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ORIGINAL PAPER

Metabolic characterization of a strain (BM90) of *Delftia tsuruhatensis* showing highly diversified capacity to degrade low molecular weight phenols

Belén Juárez-Jiménez · Maximino Manzanera · Belén Rodelas · Maria Victoria Martínez-Toledo · Jesus Gonzalez-López · Silvia Crognale · Chiara Pesciaroli · Massimiliano Fenice

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Abstract A novel bacterium, strain BM90, previously isolated from Tyrrhenian Sea, was metabolically characterized testing its ability to use 95 different carbon sources by the Biolog system. The bacterium showed a broad capacity to use fatty-, organic- and amino-acids; on the contrary, its ability to use carbohydrates was extremely scarce. Strain BM90 was identified and affiliated to *Delftia tsuruhatensis* by molecular techniques based on 16S rRNA gene sequencing. *D. tsuruhatensis* BM90, cultivated in shaken cultures, was able to grow on various phenolic compounds and to remove them from its cultural broth. The phenols used, chosen for their presence in industrial or agro-industrial effluents, were grouped on the base of their chemical characteristics. These included benzoic acid derivatives, cinnamic acid derivatives, phenolic aldehyde derivatives, acetic acid derivatives and other phenolic compounds such as catechol and *p*-hydroxyphenylpropionic acid. When all the compounds (24) were gathered in the same medium (total concentration: 500 mg/l), BM90 caused the complete depletion of 18 phenols and the partial removal of two others. Only four phenolic compounds were not removed. Flow cytometry studies were carried out to understand the physiological state of BM90 cells in presence of the above phenols in various conditions. At the concentrations tested, a certain toxic effect was exerted only by the four compounds that were not metabolized by the bacterium.

Keywords *Delftia tsuruhatensis* · Biolog · Phenolic compounds degradation · Flow cytometry · GC/MS

B. Juárez-Jiménez · M. Manzanera · B. Rodelas · M. V. Martínez-Toledo · J. Gonzalez-López
Section Microbiology, Institute of Water Research of University of Granada, Ramón y Cajal, sn., 18071 Granada, Spain

S. Crognale
Dipartimento di Agrobiologia e Agrochimica, University of Tuscia, Via San Camillo de Lellis snc, 01100 Viterbo, Italy

C. Pesciaroli · M. Fenice
Dipartimento di Ecologia e Sviluppo Economico Sostenibile, University of Tuscia, Largo dell'Università, 01100 Viterbo, Italy

M. Fenice (✉)
Laboratory of Applied Marine Microbiology, Conisma, University of Tuscia, Viterbo, Italy
e-mail: fenice@unitus.it

Introduction

Although some phenolic compounds (polyphenols), present in low concentration in various foods and beverages, are well-known healthy substances with antioxidant activity (Ng et al. 2000; Lodovici et al. 2001), in general phenols are very hazardous

pollutants. High concentration of phenols are present in effluents and wastes of oil refineries, petrochemical and coal plants, and other industrial and agro-industrial processes (Yanase et al. 1992; Garrote et al. 2004; Subramanyam and Mishra 2007). These phenolic components are often associated with phytotoxic and antimicrobial activity (Borja et al. 1995; Aggelis et al. 2003; Casa et al. 2003) and even those that exert benefits are toxic if present in higher concentration (Kamaya et al. 2006).

Nowadays, a wide range of technological treatments have been tested in order to reduce the pollution caused by phenolic compounds (Hamdi 1993). Nevertheless, due to the inhibition effects on the microbial growth, biological treatment of phenol-containing wastewater can be carried out on highly diluted effluents only (Borja 1994; Quaratino et al. 2007). Various phenols-degrading bacteria have been isolated and evaluated for the control of phenol pollution (Arrieta and Herndl 2001) but the knowledge on the degradative specificity of the involved microorganisms is still insufficient.

Marine bacteria are less investigated than those from other environments and represent an extremely rich repertoire of organisms with potential biodegradative properties for pollutant biotransformation (Munn 2004; Fenice et al. 2007). It is known that, in most of the oceans, organic matter flux into bacteria is a major pathway (Azam et al. 1983; Cole et al. 1988; Azam 1998). These marine bacteria are able to hydrolyze polymers and particles using various hydrolytic enzymes (Martinez et al. 1996; Arrieta and Herndl 2001; Arnosti et al. 2005). Hence, oceans and seas can be valid alternative niches to search microorganisms with high biodegradative properties.

Delftia tsuruhatensis, seems to have a broad metabolic diversity for the biodegradation of various recalcitrant compounds. Its degrading ability has been demonstrated for anilines (Liang et al. 2005; Sheludchenko et al. 2005), terephthalate, protocatechuate (Shigematsu et al. 2003a, b) and (*S*)-2,2-dimethylcyclopropanecarboxamide (Zheng et al. 2007). However, no extensive studies have been carried out to investigate the metabolic capacity of this bacterium in particular for its ability to metabolize phenolic compounds.

In this work, we performed a large metabolic characterization of *D. tsuruhatensis* strain BM90 testing its ability to use 95 different carbon sources. In addition, we report on its capacity to grow on a wide array of phenols, very often found in industrial and agro-industrial effluents and wastes, and to remove them from its cultural medium. Moreover, cyto-fluorimetric studies, to understand the bacterium physiological state in presence of different phenols in various conditions, are reported.

Materials and methods

Microorganism

Delftia tsuruhatensis BM90 was previously isolated from water samples collected at 90 m deep in the Tyrrhenian Sea off the coast of Giglio Island, Grosseto, Italy (Fenice et al. 2007). The strain has been deposited to the Spanish Type Culture Collection (CECT): Accession Number, CECT 7427. During the study, the strain was maintained at 4°C on Plate Count Agar slants (PCA, Oxoid, UK) and routinely sub-cultured.

Morphological, physiological and biochemical tests

Early exponential phase cells from cultures grown at 28°C were used for the morphological, physiological and biochemical tests. Morphological characterization was done using Gram stained cells. Oxidase and catalase activity were tested as previously described (Kovac 1956; Whittenbury 1964).

Metabolic competence concerning the use of 95 carbon sources (Table 1) (including carbohydrates, carboxylic acids, polymers/oligomers, amines/amides, aminoacids and other compounds) was tested by the "Biolog" system (Odumeru et al. 1999; Truu et al. 1999). Briefly: Biolog GN2 micro-plates were used to test the assimilation ability of the strain. Appropriate suspensions of BM90 (cell density OD = ±0.65 at 560 nm) were inoculated into the micro-well plates and incubated for 24 h as described in the GN2 Biolog Micro-Plate system manuals. Color development was automatically recorded using the Biolog micro-plate reader using both the 590 and 750 nm wavelength filters to measure growth and substrate oxidation, respectively. The results were interpreted with the

Table 1 Use of different carbon sources by *Delftia tsuruhatensis* BM90 as revealed by the Biolog system

Carbon source	Response
Water (control)	−
α-Cyclodextrin/dextrin	−
Tween 40/Tween 80	++
N-acetyl-D-galactosamine/*N*-acetyl-D-glucosamine	−
Adonitol/D-arabitol/D-cellobiose/L-fucose/D-galactose/i-erythritol	−
L-arabinose/D-fructose/gentiobiose/D-psicose/glycogen	±
α-D-glucose/*m*-inositol/α-D-lactose/lactulose/maltose/D-mannitol	−
D-mannose/D-melibiose/β-methyl-D-glucoside/D-raffinose/L-rhamnose	−
D-sorbitol/sucrose/D-trehalose/turanose/xylitol	−
Pyruvic acid methyl ester/succinic acid mono-methyl-ester	++
Acetic acid/formic acid/D-gluconic acid	++
Cis-aconitic acid/citric acid/D-galactonic acid lactone	−
D-galacturonic acid/D-glucosaminic acid/D-glucuronic acid	−
α- and β-hydroxybutyric acid/*p*-hydroxy phenylacetic acid	++
γ-Hydroxybutyric acid/γ-amino butyric acid	±
α-Keto butyric acid/α-keto valeric acid/D,L-lactic acid	++
α-keto glutaric acid/itaconic acid	±
Malonic acid/D-saccharic acid	−
Propionic acid/quinic acid/sebacic acid	++
Succinic acid/Br-succinic acid/succinamic acid	++
Glucuronamide/L-alaninamide/D-alanine	−
L-alanine/L-alanyl-glycine/glycyl-L-glutamic acid	±
L-asparagine/L-aspartic acid/L-glutamic acid	++
Glycyl-L-aspartic acid/hydroxy-L-proline/L-ornithine	−
L-phenylalanine/L-proline/L-threonine/L-histidine/L-leucine	++
D-serine/L-serine/D,L-carnitine	−
Urocanic acid/L-pyroglutamic acid	++
Inosine/uridine/thymidine/phenylethyl-amine/putrescine	−
2,3-Butanediol	±
Glycerol/2-aminoethanol	−
D,L-α-glycerol phosphate/α-D-glucose-1-phosphate/D-glucose-6-phosphate	−

"+", "±" and "−" indicate the full ability, the partial ability and the inability of the bacterium to grow and oxidize the various substrates

Biolog Microlog 4.2 database and software (Biolog, Hayward, CA, USA) obtaining both metabolic and taxonomic information.

Media and culture conditions

Starter cultures of BM90 in shaken flasks, obtained from PCA slants, were incubated on a rotary shaker at 28°C and 180 rpm in Nutrient Broth (NB) (Difco, USA). After 18 h of incubation, cells were harvested by centrifugation, washed and resuspended in sterile water. To obtain the requested final concentration (ca 10^6 cells/ml), measured as OD at 600 nm, the necessary amount of cell suspension was added to 500 ml Erlenmeyer flasks containing 100 ml of a medium composed of 670 mg/l Yeast Nitrogen Base (YNB) (Difco, USA) and 500 mg/l of various phenolic compounds as follows (molar concentration, mM/l, of each compound is reported in brackets):

- MA (pool of benzoic acid derivatives): 3-hydroxybenzoic acid (0.452), 4-hydroxybenzoic acid (0.452), veratric acid (0.343), vanillic acid (0.372), gentisic acid (0.405), 3,4,5-trimethoxybenzoic acid (0.294), protocatechuic acid (0.405) and syringic acid (0.315); 62.5 mg/l each.

- MB (pool of cinnamic acid derivatives): *p*-coumaric acid (0.761), ferulic acid (0.644), caffeic acid (0.694) and esculetin (0.702); 125 mg/l each.
- MC (pool of phenolic aldehyde derivatives): syringaldehyde (0.686), protocatechuic aldehyde (0.905), vanillin (0.821) and 4-hydroxybenzaldehyde (1.023); 125 mg/l each.
- MD (pool of acetic acid derivatives): 3-hydroxyphenylacetic (1.098), 4-hydroxyphenylacetic acid (1.098) and 3,4-dihydroxyphenylacetic acid (0.993), 167 mg/l each.
- ME (pool of other phenols): catechol (0.908), 4-methylcatechol (0.805), tyrosol (0.724), vanillol (0.649), and 3-*p*-hydroxyphenylpropionic acid (0.602); 100 mg/l each.
- MF (pool of all the 24 compounds): 3-hydroxybenzoic acid (0.152), 4-hydroxybenzoic acid (0.152), veratric acid (0.115), vanillic acid (0.125), gentisic acid (0.136), 3,4,5-trimethoxybenzoic acid (0.099), protocatechuic acid (0.136), syringic acid (0.106); *p*-coumaric acid (0.128), ferulic acid (0.108), caffeic acid (0.116), esculetin (0.118), syringaldehyde (0.115), protocatechuic aldehyde (0.152), vanillin (0.138), 4-hydroxybenzaldehyde (0.172), 3-hydroxyphenylacetic (0.138), 4-hydroxyphenylacetic acid (0.138), 3,4-dihydroxyphenylacetic acid (0.125), catechol (0.191), 4-methylcatechol (0.169), tyrosol (0.152), vanillol (0.136) and 3-*p*-hydroxyphenylpropionic acid (0.126), 21 mg/l each.

Filter sterilized solutions of the various phenols were added to YNB solution sterilized at 121°C for 20 min. Phenols solutions pH was adjusted, before filtration, to 7.0 using 0.1 N NaOH. Shaken cultures were carried out in triplicate at 28°C for 96 h and sterile samples withdrawn every 12 h. Controls were performed submitting non-inoculated media to the same conditions. Growth was monitored by measuring the absorbance at 600 nm with an U-2000 Spectrophotometer (Hitachi, J).

All phenolic compounds were from Fluka with HPLC grade. All other chemicals were of analytical grade.

Analytical assays

Two different methods were used to determine the concentration of phenolic compounds. The Folin–Ciocalteu method was used to measure total phenol content as reported by Singleton et al. (1999). Gas chromatographic analysis was used to determine concentration of single specific phenols as follows: inoculated and non-inoculated media were cleared by centrifugation and filtered through 0.22 μm membrane filters. Samples (10 ml) were acidified to pH 3.0 with HCl and extracted three times with diethyl ether followed by solvent removal under vacuum at 35°C. Dried extracts were resuspended in 1 ml methanol, re-evaporated to dryness and re-dissolved in 1 ml pyridine. Samples were silanized with *bis*(allyldimethylsily1)-trifluoroacetamide (BSTFA) (Fluka, UK). The phenolic compounds were measured by Gas chromatography using an Agilent 6890 Series GS System gas chromatograph fitted with a splitless injector for a low background HP-5MS fused silica capillary column (5% phenylmethylsiloxane) 60 m × 0.25 mm ID; 0.25 μm film thickness (J&W Scientific, USA). A silanized injector liner split/splitless (2 mm ID) was used. Detection was carried out with a 5,973 mass-selective single quadrupole detector (Agilent technologies, USA). GC–MS operations and data process were carried out by ChemStation software. The injector temperature was set at 250°C. The column oven temperature was held at 90°C for 1 min, then it was increased to 220°C at a heating rate of 6°C/min, then to 290°C at 10°C/min and held for 1.23 min and finally to 310°C at a rate 40°C/min and the temperature was held for 7.5 min. The total run time was 38.5 min. The transfer line, manifold and trap temperatures were 260, 40 and 210°C, respectively. The carrier gas used was helium (purity 99.999%) at a flow rate of 1.0 ml/min. Samples were injected in the splitless mode and the splitter was opened after 3 min (solvent delay time). Sample volume in the direct injection mode was 5 μl.

The conditions for electron impact ionization (EI) were: ion energy of 70 eV and the mass range scanned was 140–465 *m*/*z*. The MS was tuned everyday to *m*/*z* 69, 219 and 502 with perfluorotributylamine (PFTBA) as a calibration standard. Single ion monitoring (SIM) acquisition mode (dwell time 100 ms/ion) was used.

Standard curves for each phenol were calculated from eight different concentrations (0–742.6 mg/l in YNB medium). The concentration coefficient was >0.99 and sensitivity of the method was in the range of 1–0.0156 mg/ml of phenolic compounds.

Strain identification

BM 90 was identified by genetic analysis of the 16S rRNA gene (Pozo et al. 2002). DNA was extracted as reported by Sambrook et al. (1989). Primers fD1 and rD1 (Weisburg et al. 1991), from Sigma-Genosys (UK), were used to amplify the almost full length of the 16S rRNA gene. Fresh cultured colonies of strain BM90 grown on PCA plates were lysated adding 20 µl of a mixture of NaOH (0.05 M) and SDS (0.25%) and boiling for 15 min. The lysates were adjusted to 200 µl with sterile bidistilled water and centrifuged at 13,000 rpm for 5 min. Cleared lysates (4 µl) were used as template for amplification. PCR were done adding to the lysate 1× PCR buffer (GeneCraft, D), 1.5 mM MgCl, 200 µM dNTPs (Roche Molecular Biochemicals, D), 20 pmol of each primer, and 1 U of *Taq* polymerase (GeneCraft). Final volume of the reaction tubes was adjusted to 50 µl. Reactions were run using a GeneAmp PCR system 2400 (Perkin-Elmer, USA) following a temperature profile already described (Vinuesa et al. 1998). The amplification product was purified by agarose gel electrophoresis and extracted with QIAEX II Agarose Gel Extraction kit (Qiagen, D). The nucleotide sequence of the purified band was determined by the dideoxy chain termination method, using ABI-PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA) and automated sequencer Applied Biosystems ABI373 (Applied Biosystems). Custom additional primers were synthesised by Sigma-Genosys when needed to complete the sequence of the 16S rRNA gene of the whole DNA fragments. Sequence data were analysed using the GCG Wisconsin Package V. 10.1 Programs (Genetics Computing Group, USA) and were compared to sequences in EMBL bank using FASTA v. 3.3t07 (Pearson and Lipman 1988).

Flow cytometry analysis

The physiological state of the bacterium individual cells was characterized using multi-parameter flow cytometry in different conditions. Presence of both an intact polarized cytoplasmic membrane and active transport systems, essential for a fully functional cell, was tested by the addition of propidium iodide (PI) and 3,3-dihexylocarbocyanine iodide (DiOC6). PI binds to DNA, but cannot cross an intact cytoplasmic membrane, and DiOC6 accumulates intracellularly when membranes are polarized or hyperpolarized (Müller et al. 1999; Shapiro 2003). Thus, using the two dyes together, it is possible to verify the physiological state of individual bacterial cells submitted to various growth conditions.

Flow cytometry measurements were a variant of the method described by Reis and coworkers (Reis et al. 2005) using a Facs Vantage analyser (Becton Dickinson Immunocytometry System, USA), with 488 nm excitation from an argon-ion laser at 30 mW. Samples were immediately diluted with phosphate buffer at pH 7.0 (at least 1:2000, v/v in order to obtain ca 10^6 cells/ml) and stained with a mixture of the two fluorescent dyes, PI (Molecular Probes, P-1304) and DIOC6 (Molecular Probes, D-273). Stock solutions of each dye were as follows: 10 µg/ml DIOC6 in dimethyl sulphoxide (DMSO) and PI 2 mg/ml in distilled water. The working concentrations of DIOC6 and PI were 5 ng/ml (10 nM) and 1 µg/ml, respectively (Lopes da Silva et al. 2005). Fresh working solutions were made daily and filtered (0.2 µm, Millipore, USA) to sterilize and remove possible precipitated materials. In addition, software discrimination was set on both the light scattering properties in the forward angle direction (FALS) signal and the right angle direction (RALS) signal. Optical filters were set up to measure PI and DIOC6 fluorescence at 585 and 530 nm, respectively.

Spectral overlap between DIOC6 and PI-emitted fluorescence was compensated using the software Cell Quest™ ver 3.1 (Becton Dickinson, USA).

The physiological state of *D. tsuruhatensis* BM90 was tested on samples taken after 0, 24, 48 and 72 h of incubation in media containing 670 mg/l of YNB and 500 mg/l of different pools of phenols as follows (molar concentration, mM/l, of each compound is reported in brackets):

- MF (pool of the 24 phenols, see above): 3-hydroxybenzoic acid (0.152), 4-hydroxybenzoic acid (0.152), veratric acid (0.115), vanillic acid (0.125), gentisic acid (0.136), 3,4,5-trimethoxybenzoic acid (0.099), protocatechuic acid (0.136), syringic acid (0.106); *p*-coumaric acid (0.128), ferulic acid (0.108), caffeic acid (0.116), esculetin (0.118), syringaldehyde (0.115), protocatechuic aldehyde (0.152), vanillin (0.138), 4-hydroxybenzaldehyde (0.172), 3-hydroxyphenylacetic (0.138),

4-hydroxyphenylacetic acid (0.138), 3,4-dihydroxyphenylacetic acid (0.125), catechol (0.191), 4-methylcatechol (0.169), tyrosol (0.152), vanillol (0.136) and 3-*p*-hydroxyphenylpropionic acid (0.126), 21 mg/l each.

- MG (pool of the 18 compounds that are completely metabolised by BM90) 3-hydroxybenzoic acid (0.203), 4-hydroxybenzoic acid (0.203), veratric acid (0.154), vanillic acid (0.166), gentisic acid (0.182), protocatechuic acid (0.182), syringic acid (0.141), *p*-coumaric acid (0.170), ferulic acid (0.144), caffeic acid (0.155), protocatechuic aldehyde (0.203), vanillin (0.184), 4-hydroxybenzaldehyde (0.229), 3-hydroxyphenylacetic (0.184), 4-hydroxyphenylacetic acid (0.184), 3,4-dihydroxyphenylacetic acid (0.166), catechol (0.254) and 3-*p*-hydroxyphenylpropionic acid (0.168): 28 mg/l each.
- MH (pool of the four compounds that BM90 is not able to metabolise) 3,4,5-trimethoxybenzoic acid (0.589), esculetin (0.702), tyrosol (0.905) and vanillol (0.811): 125 mg/l each.

Moreover, two controls were added: cells cultivated in YNB (without phenols as carbon source) as control under non toxic conditions (C1) and YNB added with 5 mM Carbonyl cyanide 3-chloro-phenylhydrazone as negative control (C2). This is a proton ionophore that kills the cell producing a membrane potential collapse.

Statistical analyses

One-way analysis of variance (Anova) and pairwise multiple comparison procedure (Tukey test) were carried out using the statistical software SigmaStat 2.0 (Jandel, USA).

Results

Morphological and metabolic characterization

Morphology: colonies of strain BM90 were slightly viscous, round, convex, 0.5–2 mm in diameter (after 24 h of incubation), slightly glossy, and white to cream-colored. Bacteria were Gram-negative motile rods, with an average size of 0.5–0.6 μm × 1.8–2.5 μm.

Metabolic competences: strain BM90 was catalase and oxidase positive.

The ability to assimilate different carbon sources is reported in Table 1. The Biolog systems reports the OD at two wavelength in order to measure both the growth and the assimilation of the carbon sources in terms of color development. The results are elaborated by the Biolog software that renders the capacity of the strain to use a certain carbon source elaborating the information of the two readings. Results can only be 0, 0.5 or 1 (or "−", "±" and "+") reporting the incapacity, the slight capacity and the full capacity to use a substrate, respectively. The mere reading of the OD is not meaningful (see Biolog MicroLog System User Guide).

Delftia tsuruhatensis BM90 showed the ability to use a wide array of fatty-, carboxylic, amino-acids and some of their derivatives. Actually it was able to assimilate 36 out of the 52 compounds tested (Table 1). On the contrary, the scarce ability of BM90 to use simple sugars was evident. It was only able to partially use fructose, arabinose and psicose out of the 27 sugars tested. In addition, a certain ability to use glycogen was recorded. Although strain BM90 was unable to use glucose its ability to oxidize gluconic acid was clear (Table 1).

Identification of strain BM90

In a previous work, 95 bacterial isolates, obtained from various samples collected in the east sector of Central Tyrrhenian Sea, were preliminary plate screened for 12 extracellular enzyme activities (Fenice et al. 2007). Among them, three strains, positives for phenol-oxidase, were shaken cultured in media containing some phenols as carbon sources. Strain BM90, showing the best capacity of phenol degradation, was selected for further studies (data not shown). Before further investigations, strain BM90 was taxonomically identified.

The Biolog System could be used as a taxonomic tool due to its database containing the metabolic profile of a large number of microorganisms. BM90 metabolic profile analyzed with Biolog did not permit strain classification to the species level. The most close microorganism was *Delftia acidovorans* (70% of identity). However, *D. acidovorans* is the only specie of *Delftia* present in the Biolog database. To obtain more detailed taxonomic information, strain

BM90 identification was carried out by molecular methods.

The nucleotide sequence of the nearly complete 16S rRNA gene (1,417 bp) was determined, submitted to GeneBank and deposited with the following Accession Number: bankit1099429EU779949.

BM90 16S rRNA gene was compared to previously recorded sequences and according to the BLAST search, the sequence showed 100% similarity to the 16S rRNA of *Delftia tsuruhatensis* ZJB-05174 (Accession Number DQ864991, Zheng et al. 2007) and of *Delftia tsuruhatensis* HR4 (Accession Number AY302438, Han et al. 2005). Lower similarity was recorded with various other *Delftia* spp. (data not reported).

Growth of *Delftia tsuruhatensis* BM90 on phenolic compounds

Usually, maximum of growth (ca 2×10^9 cell/ml) of *D. tsuruhatensis* BM90 in rich medium (NB), was obtained after 16–18 h of cultivation starting from inocula of ca 10^6 cell/ml. BM90 was also able to grow (same inoculum size) in all the YNB-based media containing different pools of phenolic compounds (MA–MF) but in all these media growth was delayed and quantitatively lower (Fig. 1a–f).

BM90 grew rather fast when inoculated into MA (benzoic acid derivatives) (Fig. 1a) and MD (acetic acid derivatives) (Fig. 1d) showing maximal growth (6.6×10^8 and 9.31×10^8 cell/ml for MA and MD, respectively) after 48 h of incubation. Similar growth pattern was found in MF containing a mixture of all the phenolic derivatives (Fig. 1f). By contrast, a slower growth, with a typical diauxic profile, was observed when the bacterium was cultivated on MB (cinnamic acid derivates), MC (phenolic aldehyde derivates) and ME (other phenols) (Fig. 1b, c, e). In all these cases, the first exponential phase started between the 12th hour and the 24th hour of growth. Then, a second lag phase was evident between the 24th hour and 48th hour of growth to be followed by a further exponential growth. However, maximal cell growth was always recorded at the 72nd hour of incubation (8.15×10^8 and 6.03×10^8 cell/ml for MB and MC, respectively). It is worth to note that, always, to the diauxic growth corresponds a mirror image in the curve of phenol degradation. This indicates that phenol degradation occurs in two steps corresponding to the two bacterial exponential phases of growth.

In all cases growth of BM90 stopped when phenols removal terminated.

Degradation of phenolic compounds

Presence and concentration of the phenolic compounds in the various media was followed along the whole processes by total phenol determinations (Fig. 1) and GC/MS (Table 2). GC/MS chromatograms are shown for MF only (Fig. 2).

With the only exception of ME, the results indicated that phenols removal was always simultaneous with cell growth (Fig. 1a–f). Most of benzoic acid derivatives (MA) were completely depleted within the first 48 h, except for syringic acid that at the 48th hour was approximately one sixth of the initial concentration, and for 3,4,5-trimethoxybenzoic acid that remained almost at same level throughout the whole process. Total phenol degradation in MA was 87.6% (Table 2). Complete phenol removal was also recorded in MD (acetic acid derivatives) after 48 h. Also cinnamic acid (MB) and phenolic aldehyde (MC) derivatives were completely eliminated but only after 96 h. The only exception was esculetin that at 96 h was only slightly reduced (22.5%).

As for ME (other phenolic derivatives), the removal started after the 48 h of incubation corresponding to the second exponential phase. Only catechol and 3-*p*-hydroxyphenylpropionic acid were completely depleted by BM90 at the 96th hour. Tyrosol concentration increased both after 48 and 96 h (31.3 and 11.2%, respectively) while 4-methylcatechol and vanillol decreased after 48 h of incubation to increase again after 96 h. However, this occurred in the control also.

Total phenol degradation in MB, MC, MD and ME was 80.6, 100, 100 and 34%, respectively (Table 2).

BM90 behaviour, concerning the removal of each single phenolic compound, was similar either when they were grouped by chemical characteristics (media MA–ME) or when they were pooled together in the same medium (MF). However, in MF some compounds were completely removed at 48 h instead of 96 h: this is probably due to the lower concentration of each compound. By contrast, in MF syringaldehyde was only partially depleted (56.8%) at 96 h

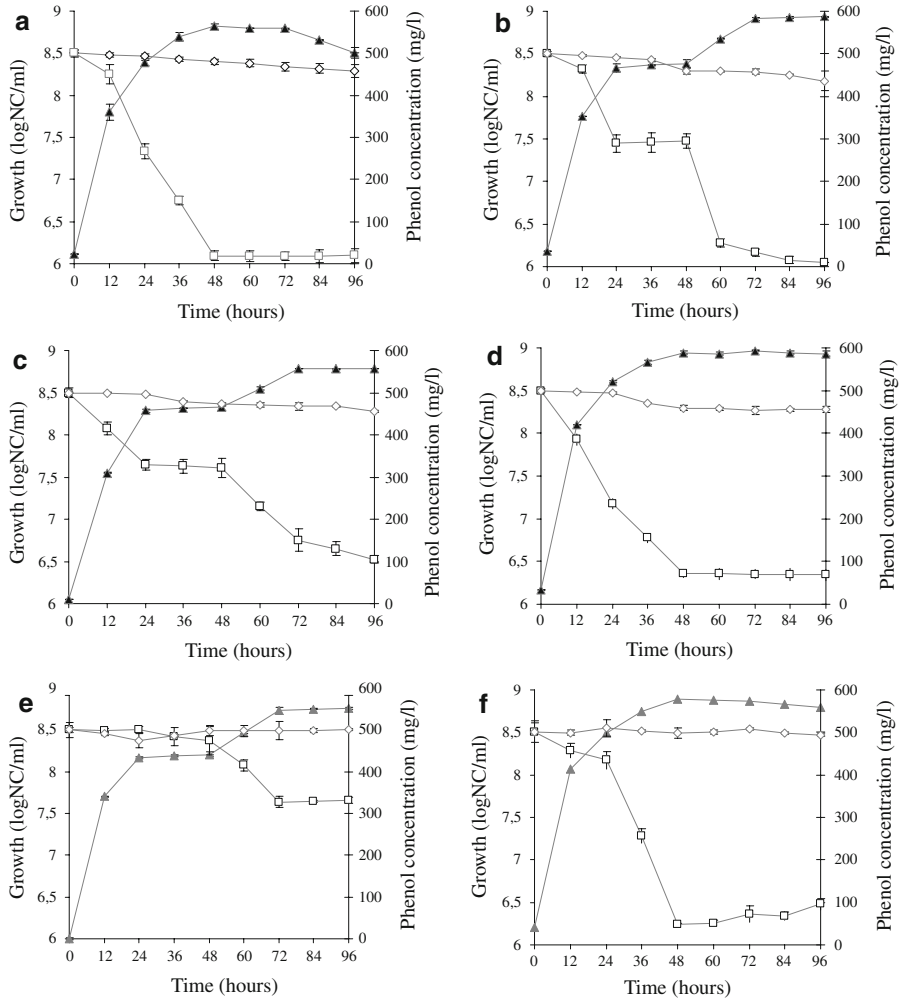

Fig. 1 Time course of growth (*filled triangle*) and phenol removal (*open square* = inoculated samples; *open diamond* = non inoculated controls) by *Delftia tsuruhatensis* BM90 in media containing different pools of phenols. **a** MA (benzoic acid derivates); **b** MB (cinnamic acid derivates); **c** MC (phenolics aldehyde derivates); **d** MD (acetic acid derivates); **e** ME (pool of others phenolics compounds), **f** MF (pool of all the 24 compounds). Results are mean of three experiments and bars represent SD

even if its concentration was lower than in MC. The concentration of 4-methylcatechol in the control increased of ca 143%. Total phenol degradation in MF was 75.3%.

Figure 2 shows the presence of the various phenols in MF at 0 and 72 h of incubation as detected by GC/MS.

Physiological studies by flow cytometry

Low DiOC6 staining and no PI staining were detected in all the media (MF–MH) at the inoculum stage (0 h) as cell membranes were intact, started to be polarized and PI was not able to enter the cell to bind the DNA (Fig. 3a). This is the typical situation of latent cells still having scarcely polarized, but whole, membrane. In MH DiOC6 was even lower (Fig. 3e) showing almost no polarization.

The opposite pattern was found for all the media after 72 h showing good staining for PI and no staining for DiOC6. This revealed depolarized, non integral membranes and PI-stained DNA (Fig. 3d, h).

After 24 h, all the samples were stained with DiOC6 and, into a much lower extent, with PI (Fig. 3b). These data suggested that some cells were starting to lose membrane polarity, and consequently beginning to die. The only exception regarded the cells grown on MH (mixture of the phenols that were not metabolized by BM90) where almost no DiOC6 was detected and the majority of the cells were stained with PI (Fig. 3f). Here, very little membrane polarization was detected, and most of the cell were

Table 2 Removal of phenol compounds by *Delftia tsuruhatensis* BM90 in different media as detected by GC/MS analysis (in brackets % of variation: in bold % of removal, in italic % of increase)

Media	Cultivation (48 h)		Time (96 h)	
	Uninoculated (control)	Inoculated	Uninoculated (control)	Inoculated
MA: benzoic acid derivates; initial concentration 62.5 mg/l each				
3-Hydroxybenzoic acid	63.82 ± 1.32^{a} (*2.1*)	<1.56^{b} (**100**)	65.13 ± 2.63^{a} (*4.2*)	<1.56^{b} (**100**)
4-Hydroxybenzoic acid	62.00 ± 0.50^{a} (**0.8**)	<1.56^{b} (**100**)	61.49 ± 1.01^{a} (**1.6**)	<1.56^{b} (**100**)
Veratric acid	62.47 ± 0.03^{a} (**0.04**)	<1.56^{b} (**100**)	62.44 ± 0.06^{a} (**0.09**)	<1.56^{b} (**100**)
Vanillic acid	60.98 ± 1.50^{a} (**2.4**)	<1.56^{b} (**100**)	59.46 ± 3.01^{a} (**4.8**)	<1.56^{b} (**100**)
Gentisic acid	61.86 ± 0.63^{a} (**1.0**)	<1.56^{b} (**100**)	61.22 ± 1.27^{a} (**2.0**)	<1.56^{b} (**100**)
3,4,5-Trimethoxybenzoic acid	61.26 ± 1.24^{a} (**1.9**)	53.05 ± 1.01^{b} (**15.1**)	60.03 ± 2.47^{a} (**3.9**)	61.69 ± 15.11^{ab} (**1.3**)
Protocatechuic acid	61.77 ± 0.73^{a} (**1.1**)	<1.56^{b} (**100**)	61.04 ± 1.46^{a} (**2.3**)	<1.56^{b} (**100**)
Syringic acid	62.49 ± 0.01^{a} (**0.01**)	10.92 ± 0.55^{b} (**82.5**)	62.49 ± 0.01^{a} (**0.01**)	<1.56^{c} (**100**)
Total	496.64 ± 3.33^{a} (0.67)	63.97 ± 1.56^{b} (87.2)	493.29 ± 6.66^{a} (1.3)	61.69 ± 15.11^{b} (87.6)
MB: cinnamic acid derivates; initial concentration 125 mg/l each				
p-Coumaric acid	144.07 ± 14.16^{a} (*15*)	134.02 ± 0.64^{a} (*7.2*)	128.26 ± 5.66^{a} (*2.6*)	<1.56^{b} (**100**)
Ferulic acid	92.49 ± 19.04^{a} (**26**)	9.81 ± 1.88^{b} (**92.1**)	169.29 ± 6.24^{c} (*35.4*)	<1.56^{d} (**100**)
Caffeic acid	185.90 ± 29.76^{a} (*49*)	115.25 ± 26.59^{b} (**7.8**)	115.38 ± 5.60^{b} (**7.6**)	<1.56^{c} (**100**)
Esculetin	92.99 ± 14.86^{a} (**25.6**)	126.00 ± 18.30^{b} (*0.8*)	154.29 ± 18.44^{b} (*23.4*)	96.79 ± 0.61^{a} (**22.5**)
Total	515.45 ± 39.69^{a} (3.0)	385.09 ± 11.10^{b} (22.9)	567.23 ± 13.48^{a} (13.4)	96.79 ± 0.61^{c} (80.6)
MC: phenolic aldehyde derivates initial concentration 125 mg/l each				
4-Hydroxybenzaldehyde	91.40 ± 14.97^{a} (**26.8**)	60.19 ± 1.78^{b} (**51.8**)	110.59 ± 2.77^{a} (**11.5**)	<1.56^{c} (**100**)
Vanillin	137.55 ± 28.18^{a} (*10*)	62.55 ± 15.36^{b} (**49.9**)	124.19 ± 32.65^{a} (**0.6**)	<1.56^{c} (**100**)
Syringaldehyde	117.52 ± 15.57^{a} (**5.9**)	65.28 ± 9.31^{b} (**47.7**)	123.55 ± 22.90^{a} (**1.1**)	<1.56^{c} (**100**)
Protocatechuic aldehyde	122.01 ± 13.55^{a} (**2.4**)	75.85 ± 4.73^{b} (**39.3**)	105.58 ± 12.28^{a} (**15.5**)	<1.56^{c} (**100**)
Total	468.49 ± 42.45^{a} (6.3)	263.87 ± 31.16^{b} (47.2)	463.89 ± 70.55^{a} (7.2)	<1.56^{c} (100)
MD: acetic acid derivates; initial concentration 166.66 mg/l each				
3-Hydroxyphenylacetic acid	191.82 ± 4.47^{a} (*15.1*)	<1.56^{b} (**100**)	143.98 ± 20.60^{c} (**13.6**)	<1.56^{b} (**100**)
4-Hydroxyphenylacetic acid	183.03 ± 9.84^{a} (*9.8*)	<1.56^{b} (**100**)	144.07 ± 20.90^{c} (**13.5**)	<1.56^{b} (**100**)
3,4-Dihydroxyphenylacetic acid	163.98 ± 2.49^{a} (**1.6**)	<1.56^{b} (**100**)	170.89 ± 0.92^{c} (*2.5*)	<1.56^{b} (**100**)
Total	538.83 ± 11.82^{a} (7.7)	<1.56^{b} (100)	458.94 ± 42.42^{c} (8.21)	<1.56^{b} (100)
ME: other phenolic derivates; initial concentration 100 mg/l each				
Catechol	140.93 ± 1.07^{a} (*40.9*)	87.36 ± 3.14^{b} (**12.6**)	98.19 ± 7.76^{b} (**1.8**)	<1.56^{c} (**100**)
4-Methylcatechol	61.27 ± 3.53^{a} (**38.7**)	70.68 ± 1.36^{ab} (**29.3**)	85.25 ± 12.18^{b} (**14.7**)	121.60 ± 9.27^{c} (*21.6*)
Tyrosol	134.17 ± 6.29^{a} (*34.1*)	131.32 ± 4.01^{a} (*31.3*)	100.85 ± 0.35^{b} (*0.8*)	111.21 ± 12.54^{b} (*11.2*)
Vanillol	30.55 ± 1.54^{a} (**69.4**)	42.72 ± 1.63^{a} (**57.2**)	87.49 ± 26.34^{b} (**12.5**)	95.96 ± 15.97^{b} (**4.0**)
3-*p*-OH-phenyl-propionic acid	101.51 ± 3.24^{a} (*1.5*)	47.95 ± 19.64^{b} (**52.0**)	96.00 ± 6.72^{a} (**4.0**)	<1.56^{c} (**100**)
Total	468.42 ± 12.60^{a} (6.3)	380.03 ± 12.77^{b} (23.9)	467.77 ± 37.09^{a} (6.4)	328.78 ± 36.89^{b} (34.2)
MF: Combination of all the 24 phenols; initial concentration 20.83 mg/l each				
3-Hydroxybenzoic acid	21.34 ± 0.74^{a} (*2.4*)	<1.56^{b} (**100**)	21.85 ± 1.48^{a} (*4.8*)	<1.56^{b} (**100**)
4-Hydroxybenzoic acid	18.61 ± 2.24^{a} (**10.6**)	<1.56^{b} (**100**)	16.39 ± 4.47^{a} (**21.3**)	<1.56^{b} (**100**)

Table 2 continued

Media	Cultivation (48 h)		Time (96 h)	
	Uninoculated (control)	Inoculated	Uninoculated (control)	Inoculated
Veratric acid	21.56 ± 0.04^{a} (*3.5*)	<1.56^{b} (**100**)	22.30 ± 0.07^{a} (*7.0*)	<1.56^{b} (**100**)
Vanillic acid	22.13 ± 1.45^{a} (*6.2*)	<1.56^{b} (**100**)	23.42 ± 2.89^{a} (*12.4*)	<1.56^{b} (**100**)
Gentisic acid	17.71 ± 0.18^{a} (**14.9**)	<1.56^{b} (**100**)	14.59 ± 0.36^{c} (**29.9**)	<1.56^{b} (**100**)
3,4,5-Trimethoxybenzoic acid	19.59 ± 0.92^{a} (**5.9**)	19.67 ± 1.03^{a} (**5.5**)	18.36 ± 1.84^{a} (**11.8**)	18.56 ± 2.17^{a} (**10.8**)
Protocatechuic acid	20.89 ± 0^{a} (*0.2*)	<1.56^{b} (**100**)	20.94 ± 0^{a} (0.5)	<1.56^{b} (**100**)
Syringic acid	20.63 ± 0^{a} (**0.9**)	<1.56^{b} (**100**)	20.43 ± 0.01^{a} (**1.9**)	<1.56^{b} (**100**)
p-Coumaric acid	14.39 ± 0.56^{a} (**30.9**)	25.53 ± 1.52^{b} (*12.9*)	7.95 ± 1.12^{c} (**61.8**)	<1.56^{d} (**100**)
Ferulic acid	17.56 ± 0.08^{a} (**15.7**)	<1.56^{b} (**100**)	14.29 ± 0.15^{c} (**31.4**)	<1.56^{b} (**100**)
Caffeic acid	18.31 ± 0.08^{a} (**12.0**)	<1.56^{b} (**100**)	15.79 ± 0.15^{c} (**24.2**)	<1.56^{b} (**100**)
Esculetin	19.33 ± 0.03^{a} (**7.2**)	22.42 ± 3.21^{a} (*7.6*)	17.84 ± 0.06^{a} (**14.3**)	24.09 ± 5.02^{a} (*15.6*)
4-Hydroxybenzaldehyde	20.59 ± 0.66^{a} (**1.1**)	<1.56^{b} (**100**)	20.35 ± 1.31^{a} (**2.3**)	<1.56^{b} (**100**)
Vanillin	19.02 ± 1.17^{a} (**8.6**)	<1.56^{b} (**100**)	17.21 ± 2.33^{a} (**17.3**)	<1.56^{b} (**100**)
Syringaldehyde	28.66 ± 1.45^{a} (*37.6*)	11.33 ± 0.32^{b} (**45.6**)	36.50 ± 2.89^{c} (*75.2*)	8.99 ± 0.13^{b} (**56.8**)
Protocatechuic aldehyde	19.49 ± 0.02^{a} (**6.4**)	<1.56^{b} (**100**)	18.15 ± 0.03^{a} (**12.8**)	<1.56^{b} (**100**)
3-Hydroxyphenylacetic acid	24.52 ± 0.35^{a} (*17.7*)	<1.56^{b} (**100**)	28.21 ± 0.70^{c} (35.4)	<1.56^{b} (**100**)
4-Hydroxyphenylacetic acid	22.35 ± 0.50^{a} (*7.3*)	<1.56^{b} (**100**)	23.87 ± 0.99^{a} (14.5)	<1.56^{b} (**100**)
3,4-Dihydroxyphenylacetic acid	18.11 ± 0.41^{a} (**13.0**)	<1.56^{b} (**100**)	15.40 ± 0.81^{c} (**26.0**)	<1.56^{b} (**100**)
Catechol	20.33 ± 0.63^{a} (**2.4**)	<1.56^{b} (**100**)	19.84 ± 1.26^{a} (**4.7**)	<1.56^{b} (**100**)
4-Methylcatechol	35.74 ± 1.00^{a} (*71.5*)	6.36 ± 1.73^{b} (**69.4**)	50.65 ± 2.00^{c} (*143.1*)	9.43 ± 1.61^{b} (**54.7**)
Tyrosol	20.81 ± 0.21^{a} (**0.09**)	27.07 ± 2.04^{b} (*29.9*)	20.79 ± 0.42^{a} (**0.2**)	27.36 ± 2.99^{b} (*31.3*)
Vanillol	24.24 ± 0.49^{a} (*16.3*)	38.51 ± 3.27^{b} (*84.8*)	27.65 ± 0.99^{a} (*32.7*)	34.76 ± 3.40^{b} (*66.8*)
3-*p*-OH-phenylpropionic acid	21.65 ± 2.23^{a} (*3.9*)	<1.56^{b} (**100**)	22.47 ± 4.45^{a} (*7.8*)	<1.56^{b} (**100**)
Total	507.58 ± 1.64^{a} (*1.5*)	150.89 ± 13.11^{b} (69.8)	515.23 ± 3.28^{a} (*3.0*)	123.19 ± 8.52^{c} (75.3)

Values are means of three replicates ± SD. Line means followed by the same superscript letters are not significantly different according to the Tukey test ($P \leq 0.01$)

dead or began to die. At the same cultivation time, C1 presented only few dying cells but the majority of them where still in latency (Fig. 3i) and started to massively die after further 24 h (Fig. 3l).

After 48 h of cultivation cell dead was even much more evident by the higher PI staining and lower DiOC6 staining (Fig. 3c). The situation was more evident in MH with almost all the cells stained with PI (Fig. 3g).

The staining profile of the cells grown on MF and MG was similar even if, in the medium containing all the 24 phenols (MF), most of the cells were still in latency after 24 h (Fig. 3m).

Figure 3n shows the cells at the inoculum stage when treated with a ionophore (C2): a typical example of dead cells with high PI staining.

Discussion

Delftia tsuruhatensis has been described as a new species in 2003 by Shigematsu et al. (2003a). This organism is commonly present in activated sludge (Shigematsu et al. 2003a; Sheludchenko et al. 2005). Nevertheless, it has been isolated from other ecological niches such as soil (Liang et al. 2005; Zheng et al. 2007) and plant rhizosphere (Han et al. 2005). Strain BM90 was isolated from seawater. It is not known yet if this organism could survive in the sea only temporary or if marine environment could be proposed as a new ecological habitat for this species. In this case, *D. tsuruhatensis* would be considered as an ubiquitous organism and this characteristic might be due to the great deal of its metabolic diversity.

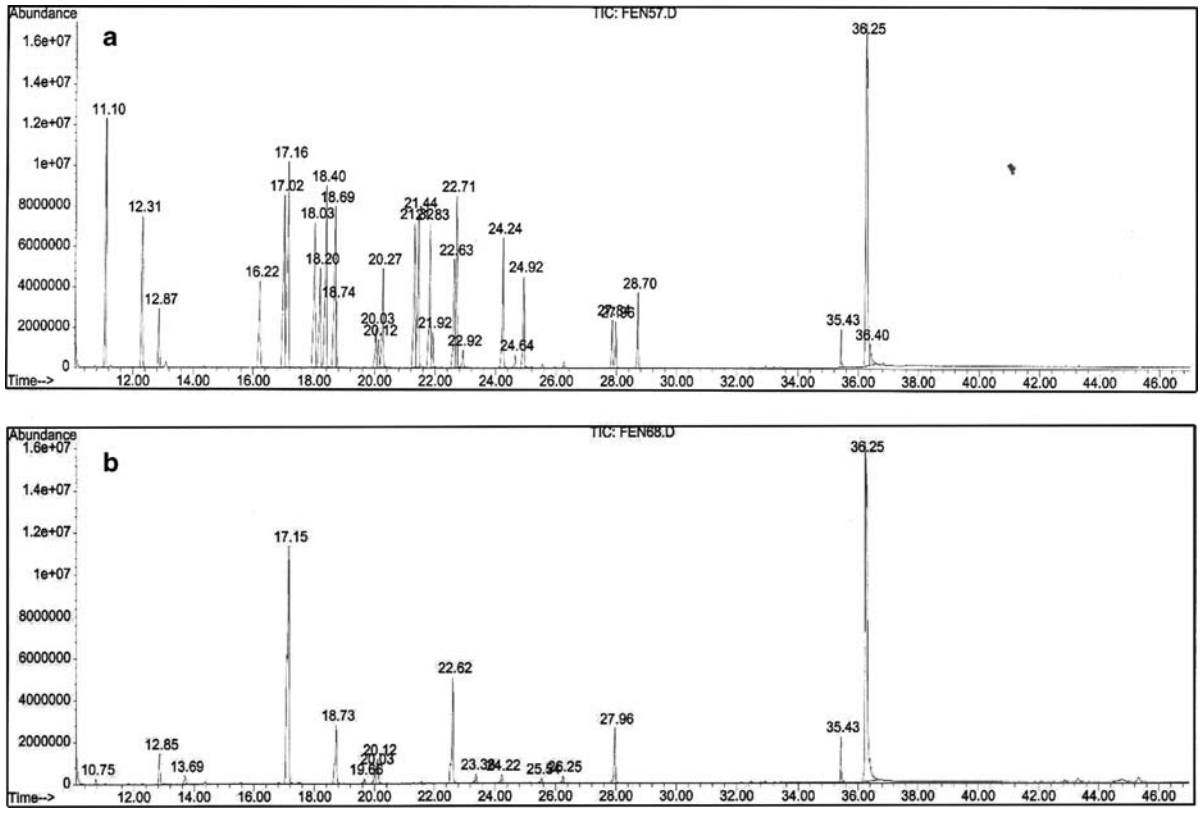

Fig. 2 GC/MS chromatogram of phenol removal by *Delftia tsuruhatensis* BM90 in MF. **a** Time 0 h; **b** Time 72 (h). Retention times (min ± 0.01): catechol, 11.10; 4-hydroxybenzaldehyde, 12.31; 4-methylcatechol, 12.87; vanillin, 16.22; 3-hydroxybenzoic acid, 17.02; tyrosol, 17.16; 3-hydroxyphenylacetic acid, 18.03; protocatechuic aldehyde, 18.20; *p*-hydroxybenzoic acid, 18.40; 4-hydroxyphenylacetic acid, 18.69; vanillol, 18.74; syringaldehyde, 20.03; veratric acid, 20.27; 3-*p*-hydroxyphenylpropionic acid, 21.32; vanillic acid, 21.44; gentisic acid, 21.83; syringic acid, 22.29; 3,4,5-trimethoxybenzoic acid, 22.63; protocatechuic acid, 22.71; 3,4-dihydroxyphenylacetic acid, 22.92; *p*-coumaric acid, 24.92; ferulic acid, 27.84; esculetin, 27.96; caffeic acid, 28.70

The rich metabolic diversity of this microorganism is validated in this work: strain BM90 showed the capacity to grow and metabolize a large number of carbon sources (43 out of 95 tested) even if compounds are only partially used. In addition, it was able to degrade a number of phenolic compounds (18 out of the 24 tested).

The metabolic competence of *Delftia* has never been investigated in details and the majority of the available information is related to *D. acidovorans*. For these reasons comparison of our strain capacities with other *Delftia* is difficult. However, our data can confirm some of those reported by other authors.

For example, BM 90 can use hydroxybutyric and propionic acid: this is somehow in accord with the work of Loo and Sudesh (2007) that demonstrated the use of these compounds by *D. acidovorans* for the production of polyhydroxybutyrate and polyhydroxyalcanoate granules.

Our strain can grow well on various aminoacids and the use of natural and unnatural aminoacids is reported also for *D. acidovorans* by Asano et al. (2001) and by Hongpattarkere et al. (2005).

In a previous work, we screened strain BM90 for its ability to release extracellular enzymes: the bacterium did not show any hydrolytic activity for various polysaccharides such as starch, chitin, cellulose and pectin (Fenice et al. 2007). In this work, the nutritional pattern obtained by the Biolog confirmed that carbohydrates are in general not used as carbon source by this strain of *D. tsuruhatensis*.

In addition, the good lipolytic activity of BM90 (Fenice et al. 2007) is confirmed here by its ability to grow on fatty acids. By contrast, BM90 did not

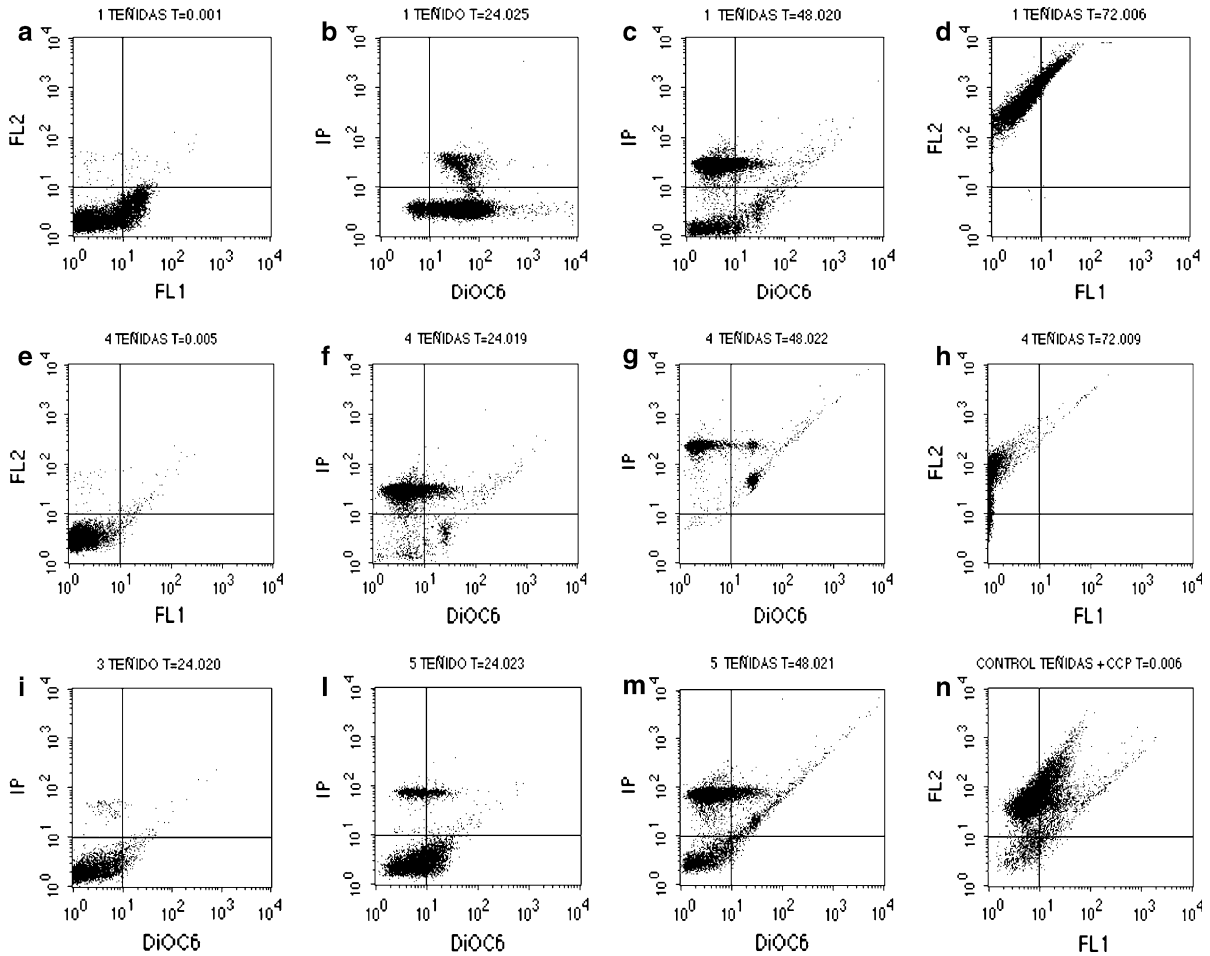

Fig. 3 Flow cytometry of *Delftia tsuruhatensis* strain BM90 stained with DiOC6 (FL1) and PI (FL2) in different media. **a**–**d** Growth in MG at 0, 24, 48 and 72 h, respectively; **e**–**h** growth in MH at 0, 24, 48, 72 h, respectively; **i** growth in MF at 24 h; **l**–**m** growth in C1 at 24 and 48 h, respectively; **n** growth in C2 at 0 h

produce extracellular proteases (Fenice et al. 2007) and this is strongly in contrast with its great preference for amino acids as carbon sources.

The capability of *Delftia* to degrade some phenolic compounds is known: for example growth of *D. tsuruhatensis* on catechol is reported by Sheludchenko et al. (2005). In addition, the bacterium could convert various aromatics (such as aniline, vanillin, ferulic acid, 2,4-dichlorophenoxyacetic acid and terephthalate) into catechol or protocatechuic acid before their ring cleavage that leads to subsequent complete degradation (mineralization) (Kahng et al. 2000; Plaggenborg et al. 2001; Shigematsu et al. 2003b; Hoffmann and Müller 2006). Typical example is the degradation of ferulic acid by *D. acidovorans*. It occurs through the conversion to vanillin, vanillic acid and protocatecuic acid before ring cleavage (Plaggenborg et al. 2001). Key enzymes of these reactions are generally the 1,2- and 2,3-dioxygenase cleaving the ring through the so-called "ortho" and "meta" pathways common in other proteobacteria (Schlafli et al. 1994). A *D. tsuruhatensis* terephthalate 1,2-dioxygenase has been purified and its gene cloned by Shigematsu et al. (2003b).

Thus, the various metabolic pathways leading to phenols degradation seem to exert a central role in the capability of *Delftia* to utilize a number of recalcitrant aromatic compounds. Nevertheless, *D. tsuruhatensis* has never been faced with such a wide array of phenols gathered in the same culture medium. Actually, the ability of BM90 to metabolize many different phenolic compounds is quite surprising. Very likely,

some of these compounds could be immediately submitted to ring cleavage by the dioxygenases system and, through the "lower pathway", transformed into pyruvate or other intermediates before possible mineralization. Others, not directly cleaved by the dioxygenases, could be transformed in different substances before ring cleavage and mineralization. However, BM90 seems able to use for its growth (carbon and energy sources) all the phenols that it removes from the cultural medium; this is somehow confirmed by flow cytometry also.

The BM90 biochemical diversity, leading to its ability to use such a variety of phenols, should be deeply investigated. However, the various metabolic pathways, involved in BM90 phenol metabolism, are probably highly interlinked and regulated by various mechanisms and/or factors. This would explain the different fate of the various phenolic compounds when they are differently mixed. In other words, BM90 behaves differently when some of the phenols are gathered with other similar derivatives (media MA–ME) or when they are all together in MF.

Even if analogous considerations could be made for other compounds, the most emblematic example concerns 4-methylcatechol. In ME, its concentration decreased after 48 h of incubation to increase again at the 96th hour. This could be due to the biotransformation of other phenols in 4-methylcatechol and to the lack of the appropriate induction of the specific degradation pathway. On the contrary, in MF, it was strongly depleted starting from the 48th hour. This could be due to the existence of a pathway, able to degrade 4-methylcatechol, but controlled (induced) by a regulatory protein activated by other phenols. 4-methylcatechol concentration in the controls (non inoculated media) slightly decreased (ME control, 96th hour) or increased (MF control).

BM90 inability to remove vanillol, tyrosol, esculetin or 3,4,5-trimethoxybenzoic acid could be a consequence of the lack of appropriate enzymatic machinery and/or regulatory proteins. Anyway, flow cytometry showed that these compound exert a certain, dose dependent, toxicity also. Almost normal growth is revealed when they are mixed with other phenols (MF) and are present in low concentration (20.83 mg/l). Strong cell inhibition was recorded in MH: in this case, in fact, their concentration was ca 6 time higher (125 mg/l).

Thus, phenol toxicity was recorded only with 4 out of the 24 compounds tested. However, in presence of all the phenols, BM90 showed a slower growth rate. A certain amount of dead cells, recorded in various media after 72 h, was probably due to lack of nutrients. In fact, similar results were obtained with C1. In this control no phenol is added and there is no carbon source: the cells remained in latency till the 72 h before starting to die.

A lot of phenolic compounds are recalcitrant and could cause pollution. In soils and waters they are originated mainly from plant decomposition or by agrochemical and industrial activities. Because of their antimicrobial and phytotoxic effect (Borja et al. 1995; Quaratino et al. 2007; Juarez et al. 2008) the application of microbial technologies for bioremediation is very often limited. In fact, only a small number of bacteria and fungi are able to degrade and use phenols as carbon source.

Our results, showing that *D. tsuruhatensis* BM90 can use various phenols as growing substrates, suggest that this organism could be considered very promising in case of phenol pollution. Experiments in this direction are in course.

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