by flow cytometry and spectrofluorometry. Appl Microbiol Biotechnol 42:653-657
6. Des Marais DJ (2003) Biogeochemistry of hypersaline microbial mats illustrates the dynamics of modern microbial ecosystems and the early evolution of the biosphere. Biol Bull 204:160-167
7. Gorenflo V, Steinbüchel A, Morose S, Rieseberg M, Scheper T (1999) Quantification of bacterial polyhydroxyalkanoic acids by Nile red staining. Appl Microbiol Biotechnol 51:765-772
8. Guerrero R, Piqueras M, Berlanga M (2002) Microbial mats and the search for minimal ecosystems. Int Microbiol 5:177-188
9. Hezayen FF, Steinbüchel A, Rehm BHA (2002) Biochemical and enzymological properties of the polyhydroxybutyrate synthase from the extremely halophilic archaeon strain 56. Arch Biochem Biophys 403:284-291
10. Hong K, Sun S, Tian W, Chen GQ, Huang W (1999) A rapid method for detecting bacterial polyhydroxyalkanoates in intact cells by Fourier transform infrared spectroscopy. Appl Microbiol Biotechnol 51:523-526
11. James BW, Mauchline WS, Dennis PJ, Keevil CW, Wait R (1999) Poly-

3-hydroxybutyrate in *Legionella pneumophila*, an energy source for survival in low-nutrient environments. Appl Environ Microbiol 65:822-827

12. Jonkers HM, Abed RMM (2003) Identification of aerobic heterotrophic bacteria from the photic zone of a hypersaline microbial mat. Aquat Microb Ecol 30:127-133

13. Kadouri D, Jurkevitch E, Okon Y (2005) Ecological and agricultural significance of bacterial polyhydroxyalkanoates. Crit Rev Microbiol 31:55-67

14. Law JH, Slepecky RA (1961) Assay of poly-hydroxybutyric acid. J Bacteriol 82:33-36

15. Madison LL, Huisman GW (1999) Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic. Microbiol Mol Biol Rev 63:21-53

16. Malcom P (1994) Desiccation tolerance of prokaryotes. Microbiol Rev 58:755-805

17. Margulis L, Ashen JB, Solé M, Guerrero R (1993) Composite, large spirochetes from microbial mats: spirochete structure review. Proc Natl Acad Sci USA 90:6966-6970

18. Martínez-Alonso M, Mir J, Caumette P, Gaju N, Guerrero R, Esteve I (2004) Distribution of phototrophic populations and primary production in a microbial mat from the Ebro Delta, Spain. Int Microbiol 7:19-25

19. Müller S, Bley T, Babel W (1999) Adaptive responses of *Ralstonia eutropha* to feast and famine conditions analysed by flow cytometry. J Biotechnol 75:81-97

20. Navarrete A, Peacock A, Macnaughton SJ, Urmeneta J, Mas-Castellà J, White DC, Guerrero R (2000) Physiological status and community composition of microbial mats of the Ebro Delta, Spain, by signature lipid biomarkers. Microb Ecol 39:92-99

21. Ostle AG, Holt JG (1982) Nile Blue A as a fluorescent stain for poly-β-hydroxybutyrate. Appl Environ Microbiol 44:238-241

22. Paerl HW, Pinckney JL, Stepper TF (2000) Cyanobacterial mat consortia: examining the functional unit of microbial survival and growth in extreme environments. Environ Microbiol 2:11-26

23. Pötter R, Steinbüchel A (2005) Poly(3-hydroxybutyrate) granule-associated proteins: Impacts on poly(3-hydroxybutyrate) synthesis and degradation. Biomacromolecules 6:552-560

24. Ramsay BA, Lomaliza K, Chavarie C, Dubé B, Bataille P, Ramsay JA (1990) Production of poly-β-hydroxybutyric-*co*-β-hydroxyvaleric acids. Appl Environ Microbiol 56:2093-2098

25. Rothermich MM, Guerrero R, Lenz RW, Goodwin S (2000) Characterization, seasonal occurrence, and diel fluctuation of poly(hydroxyalkanoate) in photosynthetic microbial mats. Appl Environ Microbiol 66: 4279-4291

26. Schlegel HG (1990) *Alcaligenes eutrophus* and its scientific and industrial career. In: Dawes EA (ed) Novel biodegradable microbial polymers. Kluwer, Dordrecht, Netherlands, pp 133-141

27. Shang L, Jiang M, Chang HN (2003) Poly(3-hydroxybutyrate) synthesis in fed-batch culture of *Ralstonia eutropha* with phosphate limitation under different glucose concentrations. Biotechnol Lett 25:1415-1419

28. Spear JR, Ley RE, Berger AB, Pace NR (2003) Complexity in natural microbial ecosystems: the Guerrero Negro experience. Biol Bull 204: 168-173

29. Spiekermann P, Rehm BHA, Kalscheuer R, Baumeister D, Steinbüchel A (1999) A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. Arch Microbiol 171:73-80

30. Vidal-Mas J, Resina-Pelfort O, Haba E, Comas J, Manresa A, Vives-Rego J (2001) Rapid flow cytometry–Nile red assessment of PHA cellular content and heterogeneity in cultures of *Pseudomonas aeruginosa* 47T2 (NCIB 40044) grown in waste frying oil. Ant Leeuw 80:57-63

31. Xavier JB, Picioreanu C, van Loosdrecht MCM (2005) A framework for multidimensional modeling of activity and structure of multispecies biofilms. Environ Microbiol 7:1085-1103

Cribado espectrofluorométrico rápido de bacterias productoras de polihidroxialcanoatos en tapetes microbianos

Resumen. El ecosistema de los tapetes microbianos se caracteriza por fluctuaciones diarias y estacionales en diversas variables fisicoquímicas, de tal manera que los microorganismos residentes deben adaptarse frecuentemente a las condiciones cambiantes de su ambiente. Se ha destacado que, en condiciones de estrés, las células bacterianas con elevado contenido en polihidroxialcanoatos (PHA) pueden sobrevivir más tiempo que las de bajo contenido. En este estudio, se utilizó la técnica del colorante rojo Nilo para seleccionar las cepas productoras de PHA de los tapetes microbianos del Delta del Ebro, y para monitorizar la acumulación relativa de PHA durante el cultivo en el laboratorio. El número de aislados heterotrofos en TSA fue de aproximadamente 10^7 unidades formadoras de colonias/g tapete microbiano. De éstas, se replicaron 100 colonias elegidas al azar cultivadas en agar mineral salino limitado en nitrógeno, al que se le añadió el colorante rojo Nilo para la detección de PHA. La fluorescencia naranja, que se produce al unirse el rojo Nilo a los gránulos de polímero en la célula, se detectó en aproximadamente el 10% de los aislados heterotrofos replicados. La cinética de acumulación de PHA en *Pseudomonas putida, P. oleovorans* y *Escherichia coli* se comparó con la de los aislados ambientales por espectrofluorometría. En todas las cepas estudiadas, la acumulación de PHA, medida como la intensi-

Crivado espectrofluorométrico rápido de bactérias produtoras de polihidroxialcanoatos em tapetes microbianos

Resumo. Os ecossistemas dos tapetes microbianos se caracterizam por oscilações diárias e estacionais em diversas variáveis físico-químicas, de forma tal que os microorganismos residentes devem adaptar-se frequentemente às condições cambiantes de seu ambiente. Se destacou que em condições de stress, as células bacterianas com conteúdos mais elevados em polihidroxialcanoatos (PHA) sobrevivem mais tempo que aquelas cujo conteúdo é menor. Neste estudo, utilizou-se a técnica do corante vermelho Nilo para seleccionar as cepas produtoras de PHA des tapetes microbianos do delta do Ebro e para monitorizar a acumulação relativa de PHA durante o cultivo no laboratório. O número de isolados heterotrofos em TSA foi de aproximadamente 10^7 unidades formadoras de colónias/g tapete microbiano. Destas, replicaram-se 100 colónias escolhidas ao acaso cultivadas em agar mineral salino limitado em nitrogénio, ao que se lhe acrescentou o corante vermelho Nilo para a detecção de PHA. A fluorescência laranja, que se produz ao unir-se o vermelho Nilo aos grânulos de polímero na célula, detectou-se em aproximadamente 10% dos isolados heterotrofos replicados. A cinética de acumulação de PHA em *Pseudomonas putida* e *P. oleovorans* foram comparadas com os isolados ambientais por espectrofluorometria. A acumulação de PHA, medida como a intensidade relativa de fluorescência, alcançou uma

dad relativa de fluorescencia, alcanzó una concentración estable a las 48 h de incubación. A las 72 h, la máxima intensidad de fluorescencia de las cepas incubadas con glucosa o fructosa fue casi siempre similar. La cepa MAT-28 acumuló más PHA que los otros aislados. Estos resultados ponen de manifiesto que los datos obtenidos a partir de aislados ambientales pueden ser mucho mejores que los que se basan en programas de simulación, y que los tapetes microbianos constituyen una excelente fuente de cepas productoras de PHA con aplicaciones industriales. [**Int Microbiol** 2006; 9(2):95-102]

Palabras clave: *Pseudomonas oleovorans* · *Pseudomonas putida* · *Escherichia coli* · poli-hidroxialcanoatos · tapetes microbianos · rojo Nilo · espectrofluorometría · cribado ("screening")

concentração estável em todas as cepas estudadas às 48 h de incubação. Às 72 h a intensidade máxima de fluorescência de cada cepa incubada com glicose e frutose foi quase sempre similar. A cepa MAT-28 acumulou mais PHA que os outros isolados. Os resultados obtidos põem de manifesto que os dados dos isolados ambientais podem melhorar notavelmente os estudos baseados em programas de simulação, e que os tapetes microbianos constituem uma excelente fonte de cepas produtoras de PHA com aplicações industriais. [**Int Microbio**l 2006; 9(2):95-102]

Palavras chave: *Pseudomonas oleovorans* · *Pseudomonas putida* · *Escherichia coli* · poli-hidroxialcanoatos · tapetes microbianos · vermelho Nilo · espectrofluorometria · crivado ("screening")