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A novel mcl-PHA produced on coprah oil by *Pseudomonas guezennei* biovar. *tikehau*, isolated from a "kopara" mat of French Polynesia

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Abstract:

Pseudomonas guezennei biovar. *tikehau* was isolated from a microbial mat on the atoll of Tikehau in French Polynesia, and is able to synthesize medium chain length poly-β-hydroxyalkanaote copolymers when grown on coprah oil. A two-step cultivation process was used and the biosynthesis of PHAs was followed along 52 h by regular culture sampling. The polyester was purified from freeze-dried cells and analysed by nuclear magnetic resonance (NMR), Fourier transform infra red (FTIR), and gas chromatography mass spectrometries. The copolyester produced by *P. guezennei* biovar. *tikehau* from coprah oil mainly consisted of saturated monomers, *i.e.* 3-hydroxyoctanoate (3HO) and 3-hydroxydecanoate (3HD), and the monomeric composition of the polyester did not change during the fermentation process. However, yield of PHAs production varied from 4% of the cellular dry weight (CDW) to 63% obtained after 36 h. Scan electron microscopy was used to study the morphology and organization of PHAs granules within the cells and revealed the presence of several granules occupying almost the entire cell volume.

Keywords: Microbial mat; "Kopara"; *Pseudomonas*; PHAs; Polyhydroxyalkanaote

1. Introduction

Marine microbial mats are laminated communities usually composed of phototrophic and chemotrophic prokaryotes. The vertical stratification of the community is representative of the response of organisms to gradients of light, oxygen, sulphide and pH in relation with their physiological requirements. Nitrogen fixation is essential for the developpement of many microbial mats; therefore, nitrogen-fixing cyanobacteria play a key-role in these ecosystems [1]. Such microbial mats are found in lagoons as in Baja California [2], in lakes as in Sinai, Egypt [3] or in Spain [4]. Benthic microbial communities are present in most shallow lakes on the rims of some atolls of French Polynesia, where they are called “kopara” by the inhabitants [5, 6] and grow as gelatinous deposits of several tens of centimetres in thickness.

These environments are known to produce a large number of viscous laminated layers in which the presence of exopolysaccharides and poly-β-hydroxyalkanoates secreted by the microbial communities has been demonstrated [7-10].

Because of their thermoplastic properties and biodegradability, bacterial poly-β-hydroxyalkanoates (PHAs) have attracted industrial interest, and have been extensively studied in the last two decades [11-14]. PHAs comprise a large class of polyesters that are accumulated in a wide variety of bacteria as carbon and energy storage material. The polymers are deposited intracellularly in the form of inclusion bodies (“granules”) and might amount up to 90% of the cellular dry weight [15-16] if the bacteria are cultivated in the presence of excess carbon and if one nutrient limits growth. The monomeric composition of PHAs depends on the bacterial strain and on the carbon source supplied. PHAs can be divided into three groups depending on the number of carbon atoms in the monomeric units: short-chain-length PHA (3-5 carbon atoms, PHA $_{SCL}$), medium-chain-length (6-15 carbon atoms, PHA $_{MCL}$) and long-chain-length (more than 15 carbon atoms, PHA $_{LCL}$). Bacterial PHAs are probably the largest group of thermoplastics known and their properties are similar to those of

petrochemicals plastics, including materials that resemble polypropylene and others that are elastomeric. Typically, PHAs with short-chain-length such as poly-3-hydroxybutyrate (PHB) are highly crystalline and stiff material, are brittle with poor elastic properties. These properties limit their range of applications, whereas longer chain are more ductile and easier to mold.

In addition to being tremendous potential substitutes for petrochemicals plastics, PHAs are produced naturally from renewable carbon sources and represent a new way of utilizing waste from low cost carbon stocks [17-21]. However, the most attractive feature of PHAs is their biodegradability to CO_2 and H_2O, resulting from the pure chirally R-configuration that create environmentally friendly plastics.

In this study we report the biosynthesis of a medium chain length PHAs with elastomeric properties by *Pseudomonas guezennei* biovar. *tikehau*, a novel bacterium that was isolated from a microbial mat on the atoll of Tikehau in French Polynesia. Under stress conditions and in the presence of coprah oil in excess, this bacterium produced a biopolymer of biotechnological interest.

2. Materials and methods

2.1 Bacterial strain

In 2006, samples of microbial mats, named “kopara”, were collected on the atoll of Tikehau (French Polynesia). These samples were inoculated in liquid medium at pH 7.6 and incubated at 25°C for 48 h. The successful cultures were subcultured on solid medium. This procedure was repeated several times until the presence of a pure monoculture was established by microscopic examination.

The isolate *Pseudomonas guezennei* biovar. *tikheau* strain TIK669 (EMBL AM922198, deposited as strain I-3766 in the Collection Nationale de Culture de Microorganismes (Institut

Pasteur, Paris, France) used in this study was isolated from a microbial mat located on the atoll of Tikehau (French Polynesia).

For phylogenetic characterization, the procedure was carried out as described elsewhere [22].

2.2. PHAs synthesis

Biosynthesis of PHAs was carried out in a two-step batch cultivation process. In the first step, the cells were inoculated at 10% (v/v) with a suspension of cells in exponential phase and grown in 5 liters fermenter (Infors, Massy, France) containing 3 L of rich marine broth medium (10g peptone, 5g yeast, 15g Sea Salts per liter distilled water). The temperature was maintained at 35°C and the pH was adjusted at 7.0 by automatic addition of 2 mol l^{-1} NaOH. The air flow was fixed at 30 L h^{-1} and the agitation rate from 200 to 800 rpm to maintain the level of dissolved O_2 at its maximum. After the cultivation for 8 h, cells were harvested by centrifugation (5000*x*g, 40 min), and transferred into 5 liters fermenter containing 3 L nitrogen-free medium (15g L^{-1}Sea salts) enriched with 20g L^{-1} of coprah oil (Pacific Way, Papeete, Tahiti), and incubated at 35°C, 200-400 rpm, and dissolved O2 maintained around 25%. The composition of coprah oil was 8% octanoic acid (C8:0), 8% decanoic acid (C10:0), 43% dodecanoic acid (C12:0), 18% tetradecanoic acid (C14:0), 11% hexadecanoic acid (C16:0), 4% octadecanoic acid (C18:0), and 8% octadecenoic acid (C18:1ω9).

During this second step, 200 mL culture fractions were collected in triplicate at after 6, 10, 14, 18, 28, 36, 42 and 52 h to follow the biosynthesis of PHAs. Cells were harvested by centrifugation (10 000*x* g, 15 min), washed-up three times with diluted sea-water, and the resulting pellets were lyophilised prior to further analysis. Cellular dry weight (CDW) was determined by weighing lyophilized sample.

2.3. Microscopic observations

Cells were observed under light microscope (model BX51, Olympus) equipped with phase-contrast oil immersion objective (X100 magnification). Actually, phase contrast microscopy is one of the easiest method to observe routinely the presence of PHAs granules in bacteria.

For scanning electron microscopy (SEM) cells were rinsed from their culture medium and a drop of the sample was laid down on a polycarbonate filter (0.2 µm, Nucleopore) immediately placed in a 13mm diameter coverglass containing 10% formaldehyde (v/v) and cells were let fixed overnight. Dried filter was mounted on scanning electron microscopy sample stub and coated in a vacuum with two layers of gold (SCD040, Balzers), each of a thickness of 100 Å. Samples were observed using a FEI ESEM Quanta 200 scanning electron microscope.

2.3. Extraction and analysis of PHAs

Freeze-dried cells recovered from the pellets were ground in a mortar and the resulting powder was extracted with chloroform for 4 h at 50°C. The PHAs-containing chloroform phase was concentrated and extracted once with water to remove residual solid particles. The organic phase was evaporated to dryness and the resulting crude extract preserved for further analyses. Purified PHAs were obtained by repeated precipitations in 10 volumes of cold methanol. To follow the yield of PHAs production regarding to the cellular dry weight, 100 mg lyophilised cells were extracted according to the above procedure, and the purified PHAs was weighed.

For the determination of PHAs monomeric composition, 7 mg polymer were subjected to methanolysis using methanol:hydrochloric acid (17:2, v/v) at 100°C for 4 h. After phase separation and successive washes with water, the organic phase was dried under nitrogen. TMSi derivation of 3-hydroxyalkanoates methyl esters was accomplished by adding 100 µL pyridine and 100 µL BSTFA to 1 mL methanolized sample. The reaction mixture was heated

at 70°C for 45 min. The TMSi derivatives of methyl esters of monomers constituents were analysed by GCMS using an Agilent 6890N chromatograph coupled to a quadrupole Agilent 5975 inert XL mass selective spectrometer, equipped with a HP-5-MS fused silica capillary column (30 m x 0.25 mm, 25 µm film thickness). A 1µL sample was injected (spit ratio 100:1) with helium as carrier gas and the temperature was programmed for the separation of peaks (60°C for one min, ramp of 4°C min^{-1} to 140°C, 15°C min^{-1} to 280°C and 5 min at 280°C). The ionising energy for MS operation was 70 eV.

^{1}H and ^{13}C NMR measurements were performed at 25°C on a BRUKER 400 DRX spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. Samples of PHAs were dissolved in deuterated chloroform. Chemical shifts are given in parts per million relative to the signal of 2,2,3,3-tetradeutero-3-(trimethylsilyl)-propanoic acid sodium.

FTIR ATR spectra were recorded on a Nicolet AVATAR 370 DTGS FTIR spectrometer. Samples of polymer were dissolved in chloroform and deposited as film on Germanium disks. Scans were performed in the range of 600 and 4000 wave number (cm^{-1}).

Differential scanning calorimetry was performed with a TA Instrument DSC. Sample of about 10 mg was encapsulated in aluminium pans and heated from –60°C to 180°C (first scan) at a heating rate of 10°C min^{-1} under 50 mL min^{-1} nitrogen purge. To determine the glass-transition temperature (Tg), the sample was maintained at 180°C for 1 min and then rapidly quenched at –60°C. Sample was then reheated from –60°C to 180°C at a rate of 10°C min^{-1} (second scan). The melting temperature (Tm) was taken at the peak of the melting endotherm and glass transition temperature (Tg) was taken as the midpoint of the heat capacity change.

3. Results and discussion

3.1. Strain identification

The sequence of the 16S rRNA encoding gene of strain TIK669 was determined (1368 bp) and deposited in the EMBL sequence database under accession number AM922198. On the basis of its phylogenetic analyses, strain TIK669 is clearly a *Pseudomonas guezennei* type strain. The only difference was the origin of the bacterium, isolated from a closed atoll of Rangiroa named Tikehau. Consequently we opted for the name *P. guezennei* biovar. *tikehau.* It has been deposited in the Collection Nationale de Culture de Microorganismes (Institut Pasteur, Paris, France) as strain I-3766.

3.2. Production of PHAs

In this study, a two-steps cultivation was applied, in which cell biomass obtained in nitrogen enriched medium was transferred to a second-step in which the medium was depleted with nitrogen and enriched with coprah oil to promote the biosynthesis of PHAs. Table 1 shows the evolution of PHAs content related to the cellular dry weight and PHAs monomeric composition during the second step of cultivation of *P. guezennei* biovar *tikehau* on coprah oil. The production of PHAs began after 6 h in coprah oil enriched medium and increased up to 63% of the CDW after 36 h when it is maximal. Intracellular structures were visible in contrast phase microscopy (magnification X100) after few hours cultivation in the second medium.

The accumulation of PHAs granules was also shown by scan electron microscopy (Fig. 1). During cultivation in nitrogen enriched medium, cells were in multiplicating phase as shown by division by constriction. Once transferred in nitrogen-free medium supplemented by coprah oil, few cell divisions were observed and intracellular inclusions granules, up to 12 per cell, appeared and occupied a very significant portion of the cell volume.

3.3. Characterization of PHAs

FTIR spectrum of PHAs produced by *P. guezennei* biovar. *tikehau* grown on coprah oil showed the typical structure of a polyester with an intense absorption peak corresponding to the stretching band of the ester carbonyl group (C=O) at 1723 cm^{-1}, and three intense bands between 2951 and 2961 cm^{-1} assigned to anti-symmetric and symmetric CH_3 and CH_2 stretching modes (Fig. 2). The C=O stretching band in the 1740-1720 cm^{-1} region could be assigned to the vibrations of the amorphous and crystalline carbonyl groups respectively [23-25]. It can be concluded from the location of the C=O ester band at 1723 cm^{-1} a crystalline polymer produced by *P.guezennei* biovar *tikehau* fron coprah oil. These data raise interesting questions with the presence of high content in hydroxyoctanoate (HO) expected to significantly lower the degree of cristallinity of this new biopolymer. Additional experiments such as X-ray diffraction are then required, taking into account the rate of cristallinity as referred in previous works [26, 27].

The ^1H NMR spectrum of PHAs $_{MCL}$ obtained from *P.guezennei* biovar. *tikehau* on coprah oil showed the signals of the methine protons (CH) at 5.2 ppm (Fig. 3). The triplet resonance at 0.89 ppm was assigned to the terminal methyl group (CH_3), and the multiplet resonance at 2.47-2.6 ppm to the methylene protons (CH_2) of C-2 carbon atom. The methylene protons of the C4 carbon atom yielded a signal at 1.58 ppm, while all other methylene hydrogens of the saturated side chains produced a signal at 1.26 ppm.

GC-MS analyses of TMSi methyl esters derivatives of PHAs allowed us to determine the monomeric composition of the PHAs obtained from *P. guezennei* biovar. *tikehau* cultivated on coprah oil. The mass spectra of TMSi derivatives of 3-hydroxyalkanoic acids exhibited characteristic fragments [$(CH_3)_3SiO^+=CHCH_2CO_2CH_3$] at *m/z* 175 and [$RCH=O^+Si(CH_3)_3$] at *m/z* [M-73] resulting from α-cleavage of the derivatized hydroxyl group (Fig. 4). The TMSi ethers do not usually show a parent molecular ion but the molecular ion related fragment at *m/z* [M-15] is quite prominent and can be used to determine the chain length of monomers.

Others fragments of lower mass were also common for the 3-hydroxyl functional group and readily assignable : *m/z* 73 [$(CH_3)_3Si^+$], *m/z* 89 [$(CH_3)_3SiO^+$], *m/z* 131 $(C_5H_{11}SiO_2)^+$, *m/z* 159 $(C_6H_{11}SiO_3)^+$ and *m/z* 133 $(C_5H_{13}SiO_2)^+$.

The composition of PHAs synthesized by *P. guezennei* biovar. *tikehau* from coprah oil was found to consist mainly of 3-hydroxyoctanoate (3HO) and 3-hydroxydecanoate (3HD), less significant amounts of 3-hydroxydodecanoate (3HDD) and 3-hydroxyhexanoate (3HHx), and low fractions of 3-hydroxybutyrate (3HB), 3-hydroxytetradecenoate (3HTDe) and 3-hydroxytetradecanoate (Table 1). The existence of a double-bond can be deduced from the molecular weight of the fragment at *m/z* [M-15] which is two amu less than that of the corresponding saturated monomer, and from the weak intensity ratio of the peak at *m/z* [M-15] to the base peak. The presence of double bonds, characterized by additional signals around 5.3, 2 and 2.3 ppm in the ^{1}H NMR spectrum and signals between 120 ppm and 135 ppm in the ^{13}C NMR spectrum (data not shown), was not detected by NMR because of the very low amount (less than 1%) of the unsaturated monomers.

From the results of DSC analysis, Tg of PHAs produced by *P.guezennei* biovar. *tikehau* grown on coprah oil was –46°C and an obvious peak of Tm appeared at 45°C.

4. DISCUSSION

In this study, a bacterium, *P. guezennei* biovar. *tikehau*, recently isolated from a microbial mat on the atoll of Tikehau in French Polynesia was shown to be capable of accumulating high yield of PHA_{MCL} using coprah oil as the sole carbon source. This bacterium produces an elastomeric PHAs copolymer with promising biotechnological applications. Moreover, the bacterium has proved its capability to use efficiently coprah oil and could be a good candidate

for production of PHAs from palm or soybean oils that are renewable, abundant worldwide and low cost carbon sources.

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Figure legends

Fig. 1. Scan electron micrograph of *P. guezennei* biovar. *tikehau*. (A) SEM image of cells in phase division when grown in nitrogen enriched medium. (B) SEM image of cells after 52 hours in free-nitrogen medium enriched with coprah oil, showing numerous PHAs granules in each cell. Bar represents 1 µm.

Fig. 2. Fourier-transform infrared spectra of purified PHAs $_{MCL}$ extracted from *P. guezennei* biovar. *tikehau* grown on coprah oil.

Fig. 3. ^{1}H NMR spectra of purified PHAs $_{MCL}$ obtained from *P. guezennei* biovar. *tikehau* during cultivation on coprah oil.

Fig. 4. Mass spectra of the TMSi derivatives of 3-hydroxydecanoic acid (3HTD) (A) and 3-hydroxydodecenoic acid (3HTDe) (B) methyl esters. The characteristic peaks and molecular ion-related fragments were assigned as described in the text.

Table footnotes

Table 1

Evolution of PHAs $_{MCL}$ monomeric composition and PHAs content of *P. guezennei* biovar. *tikehau* during the step of PHAs accumulation in nitrogen free medium and with the presence of an excess of coprah oil.

Table 1

	PHAs Monomeric composition mol%							%PHA/CDW
Time	3HB	3HHx	3HO	3HD	3HDD	3HTDe	3HTD	
6h	0.26	2.7	44.9	32	18.3	0.4	1.6	4
10h	0.14	2.9	44.9	32.2	18	0.4	1.46	35
14h		2.5	45.7	32.5	17.5	0.44	1.2	45
18h		2.5	46.6	32	17	0.6	1.2	52
28h		2.9	48	32	15.5	0.64	0.91	57
36h		2.5	49.6	32	14.7	0.5	0.8	63
42h	0.25	3	50	31.5	14.2	0.12	0.73	63
52h	0.12	3.2	51.6	30.8	13.5	0.31	0.76	62

Figure(s)

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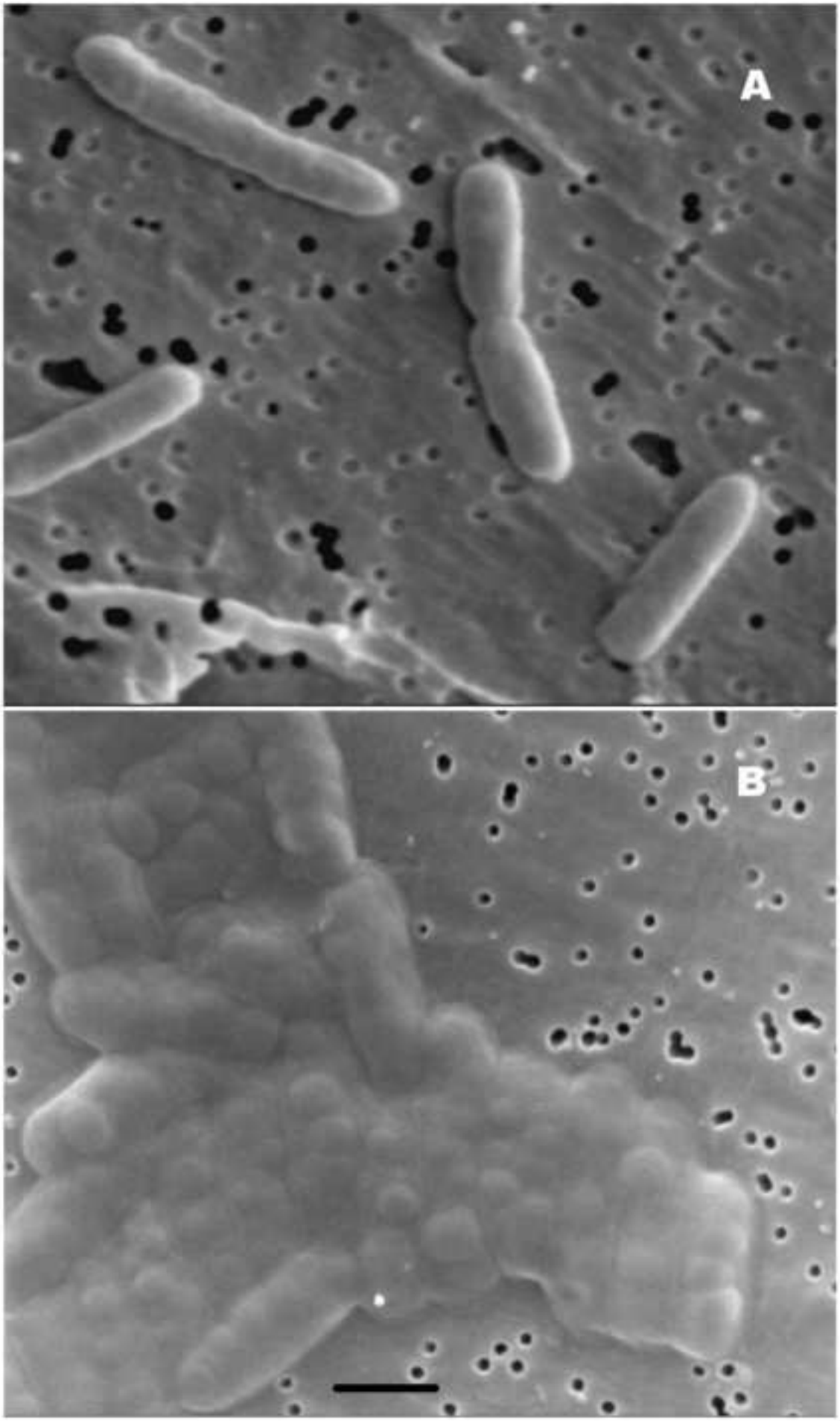

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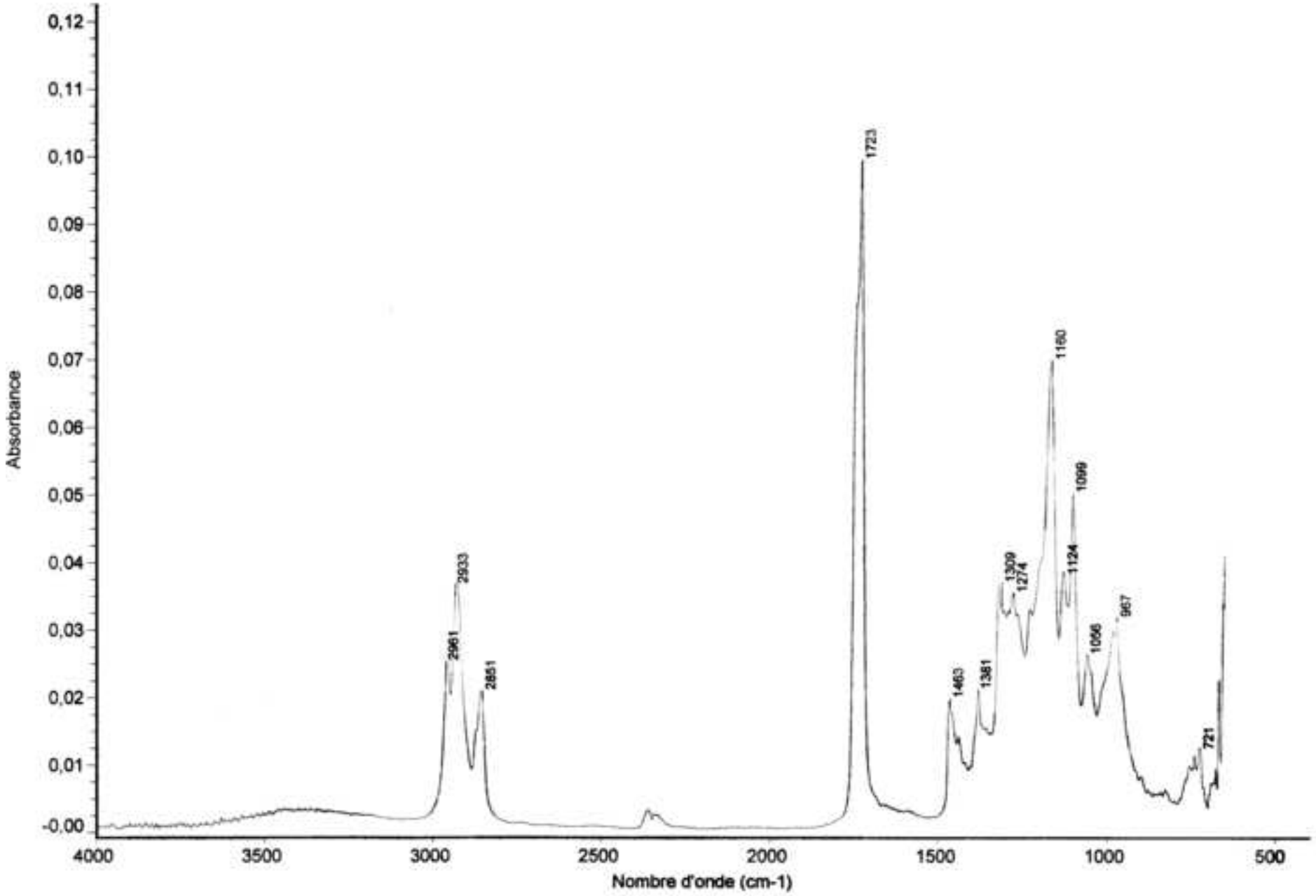

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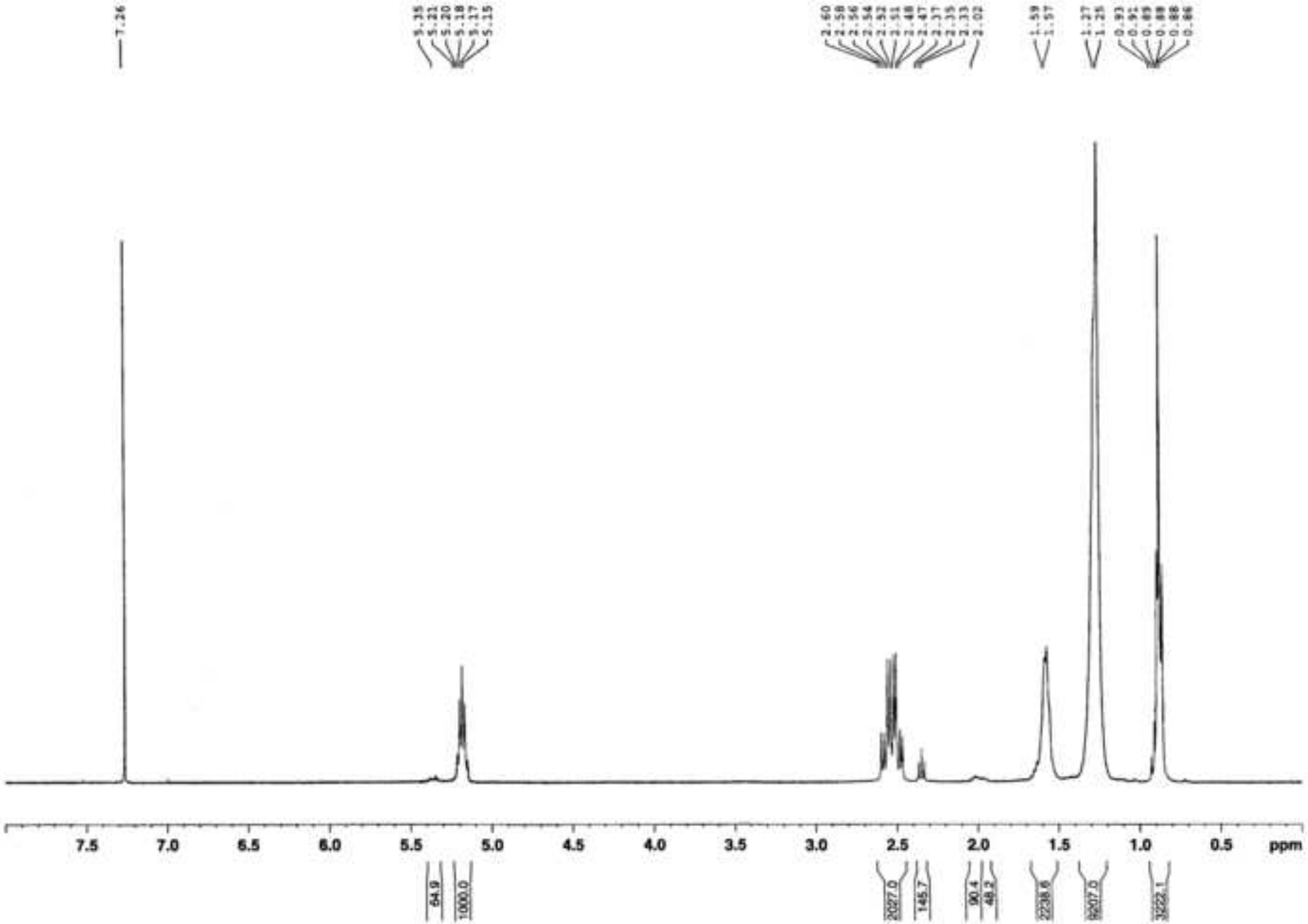

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