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ORIGINAL PAPER

Selection of a cold-adapted bacterium for bioremediation of wastewater at low temperatures

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Abstract Amongst more than 1000 isolates collected in various cold environments, the strain *Arthrobacter psychrolactophilus* Sp 31.3 has been selected for its ability to grow and to produce exoenzymes at low temperatures, its inability to grow at 37°C, its non-halophilic character and its growth versatility on various media. This non-pathogenic strain displays a strong resistance to desiccation and storage at room temperature and is suitable for the production of freeze-dried bacterial starters. When grown in a synthetic wastewater at 10°C, the strain induces a complete clarification of the turbid medium and efficiently hydrolyses proteins, starch and lipids in the broth. Furthermore, this strain has a remarkable capacity to improve the biodegradability of organic compounds in wastewater as indicated by a BOD_5/COD ratio of 0.7.

Keywords Psychrophiles · Bioremediation · Wastewater · *Arthrobacter* · Macromolecular degradation

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Introduction

Bioremediation of polluted sites is increasingly considered as a potent tool to clean and to detoxify soils and waters contaminated by unwanted residues mainly generated by human activities (Daims et al. 2006; Jorgensen 2007). This environmentally friendly process takes advantage of the remarkable capacity of microorganisms to degrade or to transform pollutants into secondary compounds that are compatible with the equilibrium of the local ecosystem. Two complementary approaches are frequently used: biostimulation consists in the supply of nutrients to the polluted environment to stimulate the metabolic activity of the indigenous microorganisms, whereas bioaugmentation is carried out by the addition of exogenous microorganisms specialized in a specific degradation capacity that is not supported by the indigenous microbial population (Vogel 1996; van Veen et al. 1997; El Fantroussi and Agathos 2005; Singer et al. 2005).

Despite of a well-demonstrated efficiency of these processes, seasonal temperature variations represent a major drawback in bioremediation (Aislabie et al. 2006). For instance, growth and metabolic activity of indigenous microorganisms are severely depressed by low temperatures during winter in temperate countries, leading to a poor biotransformation rate of pollutants and to their accumulation in case of continuous input, such as in the disposal of animal wastes in industrial farming. In this respect, bioaugmentation using psychrophilic microorganisms appears to be an appropriate alternative (Margesin and Schinner 1998) as psychrophiles are adapted to thrive efficiently at low and moderate temperatures (Margesin et al. 2002). In addition, these microorganisms synthesize cold-active enzymes possessing a much higher specific activity than that of their mesophilic homologues at

identical temperatures (Margesin and Schinner 1999, 2001; Feller and Gerday 2003; D'Amico et al. 2006). Accordingly, it is expected that psychrophilic strains can usefully complement, or possibly replace, currently used mesophilic microbes for bioremediation in the cold. We report here the selection of a cold-adapted bacterium for its biodegradative capacity in domestic wastewater, taking into account both technological and biosafety constraints for its use as a microbial starter.

Materials and methods

Microbial strains and media

All investigated bacterial isolates are maintained in the Psychrophilic Culture Collection of the Laboratory of Biochemistry (University of Liège, Belgium). The culture media (liquid or solid) differ from the original formulations by the lack of NaCl and were as follows: LB (10 g/l bactotryptone, 5 g/l yeast extract, pH 7.0), Soytone (3 g/l soytone, 0.5 g/l yeast extract, 1 g/l glucose, pH 7.0), and Schatz (1 g/l KH_2PO_4, 1 g/l NH_4NO_3, 0.2 g/l $MgSO_4{\cdot}7H_2O$, 10 mg/l $FeSO_4{\cdot}7H_2O$, 10 mg/l $CaCl_2{\cdot}2H_2O$, 2 g/l glucose, pH 7.0, Schatz and Bovell 1952). The screening of enzymatic activities on LB or Soytone plates (at 4, 10 and 20°C) was performed by adding the following substrates before autoclaving: 1% soluble starch (amylases), 0.1% calcium-precipitated casein (proteases), 0.5% emulsified tributyrine (esterases, lipases), 0.5% Na-carboxymethylcellulose, 0.01% Trypan Blue (cellulases) or 0.5% oat spelt xylan (xylanases). The synthetic wastewater Ms2a* contained 0.5 g/l $(NH_4)_2SO_4$, 1.6 g/l KH_2PO_4, 50 mg/l $MgSO_4{\cdot}7H_2O$, 50 mg/l $CaCl_2{\cdot}2H_2O$, 20 mg/l $FeSO_4{\cdot}7H_2O$, 100 mg/l Tween 80, 5 g/l starch, 5 g/l olive oil, 1 g/l casein, and 0.5 g/l bovine serum albumin, pH 7.2. The broth was homogenized with an Ultra Turrax before autoclaving. Cultures were performed at 10°C with 200 rpm orbital agitation.

Analytical methods

Gram reaction of the bacterial isolates was performed by suspending bacterial pellets in 5 mM L-alanine-4-nitroanilide, 50 mM Tris–maleate, pH 7.6 (Carlone et al. 1982). Strain freeze-drying was carried out after 7 days of culture in Soytone at 10°C. The cell pellets were washed with 1% bactopeptone, centrifuged and further suspended in 5% Neosorb 70/70 (Roquette, France) before lyophilization at −70°C during 24 h. Strain survival was monitored by plating serial dilutions of the powder in 0.9% NaCl on Soytone-agar at 10°C.

For strain identification, genomic DNA was purified using the Wizard Genomic DNA Purification kit (Promega). Amplification of the 16S rRNA gene was performed by PCR using Vent DNA polymerase and the primers 27F (AGA GTTTGATCCTGGCTCAG) and 1522R (AAGGAGGTG ATCCARCCGCA). The product was purified after agarose gel electrophoresis using the NucleoSpin Extract kit (Macherey Nagel) and sequenced on an Applied Biosystems 377 DNA Sequencer. These sequences were analysed by the program BLASTN with databases maintained at the NCBI (http://www.ncbi.nlm.nih.gov/). The biological risk category of the identified strains was assigned according to the Belgian Biosafety Server databases (http://www.biosecurite.be/) and the ATCC classification.

The synthetic wastewater Ms2a* inoculated with *A. psychrolactophilus* Sp 31.3 was assayed, after centrifugation, for proteins by the bicinchoninic acid method (Pierce), for amino acids by the ninhydrin method (Spies 1957) and for saccharide reducing ends by the dinitrosalicylic acid method (Bernfeld 1955). Lipids were extracted from 500 ml samples by dichloromethane at 37°C, the organic phase was evaporated on a Rotovap under vacuum and the residue was recovered with ethanol/ether. Total lipids were weighed after evaporation whereas free fatty acids were determined by titration with 0.01 N KOH. Chemical oxygen demand (COD) and biological oxygen demand (BOD_5) values were determined using the Nanocolor COD 160 and Nanocolor BOD_5-TT kits (Macherey-Nagel) and the Nanocolor 500D colorimeter according to the instructions of the supplier.

Results

Selection strategy

More than 1000 isolates, collected from various cold environments (Antarctica, Kerguelen Island, Spitzberg, Siberia, Canada, Laponia and deep sea samples) have been screened for their ability to produce exoenzymes involved in the biochemical degradation of major macromolecules (proteases, lipases, amylases, cellulases and xylanases) on LB plates at 4°C. Amongst this collection, about 100 isolates were selected for further screening according to the following criteria:

1. Versatile growth capacity: ability to grow on a minimal medium (Shatz medium supplemented with glucose), on Soytone (soybean hydrolysate as main nitrogen source) and on LB plates (casein digest as main nitrogen source).
2. Non-halophilic character: no salts were added to the above-mentioned media; this condition was imposed

because the strains were expected to be used in low-salt polluted media such as wastewater and to avoid oxidation of stainless steel parts of fermentation units in large-scale production of the selected isolate.

3. Cold-adapted character: ability to grow efficiently at 4, 10, and 25°C (to select cold-adapted psychrophilic or psychrotrophic strains) but not at 37°C (to avoid potentially pathogenic strains).
4. Versatile exoenzyme production: ability to produce at least two of the above-mentioned exoenzyme activities.

As this selection provided only 3 Gram-positive bacteria (TAF 33b, TAF 53b, and Sp 31.3 in Table 1), all Gram-positive strains producing at least one enzyme activity and satisfying the other conditions were retained, because this group of bacteria frequently displays a better resistance to desiccation (see below) and contains few pathogenic species. Table 1 displays the general properties of the strains retained after this selection step. It should be noted that neither cellulolytic nor xylanolytic strains satisfied the imposed criteria. The selected strains have been further identified by 16S rRNA gene sequencing to assign a biosafety level to each of them. As shown in Table 1, most bacteria belong to risk category 1, ensuring that they are non-pathogenic for humans, animals, and plants. Strains belonging to higher risk category were removed from the selection as well as those having closely related pathogenic relatives. This is an obvious biosafety requirement if the strains are to be used under non-confined conditions.

The last and most stringent selection step was the determination of the strain ability to withstand the technological conditions imposed by the preparation of a stable freeze-dried bacterial starter. Three criteria have been considered: (a) the maximal cell density obtained in liquid culture, (b) the survival after freeze-drying in the presence of a cryoprotectant, and (c) the survival in the dry powder after 8 months of storage at room temperature with a threshold value of about 5×10^9 cfu/ml. As shown in Table 1, about half of the strains produced sufficient biomass in liquid culture. By contrast, very few resisted the freeze-drying process and survived satisfactorily in the dry powder. However, the strain *Arthrobacter psychrolactophilus* Sp 31.3 displayed all the required properties and was retained for further characterization. This strain has been isolated from the sand of a freshwater pond in the vicinity of the Ny-Alesund Arctic station in Spitzberg.

Bioremediation potential of *Arthrobacter psychrolactophilus* Sp 31.3

In order to determine the efficiency of the selected strain in the degradation of the major macromolecular pollutants, *Arthrobacter psychrolactophilus* Sp 31.3 was cultivated at 10°C in a reconstituted wastewater. This medium reproduces the conditions of salinity, pH, and detergent concentration of a domestic effluent and a mixture of proteins, olive oil, and starch was also added (Ms2a* broth).

As shown in Fig. 1a, the strain has a remarkable ability to reduce the turbidity of the cell-free synthetic wastewater, indicating a strong biological activity towards the complex organic aggregates present in the medium. This clarification effect occurred during the exponential growth of the bacterium (data not shown). The strain was also able to reduce the protein concentration of the wastewater to about 50% within a few days at 10°C (Fig. 1b). This was accompanied by a strong decrease of the free amino acid concentration (Fig. 1c) that are presumably metabolized by the microorganism. The degradation of starch was monitored by the appearance of reducing groups that arise from hydrolysis of the polysaccharide. Figure 1d shows that the strain was responsible for a fast degradation of the polysaccharide within 3 days. The subsequent decay in reducing end concentration suggests that maltooligosaccharides and possibly glucose generated by starch hydrolysis were metabolized by the strain. Finally, the strain Sp 31.3 displayed a noticeable capacity to hydrolyze olive oil with 22% degradation after 11 days and 45% degradation after 22 days of culture (Fig. 2). The moderate increase of free fatty acid concentration in the cell-free medium (Fig. 2) also indicated bacterial assimilation.

The degradation efficiencies of the strains *Psychrobacter glacincola* TAF 30a, *Arthrobacter flavus* CMS 19y, and *Arthrobacter roseus* CMS 90or were investigated similarly as they also display freeze-drying tolerance. However, none of these strains outperformed the clarification and degradation capacities of *Arthrobacter psychrolactophilus* Sp 31.3, with the noticeable exception of strain TAF 30a that degraded up to 70% of olive oil in the culture medium after 22 days (Fig. 2). Co-cultures of the four strains in the synthetic wastewater were also unable to improve the performances of *A. psychrolactophilus* Sp 31.3 in pure culture.

Improvement of wastewater biodegradability by *A. psychrolactophilus* Sp 31.3

The COD (chemical oxygen demand) measures the amount of oxygen required to chemically oxidize organic compounds in water and is used as an index of water quality. The value of about 150 mg O_2/l (Table 2) recorded for the reconstituted wastewater Ms2a* (sterile or inoculated) is in the range for domestic effluents. On the other hand, the BOD_5 (biological oxygen demand after 5 days of culture at 10°C) estimates the amount of organic matter that can be biologically oxidized. As shown in Table 2 for the sterile wastewater, this index is lower than the COD value as

Table 1 General properties of some selected cold-adapted bacterial strains

Isolate	16S rDNA identification	Gram reaction	Hydrolytic activities			Risk category	Culture cell density (cfu/ml)	Survival after freeze-drying (cfu/ml)	Survival after 8 months of storage (cfu/ml)
			Protease	Lipase	α-Amylase				
TAD 20b	*Pseudomonas syringae* (*P. fragi*)	−	+	+	−	2	6×10^{10}	3×10^{10}	1×10^{5}
TAF 30a	*Psychrobacter glacincola*	−	+	+	−	1	6×10^{9}	6×10^{9}	4×10^{9}
TAF 33a	*Flavobacterium fryxellicola* (*F. frigidarium*)	−	+	+	+	1	4×10^{9}	1×10^{9}	2×10^{7}
TAF 33b	*Rhodococcus* sp. (*R. fascians*)	+	−	+	+	1 (2)	4×10^{9}	1×10^{9}	2×10^{7}
TAF 41b	*Pseudomonas* sp. (*P. marginalis*)	−	+	+	+	1 (2)	7×10^{9}	5×10^{8}	8×10^{7}
TAF 50a	*Psychrobacter* sp. (*Moraxella* sp.)	−	−	+	−	1 (2)	2×10^{9}	2×10^{8}	4×10^{5}
TAF 53b	*Arthrobacter* sp. (*A. psychrolactophilus*)	+	−	+	+	1	5×10^{9}	4×10^{9}	3×10^{5}
TAJ 14	*Pseudomonas* sp. *(P. grimontii*)	−	+	+	−	1	2×10^{9}	8×10^{8}	2×10^{5}
TAJ 39a	*Pseudomonas veronii*	−	−	+	−	1	2×10^{9}	1×10^{9}	1×10^{3}
TAJ 39b	*Pseudomonas veronii* (*P. mandelii*)	−	+	+	+	1	2×10^{9}	1×10^{9}	$<1 \times 10^{3}$
TAJ 40a	*Pseudomonas* sp. (*P. marginalis*)	−	+	+	−	1 (2)	3×10^{9}	4×10^{8}	2×10^{3}
Sp 31.3	***Arthrobacter psychrolactophilus***	**+**	**+**	**+**	**+**	**1**	$\mathbf{1 \times 10^{12}}$	$\mathbf{1 \times 10^{12}}$	$\mathbf{2 \times 10^{11}}$
CMS 19y	*Arthrobacter flavus*	+	+	−	−	1	6×10^{11}	1×10^{11}	$<1 \times 10^{3}$
CMS 21w	*Sporosarcina macmurdoensis*	+	+	−	+	1	8×10^{9}	1×10^{5}	$<1 \times 10^{3}$
CMS 26or	*Planococcus antarcticus*	+	+	−	−	1	6×10^{9}	1×10^{4}	$<1 \times 10^{3}$
CMS 53or	*Planococcus psychrophilus*	+	+	−	−	1	2×10^{10}	3×10^{6}	1×10^{3}
CMS 76or	*Kocuria polaris*	+	−	+	+	1	1×10^{12}	1×10^{11}	$<1 \times 10^{3}$
CMS 90or	*Arthrobacter roseus*	+	+	−	−	1	4×10^{11}	1×10^{11}	4×10^{10}

Bold type required to highlight properties of the selected strain

Sampling sites: *TA* Terre Adelie, Antarctica; *Sp* Ny-Alesund, Spitzberg; *CMS* Antarctic deep sea samples

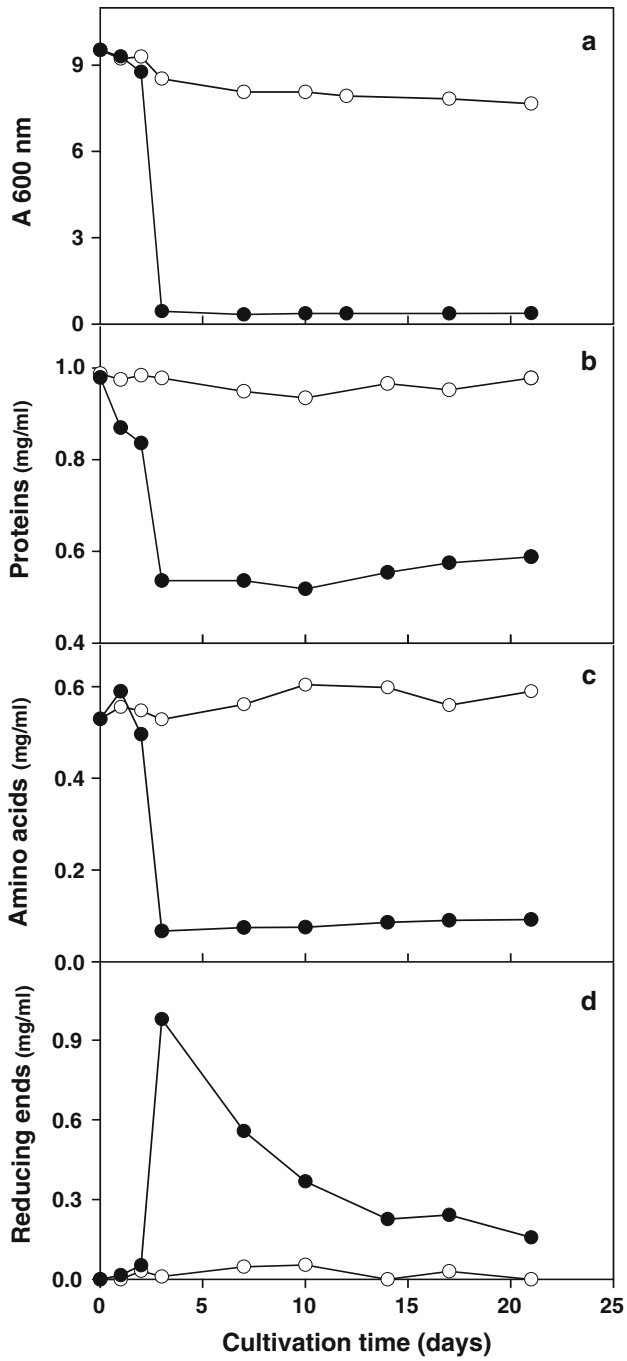

Fig. 1 Clarification, protein and starch degradation in the synthetic wastewater at 10°C. Data for the medium inoculated with *A. psychrolactophilus* (*closed symbols*) and for the sterile medium (*open symbols*). **a** Turbidity of the supernatant recorded at 600 nm. **b** Protein concentration in the supernatant determined by the bicinchoninic acid method. **c** Amino acid concentration determined by the ninhydrin method. **d** Reducing ends expressed as maltose produced, determined by the dinitrosalicylic acid method

several organic compounds are recalcitrant to biological oxidation. Interestingly, the strain *A. psychrolactophilus* Sp 31.3 significantly increased the BOD_5 value of the cell-free wastewater, leading to a BOD_5/COD ration higher than 0.7.

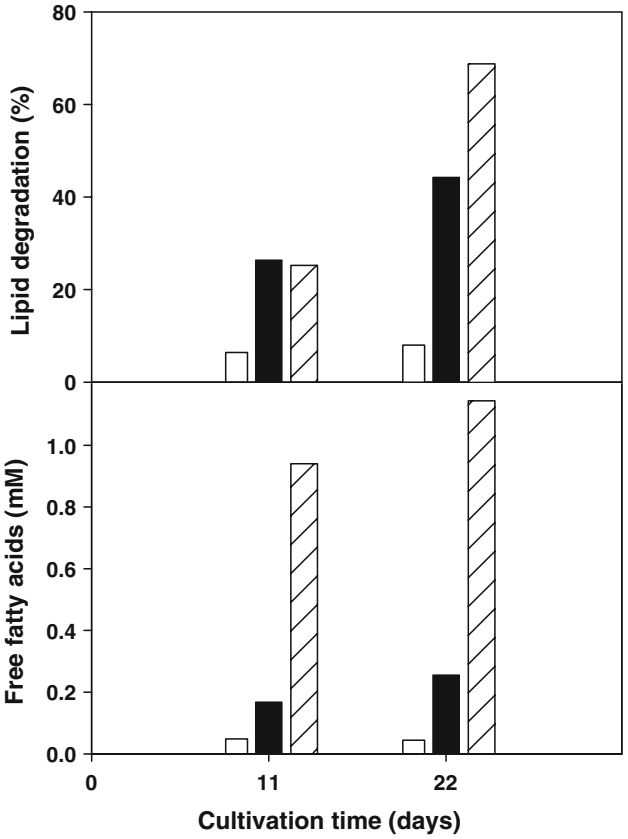

Fig. 2 Lipid degradation in the synthetic wastewater at 10°C. Data for the sterile medium (*white bars*) and for the medium inoculated with *A. psychrolactophilus* (*black bars*) or with *Psychrobacter glacincola* TAF 30a (*dashed bars*). Lipid degradation in the supernatant in percent of the initial amount (*upper panel*). Free fatty acid concentration in the supernatant (*lower panel*)

Table 2 Oxidizable organic charge (COD) and oxidative biodegradability (BOD_5) of the synthetic wastewater Ms2a* sterile or inoculated with *A. psychrolactophilus* Sp31.3

Medium	COD (mg O_2/l)	BOD_5 (mg O_2/l)	BOD_5/COD
Ms2a*	142 ± 6	43 ± 6	0.30
Ms2a* + Sp31.3	153 ± 5	112 ± 6	0.73

Considering that a ratio of 1 would correspond to a complete biodegradability, it can be concluded that the strain has a remarkable capacity to improve the biodegradability of organic compounds in the synthetic wastewater.

Discussion

Although microbial communities actively participate to the cycling of organic matter in pristine and polluted environments, the biotechnological use of these capacities in man-driven operations is generally impaired by microbial unculturability and by the complex interactions established

within these communities. Nevertheless, the selection strategy reported here allowed us to isolate a single strain displaying several properties of biotechnological significance. *Arthrobacter psychrolactophilus* Sp 31.3 is indeed a non-pathogenic Gram-positive bacterium, possessing a good resistance to lyophilization and to storage at room temperature as a dry powder. Furthermore, this strain is responsible for significant clarification, macromolecular degradation and biodegradability improvement of a synthetic wastewater at 10°C. The bacterium is therefore suitable for the formulation of a freeze-dried bacterial starter designed to improve bioremediation (via bioaugmentation) of wastewater in cold environments or during winter in temperate regions.

It should be noted that members of the *Arthrobacter* genus are known to be resistant to harsh conditions such as in polluted soils and to actively degrade recalcitrant organic compounds (Bazot et al. 2007; Sharma et al. 2007; Wang et al. 2007). The recent sequence of the genome of *Arthrobacter aurescens* has revealed several insights into the molecular origin of these useful biotechnological capacities (Mongodin et al. 2006).

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