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Slavomira Husarova

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Slovak Academy of Sciences

Slovak University of Technology

Université Blaise Pascal

NMR STUDY OF C1 COMPOUND AND SACCHARIDE
METABOLISM OF *BACILLUS* SP. 3B6, A BACTERIAL
STRAIN ISOLATED FROM CLOUD WATER.

POTENTIAL IMPLICATION FOR ATMOSPHERIC
PROCESSES.

Slavomíra Husárová

Thèse soutenue le 04-11-2011

under supervision of

Mária Matulová

Anne-Marie Delort

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SUMMARY

The discovery of the presence of living and active microorganisms in clouds is rather recent. Their survival ability under such hostile environment and their potential role in atmospheric processes are open questions in the scientific community. The objective of this thesis was to bring some answers to this recent topic. The main goal of PhD thesis was to study the metabolism of *Bacillus* sp. 3B6, isolated from cloud water, by *in situ* NMR on different substrates. Substrates represented two classes: C1 compounds (formaldehyde and methanol) and carbohydrates. Formaldehyde and methanol have been chosen since they represent major atmospheric volatile organic carbons (VOCs) and carbohydrates serve as a source of carbon for bacteria. Many of them are present in the atmosphere. Metabolism of chosen C1 compounds by *Bacillus* sp. 3B6 was compared with other three bacterial strains isolated from cloud water: *Frigoribacterium* sp., *Pseudomonas graminis*, and *P. syringae*. The metabolic routes were similar to photodegradation pathways and in a particular case C3 compounds were formed from a C1 compound. In addition, a comparison of obtained biodegradation rates with rates related to the reactivity of organic species with free radicals $\cdot\text{OH}$ (day-time chemistry) and $\text{NO}_3\cdot$ (night-time chemistry) showed that the microbiological oxidation of organic compounds has to be considered as an alternative route to radical chemistry in cloud water especially at night. Carbohydrate metabolism study of *Bacillus* sp. 3B6 followed two axes: basic metabolism of chosen sugars as carbon sources and exopolymeric substances (EPSs) production. Two substrate (glucose and

sucrose) afforded different types of EPSs. On sucrose the production of levan, fructooligosaccharides of levan and inulin type, and dianhydride of fructose DFA IV were identified in the incubation medium (IM). IM on glucose afforded a glycoprotein precipitate, 1,6-linked α -galactan, and some polyhydroxyalkanoate (PHAs) structure of which could not be identified from *in situ* NMR spectra. In fractions afforded by gradual precipitation from IM 3-hydroxybutyrate, butyric acid, glycerol, and ester of glycerols were also identified. Besides biotechnological application, identified EPSs, could have also an impact on the atmospheric environment. EPSs, which are highly functionalised molecules could influence the cloud condensation nuclei (CCN) formation by interacting with water, or due to their ability to change the “wettability” of the cell surface. In this latter case they will act by changing the CCN properties of bio-aerosols.

ABSTRAKT

Prítomnosť živých a aktívnych mikroorganizmov v oblakoch bola objavená len pomerne nedávno. Ich schopnosť prežívať v tomto nehostinnom prostredí, ako aj ich potenciálna úloha v atmosférických procesoch nie sú doteraz uspokojivo vysvetlené. Cieľom prezentovanej dizertačnej práce bolo prispieť k riešeniu tejto témy prostredníctvom štúdia metabolizmu baktérie *Bacillus* sp. 3B6 izolovanej z oblačnej vody. Jej metabolizmus bol študovaný vo väčšine prípadov priamou analýzou inkubačných zmesí získaných na rôznych substrátoch prostredníctvom *in situ* NMR spektroskopie. Použité substráty možno rozdeliť do dvoch tried: *i*) C1 zlúčeniny (metanol, formaldehyd) ako hlavní znečisťovatelia ovzdušia, *ii*) rôzne sacharidy, ktoré slúžia ako zdroj uhlíka pre baktérie a ktorých prítomnosť v atmosfére bola dokázaná. Metabolizmus metanolu a formaldehydu u *Bacillus* sp. 3B6 bol porovnaný s ďalšími tromi bakteriálnymi kmeňmi izolovanými z oblačnej vody: *Frigoribacterium* sp., *Pseudomonas graminis* a *P. syringae*. Metabolické dráhy boli podobné fotodegradácii a to hlavne v prípade tvorby C3 zlúčenín z C1 substrátu. Porovnanie takto získaných rýchlostí biodegradácií s rýchlosťami reaktivít voľných radikálov $\cdot\text{OH}$ (denné chemické procesy) a $\text{NO}_3\cdot$ (nočné chemické procesy) ukázalo, že mikrobiologické oxidácie organických látok, musia byť považované za alternatívny proces k radikálovej chémii prebiehajúcej v oblačnej vode prevažne v noci. Štúdium metabolizmu sacharidov u baktérie *Bacillus* sp. 3B6 sledovalo dve samostatné experimentálne línie: *i*) základný metabolizmus cukrov zvolených ako zdroj uhlíka, *ii*) produkcia exopolymérnych látok (EPSs). Rast baktérie na dvoch rôznych

sacharidových substrátoch (glukóza a sacharóza) viedol k produkcii rôznych typov EPSs. Analýzou inkubačného média (IM) na sacharóze boli identifikované: polysacharid levan, fruktooligosaccharidy levanového a inulínového typu a dianhydrid fruktózy DFA IV. V IM na glukóze boli identifikované: glykoproteín, 1,6- α -galaktán a polyhydroxyalkanoáty (PHA), ktorých štruktúru nebolo možné z *in situ* NMR spektier bližšie identifikovať. Vo frakciách, ktoré vznikli postupným vyzrážaním nerozpustných zložiek zmesi z IM, bol identifikovaný 3-hydroxybutyrát, kyselina maslová, glycerol a estery glycerolu. Okrem biotechnologických aplikácií, identifikované EPSs môžu zohrávať úlohu v atmosférických procesoch. EPSs, ako vysoko funkcionalizované molekuly, by mohli mať vplyv na tvorbu oblačných kondenzačných jadier (CCN) prostredníctvom ich interakcií s vodou, alebo v dôsledku ich schopnosti meniť zmáčavosť povrchu buniek. V druhom prípade sa tento efekt deje zmenou CCN vlastností bioaerosólov.

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ABBREVIATIONS

A-ATOFMS	Aircraft Aerosol Time of Flight Mass Spectrometry
ADP	Adenosine DiPhosphate
AIDA	Aerosol Interactions and Dynamics in the Atmosphere
Ara	Arabinose
ATP	Adenosine TriPhosphate
CCN	Cloud Condensation Nucleus
COSY	COrrrelation SpectroscopY
CVI	Counterflow Virtual Impactor
DNA	DeoxyriboNucleic Acid
DGGE	Denaturing Gradient Gel Electrophoresis
EPS	ExoPolymeric Substances
FADH	FormAldehyde DeHydrogenase
FDH	Formate DeHydrogenase
FOSs	FructoOligoSaccharides
Fru	Fructose
FT-IR	Fourier Transform InfraRed spectroscopy
Fuc	Fucose
Gal	Galactose
GalA	Galacturonic Acid
GalA(gly)	D-Galacturonic acid with carboxyl group amide-linked to glycine
GC	Gas Chromatography
Glc	Glucose

GlcA	Glucuronic Acid
Gln	Glutamine
GPC	Gel Permeation Chromatography
GSH	Glutathione
HPAEC-PAD	High-Performance Anion-Exchange Chromatography coupled with Amperometric Detection
HPLC	High-Performance Liquid Chromatography
HPR	HydroxyPyruvate Reductase
H ₄ MPT	Dephosphorylated tetrahydromethanopterin
HMBC	Heteronuclear Multiple-Bond Correlation spectroscopy
HSQC	Heteronuclear Single Quantum Coherence
IN	Ice Nucleus
INA bacteria	Ice Nucleation Active bacteria
InaZ	Ice nucleation activating membrane protein
Man	Mannose
ManpNAcA	2-Acetamido-2-deoxy D-mannopyranuronic acid
MCL	Malyl Coenzyme A Lyase
MDH	Methanol DeHydrogenase
MS	Mass Spectroscopy
MTK	Maleate ThioKinase
NAD ⁺	Nicotinamide Adenine Dinucleotide
NAD(P) ⁺	Nicotinamide Adenine Dinucleotide Phosphate
NMR	Nuclear Magnetic Resonance
NMVOC	Non-Methane Volatile Organic Compound
NOE	Nuclear Overhauser Effect
NOESY	Nuclear Overhauser Effect Spectroscopy
NTP	Nucleotide TriPhosphate
PBAs	Primary Biological Aerosols
PBPAs	Primary Biological Particles in Aerosols
PCR	Polymerase Chain Reaction
PDE	PhosphoDiEster

PEP	PhosphoEnolPyruvate
PFG	Pulsed Field Gradients
PGSE	Pulsed-Gradient Spin-Echo
PGSTE	Pulsed field Gradient STimulated-Echo
pMMO	Particulate Methane MonoOxygenase
PS	PolySaccharide
PURGE	Presaturation Utilizing Relaxation Gradients and Echoes
Py	Pyruvic acid acetal
QuipNAc4NAc	2,4-Diacetamido-2,4,6-trideoxy-D-glucopyranose
rDNA	Ribosomal DNA
Rha	Rhamnose
Rib	Ribose
RuMP	Ribulose MonoPhosphate pathway
sMMO	Soluble Methane MonoOxygenase
RF	Radio Frequency
ROESY	Rotating frame Overhauser Effect Spectroscopy
SOAs	Secondary Organic Aerosols
Suc	Sucrose
TCA	TriCarboxylic Acid
TLC	Thin Layer Chromatography
TMS	TriMethylSilane
TOCSY	TOTal Correlation Spectroscopy
TSPd ₄	Tetra-deuterated trimetylsilyl propionate
UDPG	Uridine DiPhosphoGlucose
UV	Ultra-Violet
VOCs	Volatile Organic Carbons
WATERGATE	WATER Suppression by GrAdient-Tailored Excitation
WET	Water suppression Enhanced Through T_1 effects
ZQF-TOCSY	Zero Quantum Filtered TOCSY

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1 INTRODUCTION

Clouds represent an extreme environment for living cells, characterised by low temperatures, acidic pH, UV radiation, and lack of nutrients. Recent studies showed that microbial communities are present and metabolically active there and provoked new investigations of their structure and functioning. This field of the research is quasi virgin and innovative. Clouds are a heterogeneous medium (solid, liquid, and gaseous phases) containing a very complex mixture of organic and inorganic compounds, and can be considered as a large reactor where many chemical reactions take place. Up to now only radical chemistry induced by solar light has been taken into account to describe atmospheric chemistry. However, microorganisms are potentially very active biocatalysts and the proposed project would like to find the answers to some questions concerning their potential role in atmospheric chemistry as an alternative to photochemical transformations, and more generally their implication in atmospheric processes.

The presented PhD work has been carried out in the frame work of a co-tutelle and of a collaborative work between a French team (SEESIB: Synthèse et Etude de Systèmes à Interêt Biologique in Clermont-Ferrand) and a Slovak team (Institute of Chemistry in Bratislava).

The studied bacteria were isolated from cloud water collected at the summit of the Puy de Dôme in free troposphere by the French team, a pioneer in this field. This site is a European place for cloud studies.

The main focus of this PhD work was to study the metabolism of *Bacillus* sp. 3B6 in detail, as it is present in many atmospheric samples. These metabolic studies have been performed using a main tool, Nuclear Magnetic Resonance (NMR, which is the domain of expertise of both French and Slovak teams). Two types of substrates have been studied: carbohydrates and C1 compounds, both of them are potentially present in cloud waters.

This study is quite interdisciplinary and requires the knowledge in various areas. This is why the bibliography presented here gives the state of the art of different fields involved in this work: clouds, microbial community in clouds, NMR in microbiology and metabolism studies; with the main focus on *Bacillus* species.

2

BIBLIOGRAPHIC STUDY

2.1 Microorganisms in Clouds: Their Role in Atmospheric Processes

2.1.1 Life Cycle of Microorganisms *via* the Atmosphere

Microorganisms are ubiquitous on the Earth including water, soil and vegetation, and they can experience a life cycle *via* the atmosphere as shown in Figure 1. The first step consists in the aerosolisation of microorganisms into the air. Detailed mechanisms involved in this process remain still unknown. However, some hypotheses have been highlighted: In the case of water, especially sea water, microorganisms are ejected by bubbling. Concerning soil, wind is obviously the major factor that brings microorganisms in the air. Bacteria can be present on soil particulates and blown up as dust. Spores of some fungi present in conidia can be easily aerosolised by wind depending on meteorological factors (Jones and Harrison, 2004). Finally, microorganisms highly present in the phyllosphere can be ejected in the air by the action of wind depending on

factors such as humidity and temperature, which allow plant leaves to dry and produce particles. Aerosols can also result from fragmentation of dried biofilms at the leaf surface where bacteria are embedded (Lindemann *et al.*, 1985; Hirano and Upper, 2000).

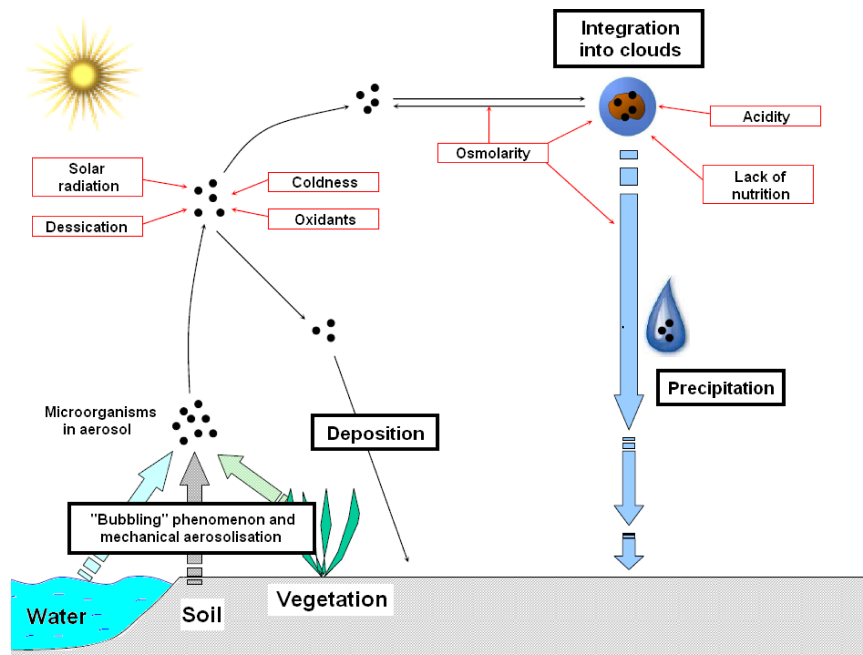


Figure 1. Life cycle of microorganisms *via* the atmosphere.

Once in the atmosphere, microorganisms are exposed to hostile conditions including solar radiation (especially UV), desiccation when they are in the air, cold, interaction with oxidants (H_2O_2 , iron, *etc...*), low quantity of nutrients, acidity, difference of osmolarity. Microorganism physiologies must be thus adapted to the life in this rather extreme environment. They can be incorporated into cloud droplets by nucleation scavenging as they have cloud condensation nuclei (CCN) or ice nuclei (IN) potential and they come back to the Earth as a part of raindrops and snowflakes by washout processes of the air or as a possible result of ice nucleation processes (Möhler *et al.*, 2007; Morris *et al.*, 2004; Sun and Ariya, 2006).

The precise mechanisms involved in CCN and IN processes are still under controversy in the scientific community (Möhler *et al.*, 2007). The

transport of microorganisms in the atmosphere has already been simulated as well as the factors influencing their residence time and the CCN and IN activities (Burrows *et al.*, 2009).

The revelation of living microorganisms integrated in cloud water recently initiated a fundamental and new scientific question by Ariya's group (Ariya *et al.*, 2002, 2004; Côté *et al.*, 2008) and our group (Amato *et al.*, 2005, 2007a): Do microorganisms play a role in atmospheric processes?

2.1.2 Number, Type, Activity, and Specificities of Microorganisms in Clouds

Clouds can be defined as atmospheric air masses, in which water is condensed around particles in solid (ice crystals) or liquid form. They represent low-temperature "aquatic" environments involved in the large-scale transport of matter and energy, and so participate in the aerial connection between ecosystems. It is assumed that the liquid and super-cooled water, from which the free tropospheric clouds are composed, provides a better temporary habitat for living airborne cells than dry air, in which desiccation can be a limiting factor for growth.

There are numerous studies on the composition of organic part of primary biological particles in aerosols (PBPA). PBPA comprise pollens, bacteria, spores, fungi, algae, protozoa, fragments of vegetal debris to name few. It was shown that bacteria and fungal spores account at the same percentage level as plant debris in atmospheric aerosols (Bauer *et al.*, 2002). Studies on PBPA structure and origin indicate that cloud droplets may provide a medium, in which bacterial cells can divide (Fuzzi *et al.*, 1997; Dimmick *et al.*, 1979; Sattler *et al.*, 2001; Ahern *et al.*, 2007; Bowers *et al.*, 2009). Very recently, Womack *et al.* (2010) introduced the

concept of biogeography of the atmosphere, suggesting a specific ecosystem of this very specific environment, which is transitory and totally opened contrary to soils and waters. To date, very few data are available concerning the microbial population in the air and clouds. The reported total number of microorganisms in cloud water ranges from about 10^3 to 10^5 cells mL^{-1} . Table 1 reports more precisely these numbers depending on the sampling sites.

Table 1. Concentration of microorganisms in atmospheric water samples.

Localisation	Type of atmospheric water sample	Concentration of bacteria	Concentration of fungi and yeasts	References
		[mL^{-1}]		
Puy de Dôme mountain, France, 1464 m.a.s.l.	Cloud	1.7×10^4 – 2.4×10^5 ^E	8.9×10^2 – 2.5×10^4 ^E	Amato <i>et al.</i> , 2007b
Rax mountain, Austria, 1644 m.a.s.l.	Cloud	2×10^4 ^C	5.9×10^3 ^C	Bauer <i>et al.</i> , 2002; 2003
Sonnblick mountain, Austria, 3106 m.a.s.l.	Cloud	1.5×10^3 ^D		Sattler <i>et al.</i> , 2001
Kleiner Feldberg mountain, Germany, 857 m.a.s.l.	Cloud		1.05×10^6 ^A	Matthias-Maser <i>et al.</i> , 2000
	Rain		3.57×10^3 ^A	
Po valley, Italy	Fog	30–150 ^B	30–60 ^B	Fuzzi <i>et al.</i> , 1997
Shizuoka and Tsukuba, Japan	Rain	1.6×10^4 – 2.4×10^4 ^A		Casareto <i>et al.</i> , 1996
Charlottesville, Virginia, USA	Rain	2×10^5 – 6×10^5 ^A		Herlihy <i>et al.</i> , 1987

^A Total number of Primary Biological Aerosol particles (PBAs)

^B CFU (colony forming units), *i.e.* cultivable microorganisms

^C Average from 3 clouds events

^D Average from 12 clouds events

^E Average from 14 clouds events

Several research groups searched for cultivable cells in fog water at low altitude, and retrieved only three bacterial genera (*Pseudomonas*, *Bacillus*, and *Acinetobacter*), and several fungi and yeasts (Fuzzi *et al.*,

1997). In tropospheric clouds more diversified cultivable populations, consisting of many bacterial phyla, were observed, namely *Alpha-*, *Beta-* and *Gamma-Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Firmicutes* belonging mainly to the genera *Pseudomonas*, *Sphingomonas*, *Staphylococcus*, *Streptomyces*, *Bacillus*, *Rhodococcus*, and *Arthrobacter* (Amato *et al.*, 2005, 2007b). An important diversity was also found among fungal (*Cladosporium* and *Trametes*) and yeast (*Cryptococcus*) communities (Amato *et al.*, 2005). Only two papers reported the description of bacterial population present in clouds using molecular tools. First, Ahern *et al.* (2007a) used direct analysis of DNA by PCR (polymerase chain reaction) amplification and cloning the 16S rDNA genes to investigate the microbial population of clouds and rainwaters collected in Scotland. Many strains belonging to the *Pseudomonas* and *Acinetobacter* genera have been identified in this way. More recently, Kourtev *et al.*, (2011) used DGGE (Denaturing Gradient Gel Electrophoresis) and sequencing 16S rRNA gene amplicons to analyse cloud bacteria from cumulus clouds in USA. The main bacterial phyla belonged to *Cyanobacteria*, *Proteobacteria*, *Actinobacteria*, and *Firmicutes*. These phyla belonged to those described before, except *Cyanobacteria*, which are described for the first time.

Although less than 1% of the bacteria and less than 50% of the fungi and yeasts isolated at the Puy de Dôme station could be cultivated; the measuring of the adenosine triphosphate (ATP) concentration in cloud samples showed that a large majority of cells counted by microscopy are actually alive (Amato *et al.*, 2007b).

Cloud water presents some specific environment with acidic pH (generally from 3 to 7), high oxidative capacity, the presence of toxic compounds such as formaldehyde, intensive light (including UV) exposure, and relatively low temperatures (5 °C in average). As a consequence, cells show specific physiological properties, which enable them to remain alive in such environment. Many bacterial colonies isolated from cloud water at the Puy de Dôme were pigmented or spore-

forming, psychrophiles and some were closely related to strains isolated from cold environments. These red, orange, and yellow pigments are actually carotenoids, which protect cells against UV damage. That decreases the fluidity of their membrane to protect them against cold conditions (Amato *et al.*, 2007b).

2.1.3 The Role of Microorganisms in Cloud Microphysics

It is well known that atmospheric aerosols influence a cloud formation and precipitation development, and thus they have important impact on the global climate, water cycling and atmospheric chemical reactions. Less is known about the contribution of Primary Biological Aerosols (PBAs) to these phenomena. PBAs comprise either living and dead cells (microorganisms, pollens, plant fragments, fungal spores, ...) or any kind of organic substances deriving from biomolecules and contributing to aerosol masses. PBAs similarly to particles of other origin, influence cloud formation and precipitation processes by participation in the following pathway: *i*) phase change from vapour to liquid, *ii*) acceleration of coalescence by large particles; and *iii*) phase change from vapour or liquid to ice. Details of these processes are described in several textbooks and articles (Pruppacher and Klett, 1997; Young, 1993; Santachiara *et al.*, 2010) and their description is beyond the scope of the study.

PBAs are a part of the organic carbon fraction of aerosols formed by mixture of thousands of organic compounds. These aerosols exhibit a wide range of molecular structures and could have a major influence on the physico-chemical, biological, climate, and health related behaviours of atmospheric aerosols (Jacobson *et al.*, 2000; Turpin *et al.*, 2000; Seinfeld and Pankow, 2003; Fuzzi *et al.*, 2006). Although they are capable to form nucleating cloud droplets and ice particles *via* physical processes (Möhler

et al., 2007), the quantification of their role in the real atmosphere, compared to that of other aerosols is not known and is under investigation by many international groups.

Clouds are formed by condensation of water vapour on the aerosol particles surface when the relative humidity (in %) exceeds the saturation with respect to the pertinent phase (water or ice). This is a kinetic process that is controlled by the size, chemical composition, and surface properties of particles. Particles that serve as nuclei for liquid cloud formation at a specified relative humidity are referred as CCN (Cloud Condensation Nuclei), while those that serve as nuclei for ice formation are called IN (Ice Nuclei). Concerning the role of microorganisms in nucleation processes, the story started a long time ago (Vali, 1974), however, experimental evidence is still missing to give final conclusions (Möhler *et al.*, 2007; Sun and Ariya, 2006; Georgakopoulos *et al.*, 2009). First, microorganisms can be considered as CCN, as a particular case of aerosol particles presenting some specific physico-chemical properties due to their biological nature. Second, IN properties of microorganisms were studied focusing on highly specialised bacteria, namely INA (Ice Nucleation Active) bacteria belonging mainly to *Pseudomonas* genus.

Insoluble biological particles, capable to retain water on their surface behave as CCN due to the presence of lipopolysaccharides or exopolymeric substances composed of polysaccharides (PS) and proteic structures, which can enhance the hydrophilic behaviour of the cell surface and thus “wettability” and hygroscopy. Data obtained by comparison of different “wetable” bacteria have shown that bacteria could participate to cloud droplet activation at similar critical supersaturation (relative humidity – 100%) conditions (Möhler *et al.*, 2007; Morris *et al.*, 2008; Petters and Kreidenweis, 2007). On the contrary, the production of surfactants, especially if they remain at the cell surface (typically for *Rhodococcus* strains), the presence of carotenoids or unsaturated fatty acids in the bacterial membrane can increase the hydrophobic nature of the bacterial surface and thus

decrease its hygroscopic property. Biosurfactants lead to a significant decrease of critical supersaturation when present in sufficient quantities (Prisle *et al.*, 2009; Ekström *et al.*, 2010). The term supersaturation refers to a solution containing higher quantity of the dissolved material than the solution formed under normal circumstances. It can also refer to a vapour of a compound that has a higher partial pressure than the vapour pressure of this compound.

Only a few studies exist, which are dealing with an activation of cloud condensation nuclei by primary biological aerosol particles and they concern bacteria. Levin *et al.* (1987) showed rapid growth of freeze-dried bacteria in saturated environment by absorption of water vapour. These data suggest that a substantial proportion of bacteria in the atmosphere act as wettable or hygroscopic particles at their sizes and they are probably frequently scavenged into drops by nucleation during the cloud formation. Franc and DeMott (1998) found different fractions of various strains of plant pathogenic *Erwinia carotovora* bacteria to activate CCN over a wide range of supersaturations. Some bacterial strains, such as *Bacillus subtilis* (Gram-positive) or *Escherichia coli* (Gram-negative), showed relatively large CCN capacity. On the surface of these cells water vapour condensation starts from a relative humidity of 85% (Lee *et al.*, 2002; Bauer *et al.*, 2003) tested bacteria cultivated from aerosol and water samples for their ability to act as CCN. As their sizes were smaller than Kelvin diameters for the respective supersaturations, results of this study indicated that the CCN activity must have been enhanced by physico-chemical properties of bacterial outer cell walls.

Ice can be formed in the atmosphere either from the vapour or from the liquid phases of water – these are distinguished as “deposition” and “freezing” (Vali, 1985). In this case, the ice nucleation pathway is catalyzed by some particle – heterogeneous nucleation. Homogenous freezing nucleation is observed at temperatures approaching $-40\text{ }^{\circ}\text{C}$, when supercooled liquid will freeze spontaneously (Tabazadeh *et al.*, 2002).

Heterogeneous ice nucleation can take on further variations in clouds depending on the mode how a particle gets into the liquid. Three modes of freezing nucleation were identified experimentally: immersion-, condensation-, and contact-freezing. Nucleation activity can be further influenced by many other effects such as pre-activation or prior exposure to low temperatures and/or humidity conditions.

Both inorganic and organic particles have been identified as atmospherically relevant ice nuclei. Mineral dusts are a general category of inorganic particles that are reported as a strong source of ice nuclei in the atmosphere (Isono, 1959; DeMott *et al.*, 2003). Biological particles, including certain bacteria, pollen, and decayed organic material have also been identified as ice nuclei (Cantrell and Heymsfield, 2005; Morris *et al.*, 2004; Schnell and Vali, 1972). Studies of certain epiphytic bacteria of *Pseudomonas*, *Xanthomonas*, and *Erwinia* have shown that these microorganisms bear a membrane protein InaZ, which is encoded by a unique gene. INaZ is the lipoglycoprotein in the external membrane that mimics the structure of ice crystals and it starts a phenomenon of water crystallisation at the temperature dependent on the arrangement of the protein at the cell surface ($-2\text{ }^{\circ}\text{C}$ for some *Pseudomonas syringae* strains, for example, against $-40\text{ }^{\circ}\text{C}$ for pure water in homogenous nucleation) (Maki *et al.*, 1974; Vali *et al.*, 1976; Szyrmer and Zawadzki, 1997; Cochet and Widehem, 2000).

Only the structural integrity of the protein is responsible for the IN activity, a dead cell is as effective as a living cell. There are Ina⁺ bacteria in the genera *Erwinia* (species *anas* and *herbicola*), *Pantoea* (species *anas*), *Pseudomonas* (species *syringae* and *fluorescens*), and *Xanthomonas* (species *campestris*). The species *Pseudomonas syringae* concerns the majority of the most active Ina⁺ strains. This species is phytopathogenic as INaZ protein promotes the freezing of water on the plant surface, deteriorating walls and plant to facilitate bacterial attack. In fungi species, *Fusarium avenaceum* is almost as effective as Ina⁺ bacteria. This property gives them better protection against desiccation

and, in addition, the greater cellulolytic efficiency against their hosts.

The presence of microorganisms with so unusual physical properties in clouds can suggest their significant role in the droplets and crystals formation, particularly by initiation of these phenomena. However, clear evidence of the exact role of microorganisms in ice formation is still under controversy. Scientists have tested different strategies to answer this question. The first strategy is based in creating “artificial clouds” in cloud chambers by spraying bacterial isolates or commercial product Snowmax® (derived from *Pseudomonas syringae*, largely used in ski resorts to make artificial snow) and observing the formation of ice crystals (Möhler *et al.*, 2007). One of the cloud chambers named AIDA (Aerosol Interactions and Dynamics in the Atmosphere) is presented in Figure 2.

The second strategy is based on direct study of cloud and precipitation samples. For instance, very interesting results were obtained by Christner *et al.* (2008) who examined the concentration and nature of IN over a large number of rain and snow samples collected in various locations: Montana and Louisiana (USA), the Alps and Pyrenees (France), Ross Island (Antarctica), and Yukon (Canada). They found that half of the samples were ice nucleation active at temperatures ≥ 5 °C based on immersion freezing testing. Väitilingom (2011, PhD thesis) also isolated various INA strains from cloud water collected at the Puy de Dôme station, including a fungus *Fusarium avenaceum*, and bacteria *Pseudomonas syringae* sp. 38B10, sp. 32B72, sp. 13B2, *P. graminis* 38B9, *P. fluorescence* 36B3, and *Xanthomonas campestris* 32B33. Note that the strain *P. syringae* sp. 32B74 is extremely active (INA temperature -3 °C, 10^{-8} INA cell⁻¹).



Figure 2. AIDA chamber: volume of the aerosol vessel is 84 m³. Insulated box regulates the temperature in the range $-90\text{ }^{\circ}\text{C}$ to $+60\text{ }^{\circ}\text{C}$ (temperature control within $\pm 0.3\text{ }^{\circ}\text{C}$).

Finally, Pratt *et al.* (2009) detected *in situ* biological particles in ice-crystals in clouds sampled by aircraft at high altitude in Wyoming (7 to 8 km, temperature -31 to $-34\text{ }^{\circ}\text{C}$). The chemical composition of individual CVI (Counterflow Virtual Impactor) ice residual particles was measured by A-ATOFMS (Aircraft Aerosol Time of Flight Mass Spectrometry). This technique allowed showing that biological particles accounted for 33% of the residue and mineral dust particles for 55%. Their results are consistent with the presence of bacterial cells in cloud ice-crystals present at high altitude.

2.1.4 The Role of Bacteria in Cloud Chemistry

Airborne microorganisms may have a potential role in cloud chemistry as biocatalysts because they contain multitude of enzymes organised in different metabolic pathways. As it was shown, they can be activated into

cloud droplets (Möhler *et al.*, 2007) and once there, they could interact with nitrogen, sulphur, and organic compounds present in aqueous phase. Thus, they can modify multiphase cloud chemistry by entering into a competition with photochemistry and multiphase processes (dissolution, reactivity, scavenging, and deposition).

The aqueous phase of clouds is actually a very complex chemical reactor. Table 2 presents the average concentrations of main carboxylic acids and inorganic compounds present in cloud waters collected at the Puy de Dôme summit (Marinoni *et al.*, 2004).

Table 2. Content of most important dicarboxylic acids, cations and anions in cloud water measured by Ionic Chromatography (IC).

Compound	Cloud water sampled at the Puy de Dôme station	
	min	max
concentration (μM)		
Acetate	0.6	48.7
Formate	0.8	71.4
Succinate	0.1	4.1
Oxalate	0.1	17.4
Cl^-	0.5	1948.9
NO_3^-	3.4	766.8
SO_4^{2-}	1.9	369.5
Na^+	0.4	281
NH_4^+	6.3	1801.7
K^+	0.1	124.1
Mg^{2+}	0.2	45.6
Ca^{2+}	0.3	74.8
pH	3.9	7.6

Puy de Dôme mountain is a reference site to study clouds since the top of the mountain is often cloudy and it offers the possibility to sample air masses from various origins. Clouds are classified as a function of their origins (two main categories: continental and marine) and present significant differences in their chemical composition. For instance the pH is lower for higher NO_3^- , NH_4^+ , and SO_4^{2-} concentrations in continental

air masses compared to oceanic air ones. It can be noted that pH can be rather acidic for microbial life.

Organic and inorganic compounds present in cloud water result from various multiphase processes than are summarised in Figure 3.

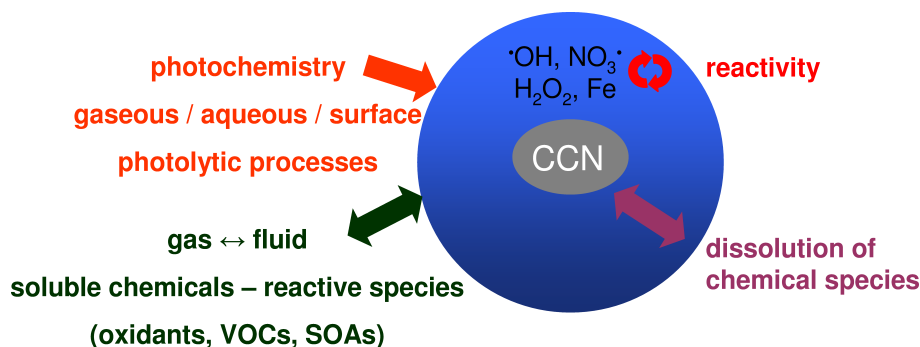


Figure 3. Simplified scheme of the multiphase cloud chemistry processes taking place in clouds. CCN – cloud condensation nucleus; VOCs – Volatile Organic Carbons; SOAs – Secondary Organic Aerosols.

Chemical compounds that are present in the atmospheric aqueous phase can result from various sources. First, chemicals present in the solid phase (aerosol particles that acted as cloud nuclei) can be dissolved in the aqueous phase of clouds. Second, soluble chemicals present in the gaseous phase can exchange with the liquid phase of clouds and thus be transferred to this phase. These gaseous chemicals can be primary emitted from the natural or anthropogenic sources and/or are produced through atmospheric gaseous reactivity. For example, VOCs (Volatile Organic Carbons) are soluble in the cloud droplet and photoreactivity in the gaseous atmospheric phase can lead to the production of a myriad of oxygenated compounds that can enter in the aqueous phases. These compounds in the gas phase can also lead to the formation of so-called “Secondary Organic Aerosols” (SOAs) (Hallquist *et al.*, 2009) that can potentially act as CCN and be transferred in the atmospheric aqueous phase. Finally, in the liquid phase, chemical reactions can also occur;

clouds are oxidative media and the reactivity is driven by two main radicals ($\cdot\text{OH}$, $\text{NO}_3\cdot$) produced by the presence of strong oxidants such as iron and H_2O_2 and by the mass transfer from the gas and liquid phases (Deguillaume *et al.*, 2005; Tilgner *et al.*, 2005, 2010). These radicals result from photochemical reactions or from the Fenton-type reactions.

In this context, the organic matter represents a significant fraction of the soluble matter in cloud droplets (Hadi *et al.*, 1995; Saxena and Hildemann, 1996; Fuzzi *et al.*, 2002). Among them, carboxylic acids represent 9% of the dissolved organic carbon in the cloud droplets (Marinoni *et al.*, 2004). The carboxylic acids can be produced in the gaseous phase and dissolved in the aqueous phase (main source of acetic and formic acid); they can also result from the dissolution of soluble particles (main source of acids oxalic, succinic, malonic, and maleic acids, ...); or produced *via* the aqueous phase reactions (Chebbi and Carlier, 1996; Goldstein and Galbally, 2007; Sellegri *et al.*, 2003; Ervens *et al.*, 2004). Due to the presence of free radicals such as $\cdot\text{OH}$ in the aqueous phase, the oxidation of organic matter can be considered as an important source of carboxylic acids; they also represent one of their main sinks (Ervens *et al.*, 2003; Herrmann, 2003, 2010; Karpel Vel Leitner and Doré, 1997; Warneck, 2003).

Among these VOCs, methanol is the second most abundant organic molecule in the atmosphere after methane and it is the predominant oxygenated compound in the mid to the upper troposphere (Singh *et al.*, 2000, 2001). 11 to 20% of the methanol in the atmosphere is of anthropogenic origin and from oxidation taking place in the atmosphere, while the major part (80 to 90%) is of biogenic origin, mainly issued from plant metabolism (Tie *et al.*, 2003; Brunner *et al.*, 2007). Formaldehyde is also a key atmospheric VOC since it is an important indicator of so-called Non-Methane Volatile Organic Compound (NMVOC) emissions and photochemical activity. Formaldehyde is a primary emission product from biomass burning and fossil fuel combustion, but the photochemical oxidation of methane and non-methane hydrocarbons is its main source in

the atmosphere.

Methanol and formaldehyde are soluble in water and thus they are efficiently transferred into atmospheric waters (Henry's law constants equal to $2.2 \times 10^2 \text{ M atm}^{-1}$ and 10^3 M atm^{-1} respectively) (Sander, 1999). Very few papers reported methanol concentrations in atmospheric waters. It was found at $0.7 \text{ }\mu\text{M}$ concentrations in rain (Snider and Dawson, 1985). Formaldehyde concentration varies in a very wide range of values: from $0.001 \text{ }\mu\text{M}$ in rainwater in rural areas (Kieber *et al.*, 1999) to $710 \text{ }\mu\text{M}$ in highly polluted fog (Jacob *et al.*, 1984). The average concentration measured in cloud water at the Puy de Dôme station is $2.9 \text{ }\mu\text{M}$ (Parazols, 2007) and depends on the air mass origins. Pashynska *et al.* (2002) have shown that compounds belonging to various classes of saccharides are important constituents of ambient atmospheric aerosols. Although the concentration of these saccharides has not been measured in cloud waters, their occurrence is highly likely due to their of low polarity and high solubility in water. The origin of saccharides present in atmospheric aerosols can be different. Monosaccharides as glucose, fructose, xylose and disaccharides sucrose and trehalose can be released into the atmosphere by microorganisms, plants, and animals (Pashynska *et al.*, 2002). Fungi, lichens, and bacteria were found to produce sugar alcohols, such as arabitol, mannitol, and glucitol (Simoneit *et al.*, 2004; Medeiros *et al.*, 2006). Levoglucosan derived from cellulose, galactans and mannans, all are released by burning of biomass (Medeiros, 2006; Simoneit *et al.*, 1999). Type and quantities of sugars present in the aerosol were found changing depending on the year period (Medeiros, 2006). Moreover, biogenic organic carbon in aerosols can be produced also by photochemical reactions with volatile hydrocarbons and thus it can form a secondary organic aerosol. In recent years, an increased interest in characterisation of polar organic constituents in atmospheric particulate samples – including acids, alkanols, aldehydes, and sugar derivatives, has been observed. Short chain dicarboxylic acids, carbohydrates and their alcohols were two largest identified groups of compounds. The presence of carbohydrates in PBPA have been shown

by Wang *et al.* (2006), Duarte *et al.* (2008), Jia *et al.* (2010a, 2010b). Composition of PBPA fixed attention of scientists and thus numerous studies appeared dealing with analytical methods enabling detection and quantification of water soluble organics in atmospheric aerosols. For example sugar compounds have been detected in atmospheric aerosols by LC-MS liquid chromatography combined with positive electrospray ionisation mass spectrometry (Wan and Yu, 2006), High-performance anion-exchange chromatography coupled with amperometric detection (HPAEC-PAD) (Caseiro *et al.*, 2007) and GC-MS (Pietrogrande and Bacco, 2011). A critical review of advanced analytical techniques for water-soluble organic matter from atmospheric aerosols, which includes also FT-IR and NMR spectroscopy, was published by Duarte *et al.* (2011). Table 3 summarises the ambient concentration of saccharides and other compounds in the atmosphere presented by Wang *et al.* (2011).

Table 3. Ambient concentrations of saccharides, sugar-alcohols and vanillin during the haze and none-haze days, ng m^{-3} (haze event caused by wheat straw burning) (Wang *et al.*, 2011).

	June 1–5	June 5–17	October 12–24
	Haze ($n = 1$)	None-haze ($n = 3$)	None-haze ($n = 3$)
Levogluconan	4030	204 ± 129	689 ± 243
Fructose	72	32 ± 2.0	32 ± 13
Glucose	186	49 ± 27	44 ± 8.3
Sucrose	68	177 ± 24	31 ± 19
Trehalose	20	21 ± 0.7	17 ± 1.9
Arabitol	195	16 ± 2.4	21 ± 3.6
Mannitol	123	26 ± 3.9	24 ± 4.2
Inositol	19	4.6 ± 0.3	7.0 ± 2.8
Glycerol	207	41 ± 4.8	44 ± 11
Vanillin	16	0.5 ± 0.2	2.8 ± 0.9
Subtotal	4938	571 ± 125	919 ± 280

The presence of metabolically active air-borne microorganisms suggests organic compound transformation in clouds and thus a scientific emerging question rose about their role in the chemistry of clouds (Ariya and Amyot, 2004; Delort *et al.*, 2010).

Few studies tried to analyse biodegradation efficiency under cloud conditions. Herlihy *et al.* (1987) were the first who studied the degradation of formic and acetic acid by bacteria in rainwater. Ariya *et al.* (2002) and Côté *et al.* (2008) observed that several dicarboxylic acids detected in the atmosphere can be efficiently degraded by airborne bacteria and fungi using ^{13}C NMR. Amato *et al.* (2005) showed that bacterial strains present in cloud water contained the enzymatic equipments required for the degradation of some atmospheric compounds including formate, acetate, lactate, methanol, and formaldehyde. A more detailed study (Amato *et al.*, 2007c) was based on 60 microbial strains incubated at 27 °C as pure cultures with one single substrate. Using *in situ* ^1H NMR it could be shown that their metabolic pathways presented high similarities with chemical reactions common to atmospheric radical chemistry (Figure 4). This work showed that microbial metabolism can be a sink but also a source of organic compounds for the atmospheric chemistry.

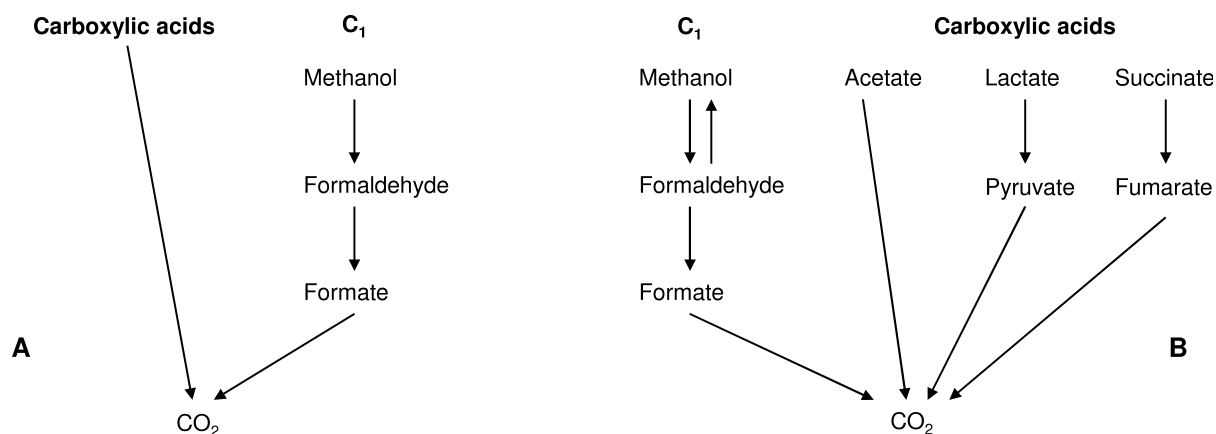


Figure 4. Similarities between photochemical reactions (A) and microbial metabolism (B).

These first papers mainly showed the ability of microbial strains to degrade organic compounds in clouds. However, in order to evaluate their effective contribution in atmospheric chemistry a quantitative

comparison of the contribution of this microbial activity with that of radical chemistry is necessary.

Biodegradation rates of dicarboxylic acids served to Ariya *et al.* (2004) for the estimation of their lifetimes (few days, see Table 4). They were comparable to that issued from the reactivity of the major atmospheric oxidants ($\cdot\text{OH}$, O_3 , and $\text{HO}_2\cdot$). However, it should be noted that the experimental conditions used by Ariya *et al.* (2002) were rather unclear and far from real cloud environment: the temperature was not controlled and close to room temperature, the isolated microorganism were from the air and not from cloud waters.

In order to be closer to cloud water conditions, Vaïtilingom *et al.* (2010), used artificial cloud water that mimicked the composition of real cloud water collected at the Puy de Dôme station. *P. graminis*, a strain isolated from cloud water, was incubated at 5 °C and 17 °C. The colder temperature (5 °C) corresponds to the mean annual temperature measured at the Puy de Dôme summit, while 17 °C is approximately the maximal temperature observed there when a cloud forms (see <http://www.obs.univ-bpclermont.fr/SO/mesures/pdd.php>). *P. graminis* strain was able to degrade acetate, succinate, and formate but not oxalate. The relative contribution of microbial metabolism to the degradation of acetate, succinate, and formate was compared with that of radical chemistry involving $\cdot\text{OH}$ and $\text{NO}_3\cdot$ reactivity (Ervens *et al.* 2003; Herrmann, 2003). Figure 5 shows that during the day $\cdot\text{OH}$ activity is dominating; bacteria have only a small influence. On the contrary, during the night, when $\cdot\text{OH}$ are no longer present, microorganisms could be the main actors of cloud chemistry.

Table 4. Lifetimes of some dicarboxylic acids present in the atmosphere resulting from the reactivity of $\cdot\text{OH}$, O_3 , and $\text{HO}_2\cdot$ and from the metabolism of atmospheric microorganisms (Ariya *et al.*, 2002).

Carboxylic acid	Oxidant	Kinetic constant k [$\text{M}^{-1} \text{s}^{-1}$]	Lifetime [days]	Reference
	$\cdot\text{OH}$ (aq)			
Oxalic		1.0×10^7	16–64	Getoff <i>et al.</i> , 1971
Malonic		1.6×10^7	6–24	Walling <i>et al.</i> , 1973
Succinic		3.1×10^8	3–12	
Glutaric		8.3×10^8	1.3–5.2	Cabelli and Bielski, 1985
Adipic		2.0×10^9	0.1–0.4	
Pimelic		3.5×10^9	0.03–0.12	
	$\text{HO}_2\cdot$ (aq)			
Oxalic		<0.2	>964	
Malonic		<0.3	>643	Scholes and Willson, 1967
Succinic		<0.25	>771	
	O_3 (aq)			
Oxalic		2.7×10^{-2}	428669	
Malonic		5.5	2104	
Succinic		6.7×10^{-4}	17274737	
Glutaric		1.3×10^{-3}	8903134	Nepotchatykh and Ariya, 2002
Adipic		1.7×10^{-3}	6808279	
Pimelic		4.4×10^{-3}	2630471	
Pinic		2.5×10^{-2}	469963	
	Microorganisms		Lifetime [days]	
Oxalic			∞	
Malonic			1.5	Ariya <i>et al.</i> , 2002
Succinic			10	
Glutaric			5	

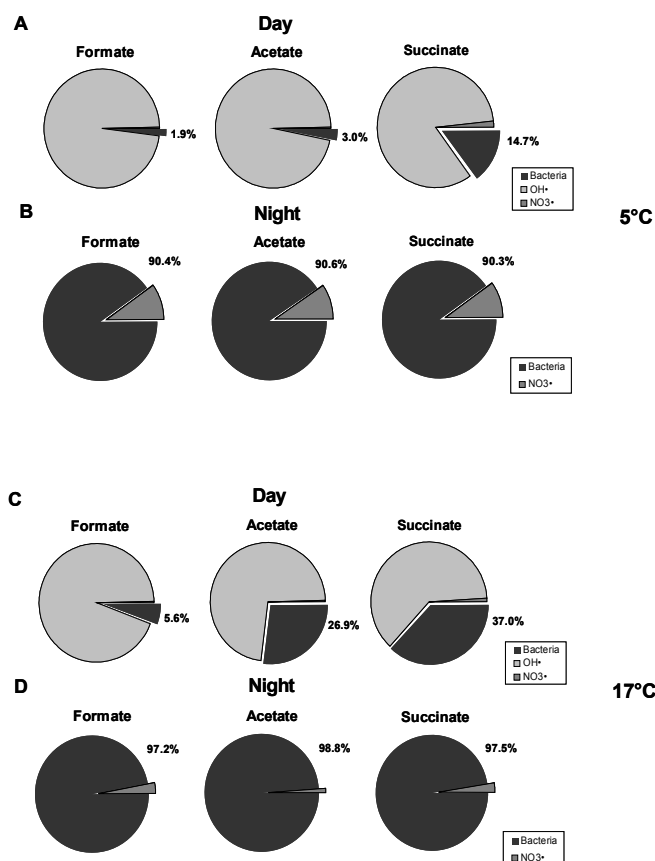


Figure 5. Estimated relative influence of bacterial activity (in black) and free radicals (hydroxyl $\cdot\text{OH}$ and nitrate $\text{NO}_3\cdot$) on the degradation of formate, acetate, and succinate in cloud water at 5 °C (A) during the day and (B) during the night and at 17 °C (C) during the day and (D) during the night. During night-time (*i.e.* in the absence of photochemical reactivity), $\cdot\text{OH}$ radicals are not present and were not considered. The following values of concentrations have been used for calculations: [bacteria] = 8.4×10^4 cell mL^{-1} ; [$\cdot\text{OH}$] = 1.2×10^{-13} mol L^{-1} (day-time); [$\text{NO}_3\cdot$] = 1.5×10^{-14} mol L^{-1} ; [formate] = 14.5×10^{-6} mol L^{-1} ; [acetate] = 20×10^{-6} mol L^{-1} ; [succinate] = 1.5×10^{-6} mol L^{-1} . (From Väitilingom *et al.*, 2010).

Very recently, Väitilingom *et al.* (2011) has extended this approach to a series of 17 strains incubated in artificial cloud waters mimicking both marine and continental clouds (Table 5). They showed that by the change of temperature from 17 °C to 5 °C, the biodegradation rate decreased from a factor 2 to 5. The composition of cloud water and more specifically the pH did not have an influence on biodegradation rates. The obtained biodegradation rates were then compared with those obtained from photochemical reaction performed in the same artificial media in two different irradiation systems (*P1* and *P2*), or from theoretical calculations

using two different $\cdot\text{OH}$ concentrations. Table 5 shows results obtained at 17 °C. They show that oxalate is not degraded by microorganisms, only by photochemical processes. The rates of photodegradation and biodegradation are in the same range of order. Depending on the chosen photochemical scenarios, the microbial activity has a major or a less important contribution to the transformation of organic acids in cloud water.

Finally, in order to validate their results concerning the biodegradation rates, Väitilingom *et al.* (2011) conducted experiments using real cloud water with complex composition containing the whole endogenous microflora without any strain selection. The biodegradation rates obtained in real cloud water were very similar to those obtained in artificial cloud water.

Table 5. Estimated rates of photo- and biodegradation in artificial cloud water at 17 °C (Väitilingom *et al.*, 2011).

Medium or compound	17 °C	Rate in artificial cloud (M s^{-1})			
		Acetate	Formate	Succinate	Oxalate
Marine	P1	0	$7.1 (\pm 2.2) \times 10^{-10}$	0	$2.7 (\pm 1.2) \times 10^{-10}$
	P2	0	$2.4 (\pm 0.1) \times 10^{-11}$	0	$2.2 (\pm 0.9) \times 10^{-11}$
	Microbial*	$5.3 (\pm 9.1) \times 10^{-11}$	$1.1 (\pm 1.8) \times 10^{-11}$	$1.9 (\pm 3.2) \times 10^{-11}$	0
Continental	P1	0	$7.4 (\pm 2.2) \times 10^{-10}$	0	$3.4 (\pm 0.9) \times 10^{-10}$
	P2	0	$1.5 (\pm 0.4) \times 10^{-10}$	0	$1.9 (\pm 0.7) \times 10^{-10}$
	Microbial*	$6.7 (\pm 1.4) \times 10^{-11}$	$1.4 (\pm 2.3) \times 10^{-10}$	$1.5 (\pm 1.8) \times 10^{-11}$	0
Marine and continental	$\cdot\text{OH}$ (1×10^{-12} M)	$1.2 \times 10^{-9} \dagger$	$3.2 \times 10^{-8} \ddagger$	$6.6 \times 10^{-10} \ddagger$	$3.2 \times 10^{-10} \ddagger$
	$\cdot\text{OH}$ (1×10^{-14} M)	$1.2 \times 10^{-11} \dagger$	$3.2 \times 10^{-10} \ddagger$	$6.6 \times 10^{-10} \ddagger$	$3.2 \times 10^{-12} \ddagger$

* Cell concentration used was 8×10^7 cells L^{-1} (mean value in the cloud water sampled at the Puy de Dôme station (Amato *et al.*, 2007c))

† (Chin and Wine, 1994)

‡ (Ervens *et al.*, 2003)

All these results are consistent with a potential contribution of microorganisms to atmospheric chemistry. However, to know a real contribution of microbiological processes relative to photochemical processes in cloud chemistry, an application of numerical process models is necessary (Deguillaume *et al.*, 2008). These process models will take into account the exchange existing between the interstitial phase (gases and particles), cloud droplets and crystals. To simulate more realistic clouds, these models will introduce both biological and radical reactions and modify different parameters (kinetic constants of degradation, number of cells, chemical composition, temperature, pH, light, flux, ...) (Georkakopoulos *et al.*, 2009; Leriche *et al.*, 2007; Deguillaume *et al.*, 2010).

2.2 Metabolism of Carboxylic Acids and C₁ Compounds, Major VOCs Present in Atmospheric Waters

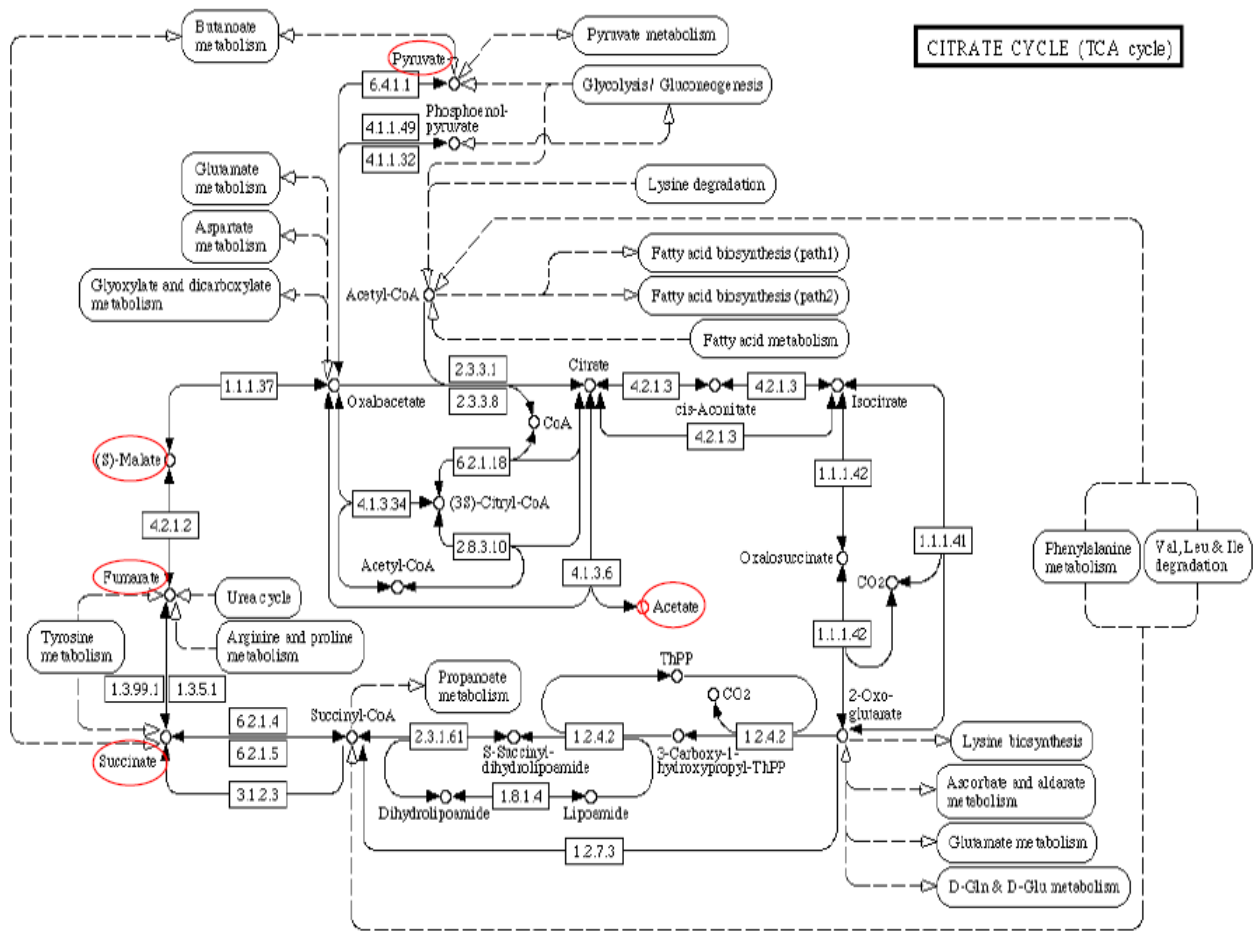
This chapter is dedicated to a brief description of metabolic routes related to the degradation of the major carboxylic acids and C₁ compounds of interest, present in cloud water. Only metabolisms of aerobic heterotrophic microorganisms will be considered here.

Metabolism is defined as an interconnection of complex biochemical reactions catalysed by enzymes. Heterotrophic aerobic organisms use all nutrition obtained from their solid or liquid environment, and use oxygen as final electron acceptor from breathing. Different substrates or intermediates of microbial metabolism have been found in clouds, and they might be potentially further used as substrates by cells. Some metabolic pathways represent the central part of the energy production, such as the glycolysis and the Krebs cycle (also called the citrate cycle or

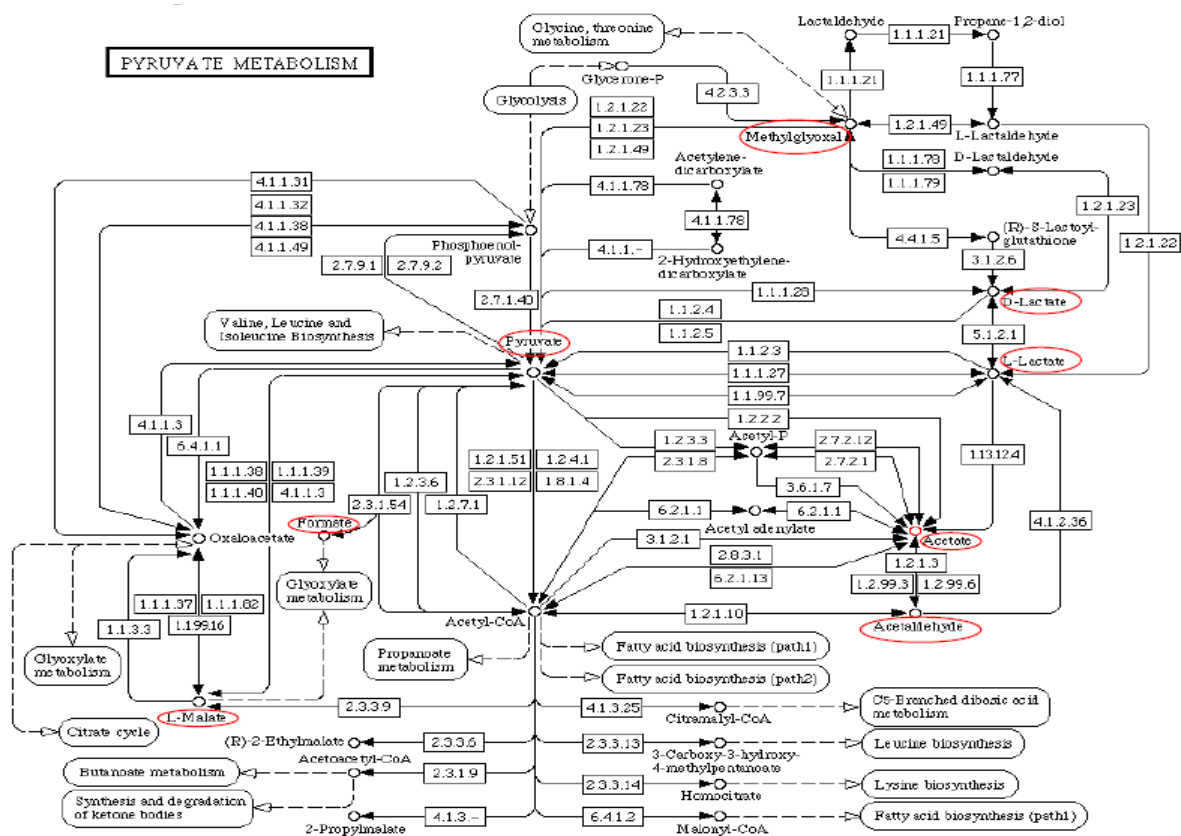
cycle of tricarboxylic acid, TCA).

2.2.1 Metabolism of Carboxylic Acids

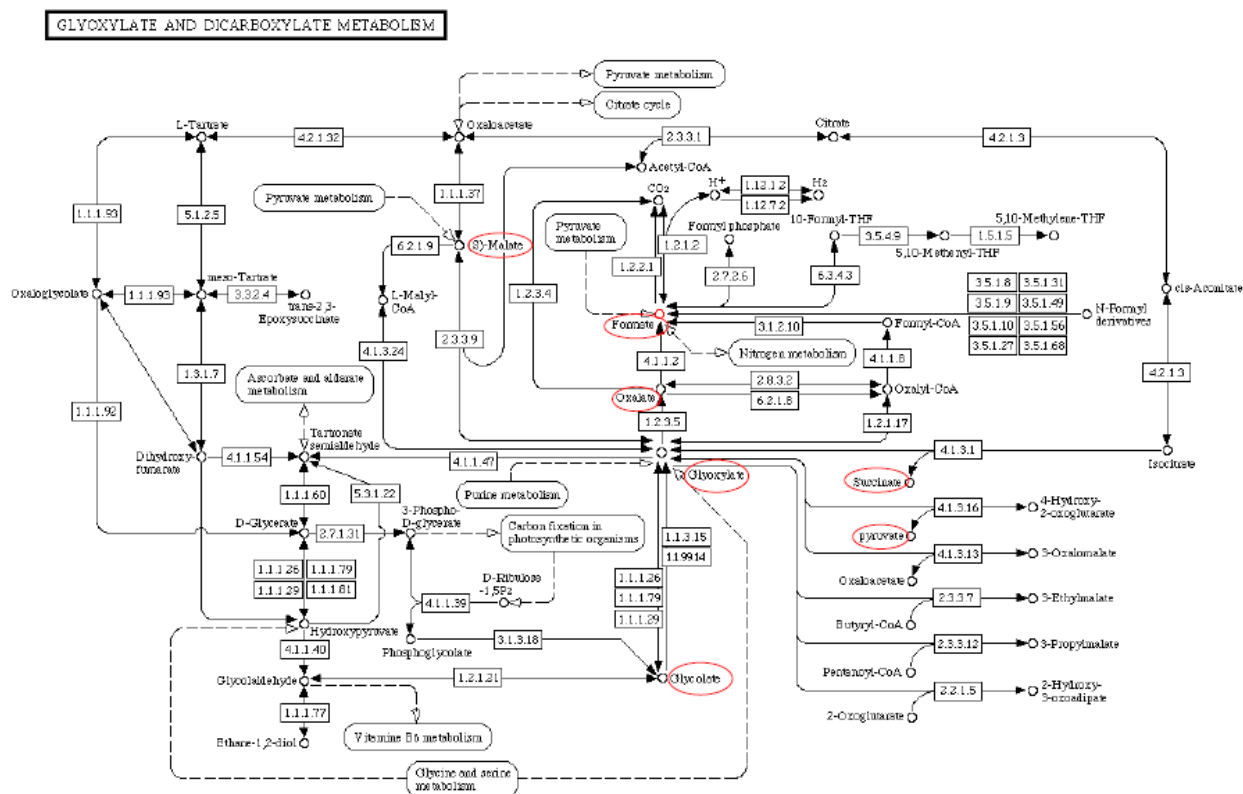
Metabolic pathways, which could be involved in the metabolism of bacteria in the clouds, are presented here (See Schemes 1, 2, 3, and 4). They are reproduced from the KEGG metabolic pathway database with free access (KEGG pathway database <http://www.genome.jp/kegg/pathway.html>). This database collects most of known metabolic pathways identified in a whole collection of organisms and it contains also information about associated enzymes. Numbers in schemes identify enzymes involved in reactions. Database allows a search according organism or enzymes. Each bacterium has its own pool of enzymes. Although general rules exists (principal reactions are generally conserved), involved enzymes can vary and lateral pathways may not exist. Studies are generally necessary to determine metabolic pathways for a given bacterium or species.



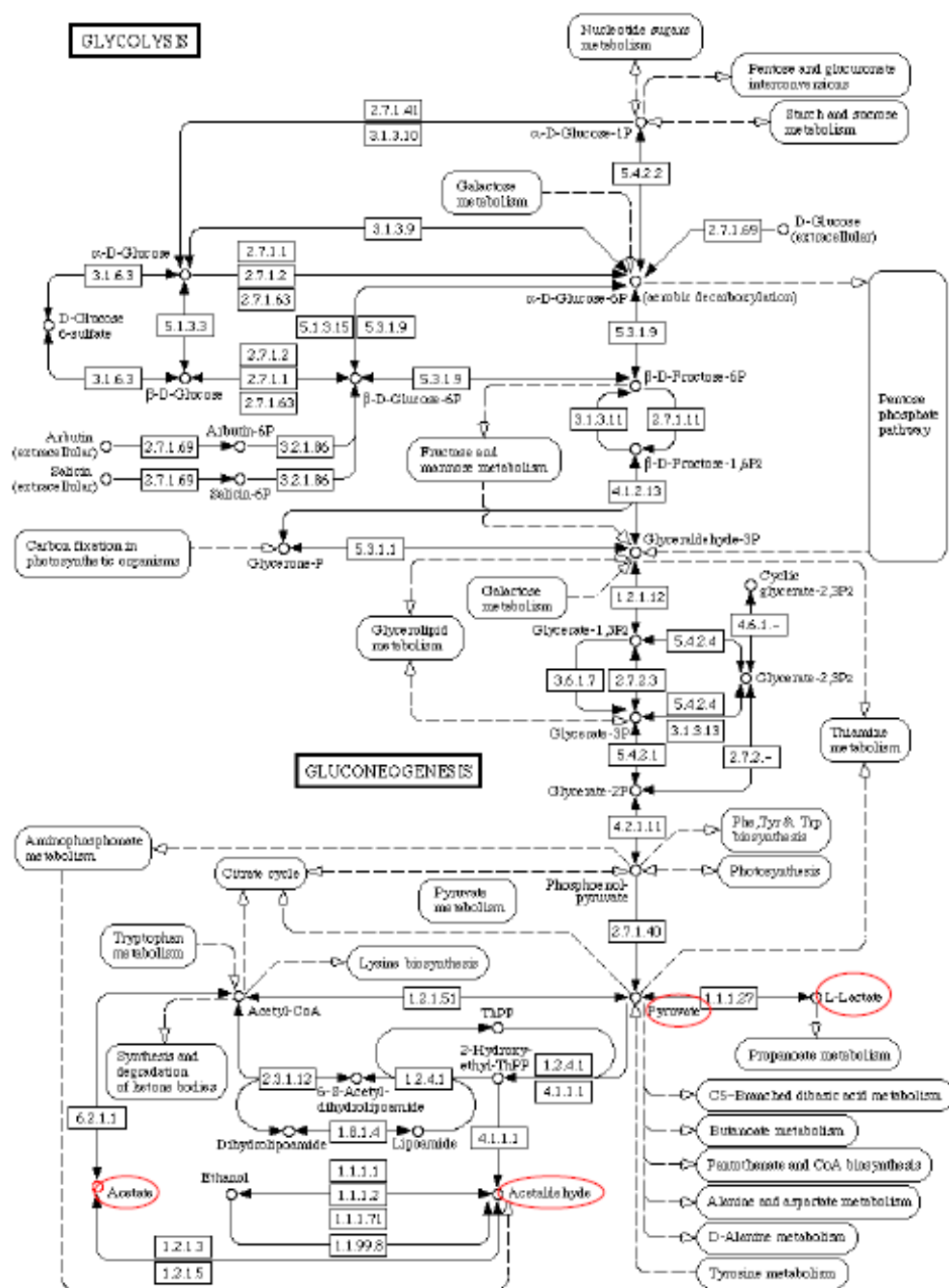
Scheme 1. Metabolic pathways involved in bacterial metabolism. Citrate cycle (TCA cycle).



Scheme 2. Metabolic pathways involved in bacterial metabolism. Pyruvate metabolism.



Scheme 3. Metabolic pathways involved in bacterial metabolism. Glyoxalate and dicarboxylate metabolism.



Scheme 4. Metabolic pathways involved in bacterial metabolism. Glycolysis.

2.2.2 Metabolism of C₁ Compounds

Some microorganisms can transform single carbon compounds (C₁ compounds). Among them, methylotrophs are microorganisms, which metabolism is based on their assimilation of C₁ compounds. Most of them are aerobic and compatible with droplets in clouds. Methylotrophs are microorganisms very disseminated in the environment (aquatic and terrestrial media) and they are very important in humid zones, tundras. Methylotrophs should be found among the *Alpha-*, *Beta-*, and *Gamma-Proteobacteria* and among Gram-positive bacteria. In addition, many microorganisms are facultative methylotrophs, and therefore according to conditions they can operate on the same substrates as obligatory methylotrophs. For the energy production purposes, if C₁ compounds more reduced than formaldehyde (methane, methanol, methylated amines, ...) are metabolised by bacteria, they are generally oxidised to formaldehyde, then to formate, and finally to CO₂. Formaldehyde can also join central metabolism of the cell and participate in the synthesis of cell constituents. Each C₁ compound is susceptible to be found in the cloud waters, if formaldehyde is present. Formaldehyde is in the centre of this type of metabolism. C₁ compound metabolic routes can be found in KEGG, however, we have decided to describe each step of this metabolism in detail later in this chapter as these compounds will be studied in this work. The different enzymes, co-enzymes, and their modes of action are described in detail.

Oxidation of Methane to Methanol and Formaldehyde

The methanotrophs are methylotrophs, which are able to use methane as a sole source of carbon. The methanotrophs can form lipidic intracytoplasmic membranes, which are unusual for bacteria. Depending

on their physiology, the arrangement of these membranes and the path of formaldehyde assimilation, two categories of methanotrophs have been established. The methanotrophs type I (*Methylomonas*, *Methylococcus*, *Methylobacter*) using ribulose monophosphate (RuMP) pathway, and type II (*Methylosinus*, *Methylocystis*) using serine pathway. Figure 6 shows details of the oxidation of methane into methanol, which is oxidised to formaldehyde and afterward it joins the metabolism of single carbon compounds (Hanson and Hanson, 1996).

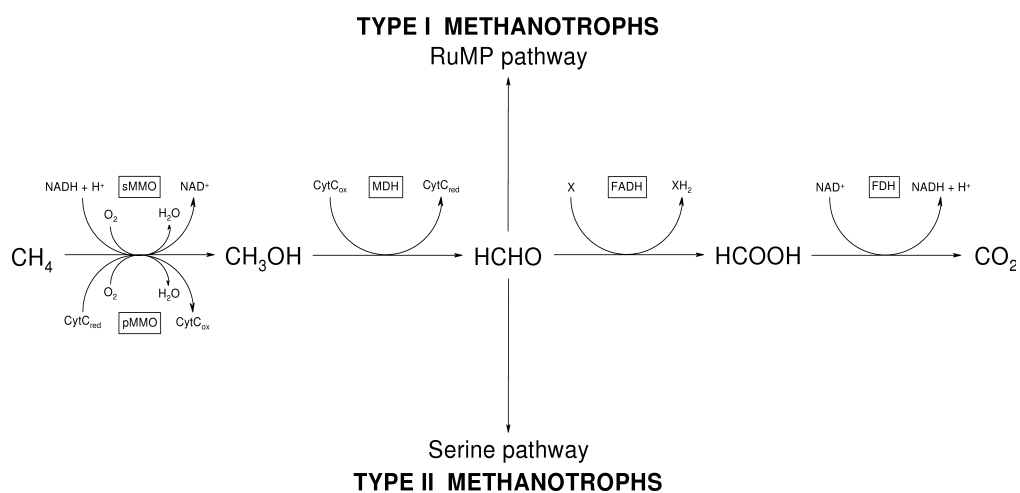
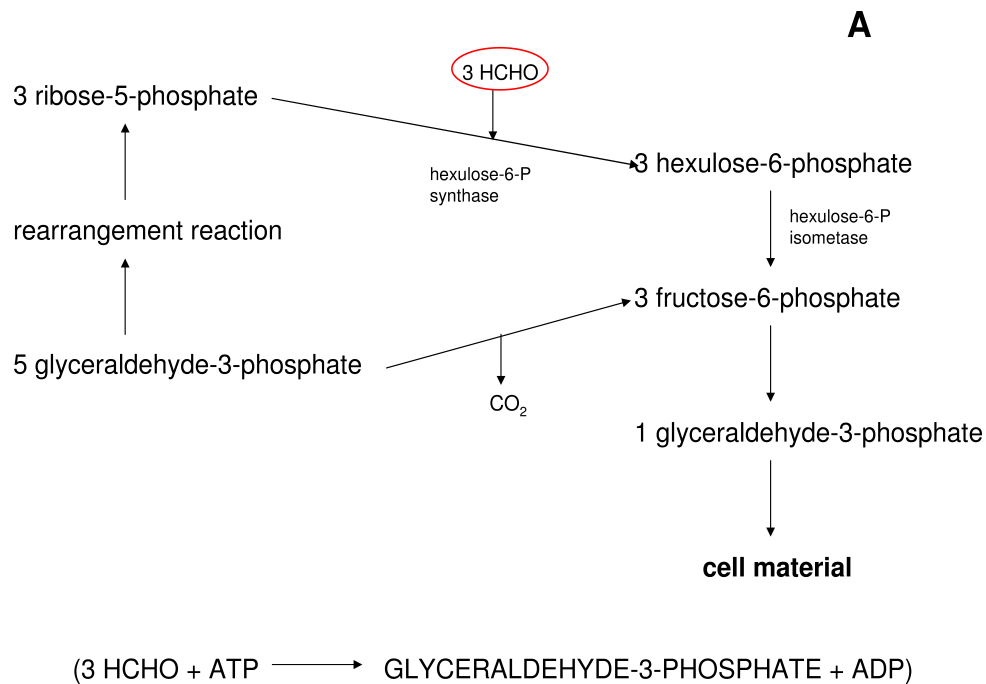


Figure 6. Oxidation pathway of methane to methanol and formaldehyde. SMMO – soluble methane monooxygenase; pMMO – particulate methane monooxygenase; MDH – methanol dehydrogenase, FADH – formaldehyde dehydrogenase, FDH – formate dehydrogenase (Hanson and Hanson, 1996).

The oxidation of methane into methanol can be performed by two forms of enzyme, a soluble form, the sMMO, and a form bound to intracytoplasmic membranes, the pMMO (for “particulate” MMO). MMO requires the presence of oxygen and only the sMMO is dependent on the presence of NAD⁺. The methanol dehydrogenase (MDH) oxidises methanol to formaldehyde. A cytochrome *c* is required as electron acceptor (Jiang *et al.*, 2010).

Formaldehyde Assimilation

Formaldehyde can be assimilated by the cell and participates in the formation of the cellular structures. The cyclic pathways of RuMP and serine can be used (Figure 7). In general, RuMP pathway is more effective and more common than that of serine. The latter is known in methanotrophs type II and in facultatively methanotrophs, which is activated in the presence of methanol (Trotsenko *et al.*, 2008).



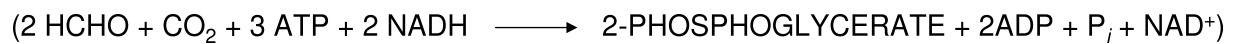
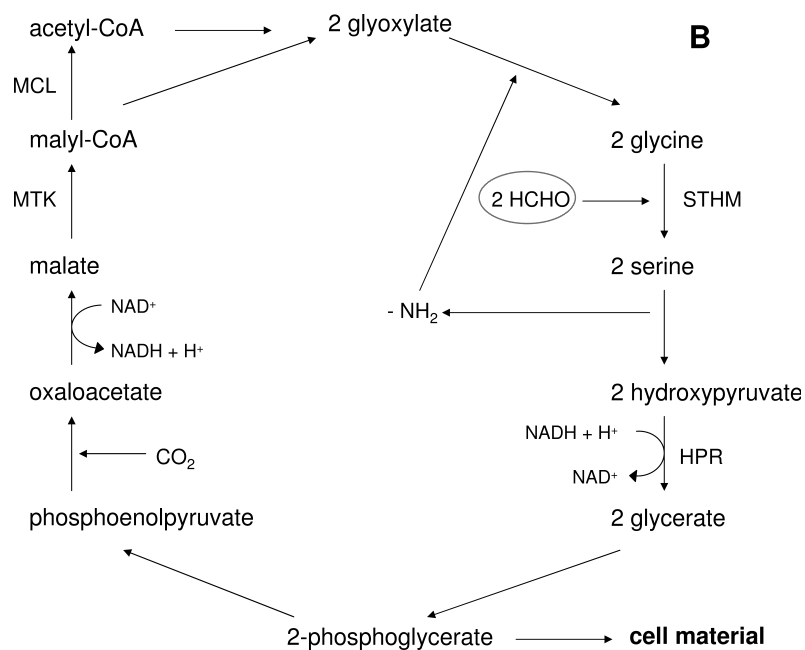


Figure 7. Pathways of formaldehyde assimilation by (A) the RuMP pathway and (B) path of serine. STHM – serine hydroxymethyl-transferase; HPR – hydroxypyruvate reductase; MTK – maleate thiokinase; MCL – maly l coenzyme A lyase (Hanson and Hanson, 1996).

Formaldehyde Disproportionation

The disproportionation of formaldehyde to methanol and formate can proceed in 2 ways (Figure 8). The first involves a NAD⁺ dependent oxidoreductase. It results either to the oxidation of formaldehyde to formate by simultaneous reduction of NAD⁺, or to methanol by reduction of NAD⁺. The second way, also called Cannizaro's reaction is catalysed by formaldehyde dismutase (Kato *et al.*, 1984) and it does not require the presence of cofactor. It is induced by the presence of formaldehyde in the medium (Hektor *et al.*, 2000; Mitsui *et al.*, 2005).

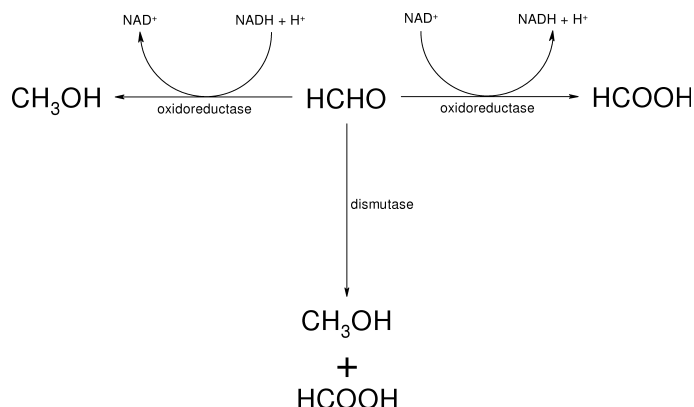
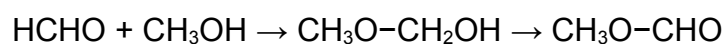


Figure 8. Disproportionation pathways of formaldehyde to formate and methanol, catalysed by NAD^+ -dependent oxidoreductase or formaldehyde dismutase.

Reaction of Formaldehyde with Methanol

Formaldehyde can recombine with methanol leading to the formation of methylformate, following the reaction (Mason *et al.*, 1986; Delort, 2006):



Oxidation of Formaldehyde to Formate

The oxidation of formaldehyde to formate takes place in the cytoplasm by action of both, free or to the membrane bound enzymes. There are several pathways that can lead to the formation of formate from formaldehyde. Three of them are described in the review of Vorholt (2002). They are shown in Figure 9. Each of them requires a specific cofactor, which binds to the molecule of formaldehyde and NAD(P)^+ .

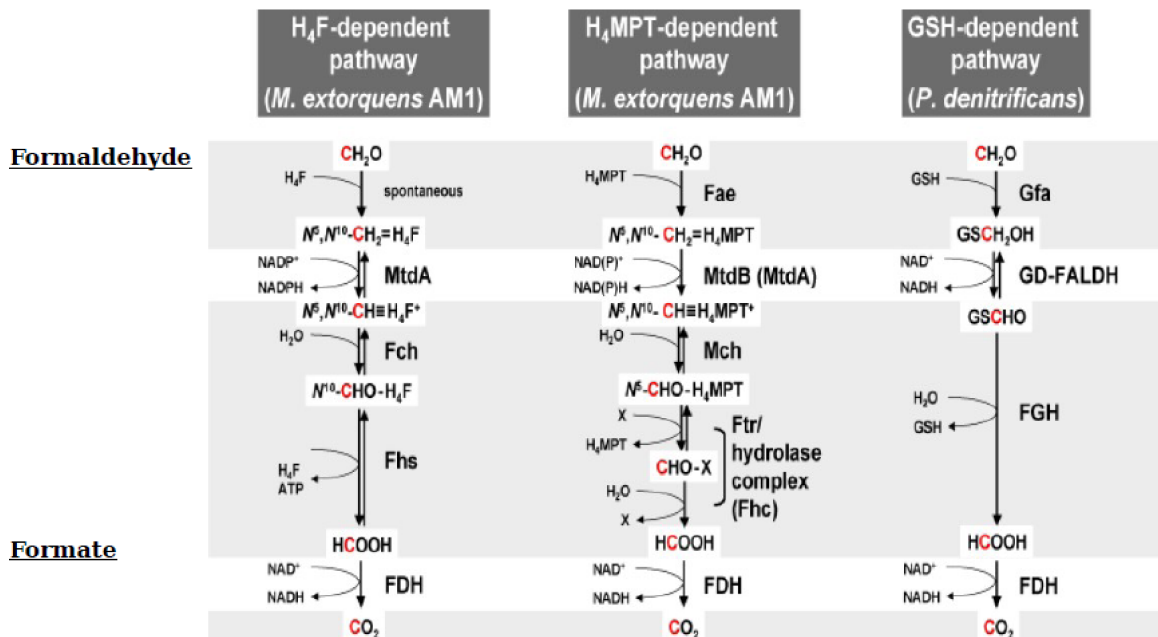
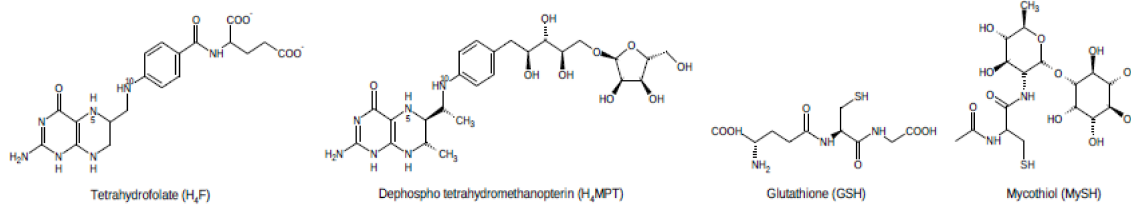
Cofactors:

Figure 9. Different linear pathways of formaldehyde oxidation in *Methylobacterium extorquens* AM1 and *Paracoccus denitrificans* and their associated cofactors. MtdA – NADP-dependent methylene-H₄MPT dehydrogenase; Fch – methenyl-H₄F cyclohydrolase; Fhs – formyl-H₄F synthetase; FDH – formate dehydrogenase; Fae – H₄MPT-dependent formaldehyde activating enzyme; MtdB – NAD(P)-dependent methylene-H₄MPT dehydrogenase; Mch – methenyl-H₄MPT cyclohydrolase; Ftr – formyltransferase; Fhc – Ftr/hydrolase complex; Gfa – glutathione-dependent formaldehyde activating enzyme; GD-FALDH – NAD⁺- and glutathione-dependent formaldehyde dehydrogenase; FGH – formyl-glutathione hydrolase; X – unknown cofactor (Vorholt, 2002).

The path H₄F-dependent requires the presence of tetrahydrofolate. It is mainly present in bacteria, for which the serine pathway represents only the part of formaldehyde assimilation, and is therefore relatively little frequent. The H₄MPT-dependent path is associated with the presence of dephosphorylated tetrahydromethanopterin. This path was observed in anaerobic methanogenic bacteria, sulfate-reducers, and in some

Archaea. The third path, GSH-dependent, is mostly widespread, both in bacteria that in yeasts, plants, and mammals, and most often it is used to detoxify the organism from formaldehyde. It requires the presence of glutathione (GSH) or mycothiol (myshi).

In each case, the oxidation reaction is initiated by the binding between the cofactor and formaldehyde. This activation is spontaneous in the case of the H₄F-dependent path. For the other two paths, this binding is glutathione-dependent in certain bacteria, or it is caused by an activation of other formaldehyde activation enzymes: glutathione-dependent or H₄MPT-dependent. These activation enzymes allow creating complexes with free formaldehyde in the cell and thus to prevent its interaction with molecules that can cause damages (*e.g.*, DNA, *etc.*).

Above-mentioned facts give the evidence that there is no universal way how formaldehyde is used by microorganisms. Several ways exist and often coexist in the same cell, whether for carbon assimilation, energy production, and detoxification.

2.3 NMR as a Tool to Investigate the Metabolism of Cloud Microorganisms

Previous studies related to the metabolism of molecules of interest found in the atmosphere have been performed using Nuclear Magnetic Resonance (NMR) (Ariya *et al.*, 2004; Amato *et al.*, 2005; 2007c). We propose to give some insight in this technique, which is a method of choice to study microbial metabolism.

NMR is a non destructive method, enabling thus the analysis of complex intracellular or incubation media (*in situ* NMR) but also the analysis of the metabolism of living cells (*in vivo* NMR) and its monitoring over the

time. NMR offers an important alternative to most commonly used analytical techniques such as mass spectroscopy, HPLC, GPC, enzymatic assays, or the use of radio markers (^{14}C , ^3H). ^1H , ^{13}C , ^{31}P , and ^{15}N are the most frequently observed nuclei in the metabolism studies either due to their natural abundance or the availability of enriched substrates. However, some other nuclei such as ^{23}Na and ^{39}K can be used for special purposes such as the measurement of trans-membrane gradients. Numerous NMR studies of microorganism metabolism have been reviewed (Grivet and Delort, 2009; Grivet *et al.*, 2003; Portais and Delort, 2002). The majority of them were performed on supernatants (incubation media) of bacterial cultures or cell extracts.

For *in situ* studies, the pre-treatment of biological samples is often necessary, *e.g.* separation of cellular fractions, pH adjustment, freeze-drying and subsequent dissolution in D_2O , partial purification of certain metabolite, *etc.* When *in vivo* NMR is performed, specific apparatus must be used to maintain the cells alive in the NMR spectrometer. Figure 10 presents the system of perfusion described by Choraó *et al.* (2009) that has been used in this work.

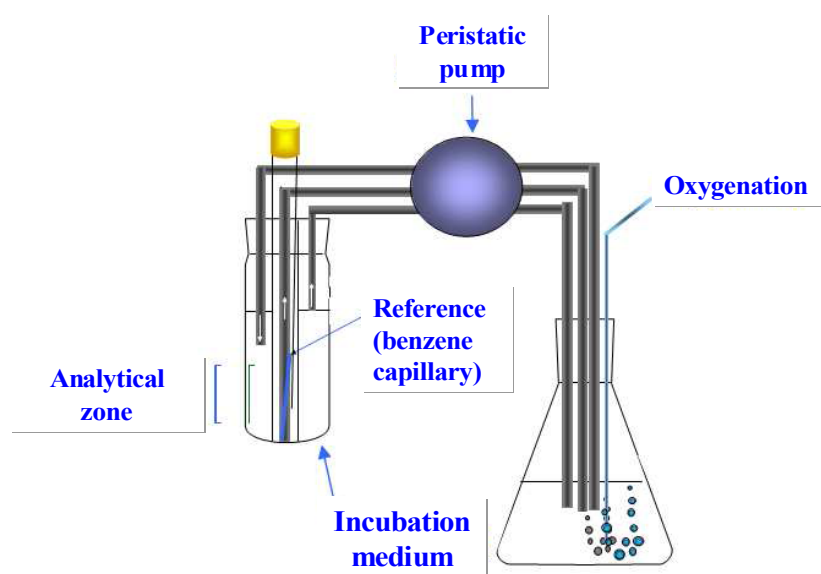


Figure 10. Perfusion system for *in vivo* NMR experiments described by Choraó *et al.* (2009). Bacteria are perfused with the incubation medium containing 10% of D_2O and

saturated with air. The perfusion rate is controlled by a peristaltic pump (flow rate 20 mL min⁻¹). Bacterial suspension circulates continuously in the system. Benzene is used as an internal reference to calibrate chemical shifts (128.39 ppm relative to TMS (0 ppm)) in a sealed capillary placed in a 10 mm sample tube.

2.3.1 ¹H NMR

The natural abundance of ¹H is close to 100% and thus makes the proton NMR technique the most sensitive: A tagging of the molecules is not necessary. It is a quantitative technique but low range of proton resonance (~15 ppm) may lead to overlapping of peaks. Very important drawback in biological samples studies is the presence of a huge signal of water. Thus, techniques enabling its efficient suppression are of great importance because of the residual water signal adversely affect appearance and subsequent analysis of NMR spectra. A lot of methods allowing the water signal suppression have been developed. The most frequently used techniques are described below.

Presaturation methods (Hoult, 1976) based on irradiation of water signal by a low energy radiofrequency, are frequently used in the case of samples with higher quantity of metabolites. However, usually in biological samples the energy used for the presaturation cannot be sufficiently strong to suppress efficiently the residual water signal without a partial saturation of signals with the resonance frequency in its neighbourhood and without the baseline distortion of the spectrum.

By far, the most often-used water suppression techniques use *pulsed field gradients* (PFGs) to rapidly attenuate the H₂O resonance, winding any transverse magnetisation into a tight spatial helix oriented along the PFG axis, and causing the integrated magnetic flux through the NMR receiver coil to nearly vanish for the water magnetisation. A field-gradient pulse is a pulse or a period, during which the magnetic field is made deliberately

inhomogeneous. It includes gradient-enhanced coherence transfer pathway selection, which was incorporated into sequences as WATERGATE, excitation sculpting, WET, different modifications of WATERGATE, and PURGE. All these PFG-based methods have their individual advantages and drawbacks, and may not be applicable in all situations depending on the details of underlying pulse sequence and characteristics of the sample itself (Nguyen *et al.*, 2007).

Modern high-resolution NMR probes contain besides the RF coils additional coils that can be fed by a direct current. The coils are built so that a pulse (~ 1 ms long) of direct current creates a gradient of the B_0 field along z (x or y , these refer to the lab coordinate system), as if the z (x/y) shims were mis-set so that the line-widths would be ~ 100 kHz. A signal of 100 kHz line width will be completely dephased after 1 ms. 200 μ s after the gradient pulse, the B_0 field is homogeneous again but the magnetisation is still dephased. Therefore, if one applies another gradient after some time, the magnetisation can be refocussed if the gradients have the right ratio.

Mo and Raftery (2008) have improved pulse sequence for residual water suppression – WET180 (WET with 180° pulse-toggling), which is proposed to cancel the water contribution to the residual solvent signal. This pulse sequence uses shaped, selective pulses and magnetic field gradient pulses to suppress one or more solvent signals. It provides very efficient suppression with excellent selectivity. The WET sequence was originally developed for the suppression of the effects of T_1 and B_1 inhomogeneity during the *in vivo* spectroscopy (Frahm *et al.*, 1990; Bottomley, 1987). The WET technique uses a series of variable-tip-angle solvent selective RF pulses, where each selective RF pulse is followed by a dephasing field-gradient pulse (Smallcombe *et al.*, 1995). The B_1 insensitivity shares the same refocusing mechanism. For the two-pulse B_1 insensitive WET sequence, positive longitudinal magnetisation components are generated by the 79.7° excitation pulse and then start to refocus near the null after being inverted by the 110.2° pulse (Ogg *et al.*,

1994).

WATERGATE (WATER suppression by GrAdient Tailored Excitation) sequence is very efficient in suppression of water signal. The sequence comprises of selective inverse pulse surrounded by two gradients of equal magnitude and duration. Its drawback is the suppression of signals surrounding the water signal. The sequence is frequently used in protein and nucleic acids structural studies (Piotto *et al.*, 1992; Sklenář *et al.*, 1993). The original pulse sequence was composed of an initial 90° pulse bringing all magnetisation into the *xy*-plane. A gradient is used to encode the coherences, and refocusing is employed by a non-selective 180° hard pulse surrounded by two 90° shaped pulses of opposite phase. After the refocusing pulses, another identical time period was used containing a final gradient of equal amplitude. Solute was effectively encoded by the first gradient, reversed, and decoded by the final gradient. The type of selective pulses, amplitudes, and durations determined the efficacy and selectivity of the solvent suppression. Optimisation could be achieved by modifying the type of shaped pulse and its duration (McKay, 2009). Nowadays, WATERGATE is often integrated into various pulse sequences as a solvent suppression. For example, Price *et al.* (2002) developed PGSE-WATERGATE (Pulse Gradient Spin Echo WATERGATE) sequence by integrating the WATERGATE unit into original Hahn spin echo based PGSE sequence. By its application effective solvent signal suppression was achieved. PGSTE-WATERGATE was applied by Zheng *et al.* (2008) for measuring diffusion coefficients in aqueous solution, where WATERGATE provides excellent solvent suppression without any phase distortions. The progress in water/solvent suppression techniques is clearly visible in many available reviews (Zheng and Price, 2010; Cook, 2004; Tsang and Rance, 1996).

In microbiology, ¹H NMR has been used in numerous of studies concerning carbohydrate metabolism but also xenobiotic degradation (pollutants, food additives) (Grivet and Delort, 2009). In the case of xenobiotics, proton is often the only nucleus to be used due to low

concentrations of these compounds (Figure 11) and the unavailability of commercially labelled compounds.

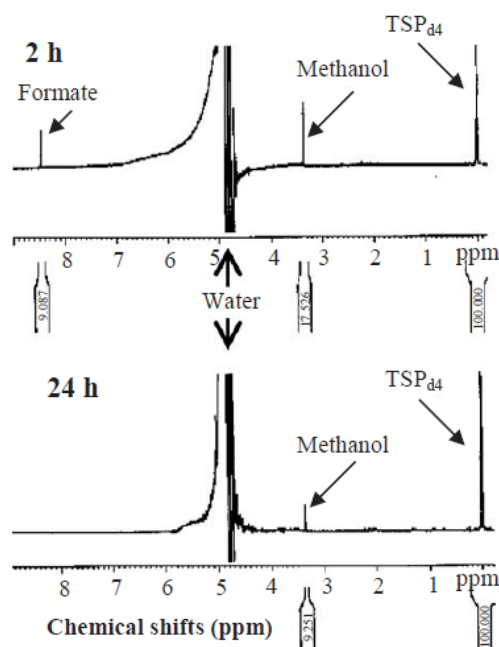


Figure 11. ^1H NMR spectra of the incubation medium of *Pseudomonas* sp. with formaldehyde and methanol as substrates collected after 2 and 24 hours of incubation. Formaldehyde resonance is hidden by the huge residual water signal. TSP_{d_4} is the internal reference (from Amato *et al.*, 2005).

^1H NMR is also extremely useful in indirect ^{13}C (or X) label detection in produced compounds and the $^{13}\text{C}/^{12}\text{C}$ ratio quantification as well as the ^{13}C label distributions. Obtained information is often used in studies of metabolic fluxes (see Grivet and Delort, 2009 for review) and it is a key point to carbohydrate metabolic cycles studies for instance (Portais and Delort, 2002).

Finally, ^1H NMR covers also growing area of metabolomics (Grivet and Delort, 2009). This approach allows comparing the whole metabolome of genetically different microbial strains (biodiversity, mutants, *etc.*) but also to investigate the global response of microorganisms to various stresses (case of environment microbes) or to different diets (case of gut

microbes).

2.3.2 ^{13}C NMR

A limiting factor for the use of ^{13}C NMR in microbiological studies is a low natural abundance of ^{13}C isotope ($\sim 1\%$), which does not allow its direct detection due to the low concentration of metabolites. Therefore, utilisation of the ^{13}C labelled substrates is a widely used technique in microbial metabolism studies. Besides an increased sensitivity, it enables the monitoring of the ^{13}C label fate in metabolites created by microorganisms during the incubation. Detailed analysis of various isotopomers allows metabolic pathways identification and flux studies. Numerous examples of the microbial metabolism studied by ^{13}C NMR were shown in several reviews (Grivet *et al.*, 2003; Grivet and Delort, 2009).

Since C_1 compound (methanol and formaldehyde) metabolism is of major interest in this PhD work, we would like to give here some examples of ^{13}C NMR studies using ^{13}C labelled substrates. Most of them are cited by Delort (2006).

Figure 12 presents the transformation of ^{13}C -formaldehyde (~ 82 ppm) into different compounds, including ^{13}C -formate (~ 70 ppm) and ^{13}C -methanol (~ 50 ppm) by *E. coli* (Hunter *et al.*, 1984) studied by *in vivo* ^{13}C NMR. Signals at ~ 60 – 70 ppm correspond to compounds with longer chain ($\text{R}-\text{CH}_2-\text{R}'$) formed by the assimilation of formaldehyde. They were assigned to glycerol (propane-1,2,3-triol), propane-1,2-diol, and propane-1,3-diol.

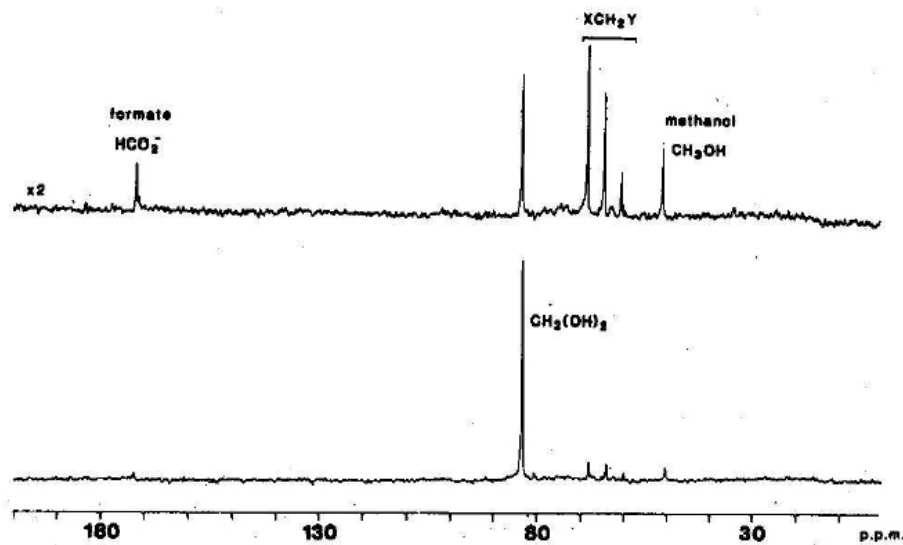


Figure 12. ^{13}C NMR spectrum of a suspension of *E. coli* supplemented for 4 minutes with 10 mM ^{13}C -formaldehyde, after 20 minutes (below); after 3 hours (above) (Hunter *et al.*, 1984).

Spectra, in the Figure 13 show the ^{13}C labelled methanol degradation by *B. methanolicus*. Simultaneously with the methanol consumption the three peaks of metabolites appeared: ^{13}C -formaldehyde, ^{13}C -methanol, and 1- ^{13}C]hexose-phosphate (Figure 13). The assimilation of methanol into the RuMP pathway is presupposed according to the presence of 1- ^{13}C]hexose-phosphate, to which methanol is incorporated during the first step of this pathway (Pluschkell and Flickinger, 2002).

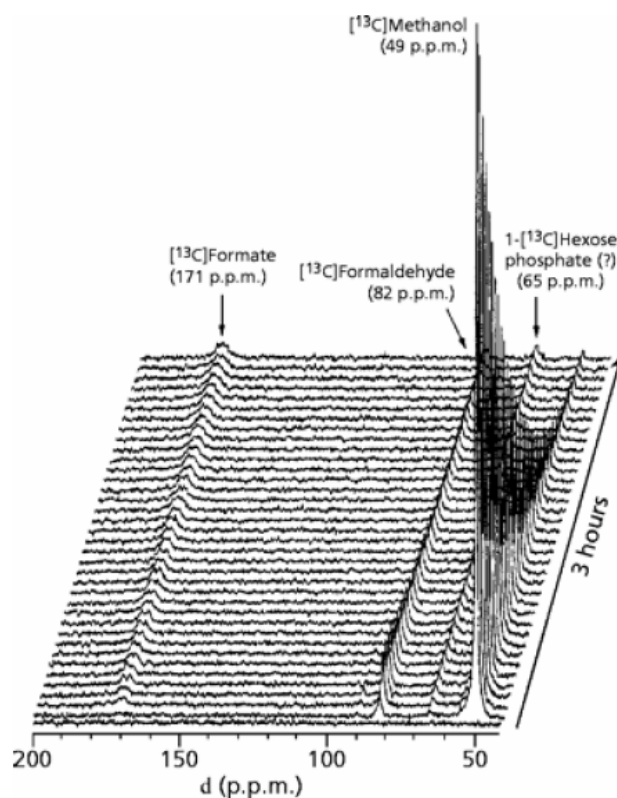


Figure 13. ^{13}C NMR analysis of the kinetics of ^{13}C -methanol degradation in a cell suspension of *B. methanolicus* (Pluschkell and Flickinger, 2002).

2.3.3 ^{31}P NMR

High sensitivity of ^{31}P due to a high natural abundance of this isotope is an advantage for the studying of a great number of biological processes by NMR: Energetic state of biological systems (*in vivo* and *in vitro* analysis), measurement of intracellular pH, and quantification of phosphorylated metabolites (Grivet, 2001; Grivet and Delort, 2009; Grivet *et al.*, 2003). Identification of some intracellular phosphorylated metabolites including polyphosphates, nucleotide triphosphates (NTPs) (mainly ATP and sugar phosphates) can be carried out directly by *in vivo* ^{31}P NMR experiments (Lohmeiner-Vogel *et al.*, 2004) (see Figure 14).

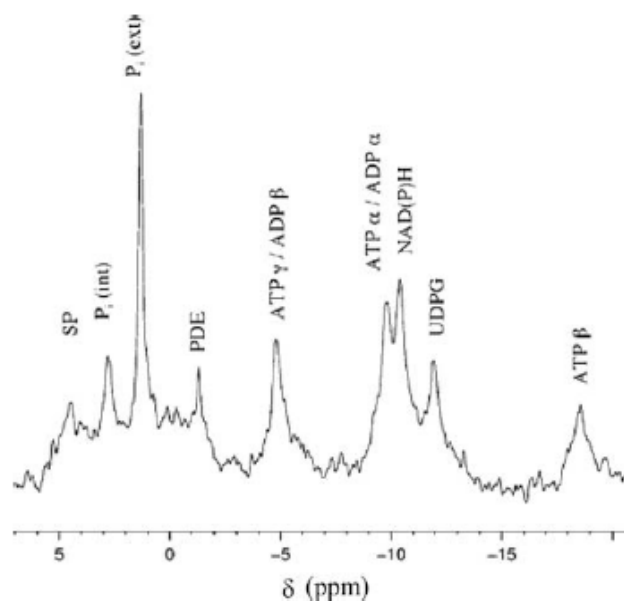


Figure 14. Example of *in vivo* ^{31}P NMR spectrum recorded at 161.8 MHz on the strain *E. coli* HB101(pUC8). SP – sugar phosphomonoesters; P_i (int), P_i (ext) – intracellular and extracellular pools of inorganic phosphate, respectively; PDE – phosphodiester; UDPG – uridine diphosphoglucose (Lohmeiner-Vogel *et al.*, 2004).

The most interesting aspect of *in vivo* ^{31}P NMR application is the measurement of intracellular pH in living cells. This method is based on the pH dependence of the ^{31}P chemical shift of phosphorylated compounds. The appearance of several “inside” inorganic phosphate signals is a proof of the existence of several cellular compartments or cell populations, each with a distinctive pH. Principle of the pH measurement by *in vivo* ^{31}P NMR is based on the chemical exchange between the various protonated forms of inorganic phosphate P_i . One of these equilibrium is of particular biological interest because its related pK_a is in the range of the intracellular natural pH ($\text{pK}_a = 7.0$, pH varies from 5 to 11). This exchange is very fast and therefore gives rise to only one average ^{31}P resonance whose chemical shift is directly dependent on the ratio of protonated/non protonated phosphate species. In other words, it is dependent on pH value. It is important to construct a calibration curve correlating pH and ^{31}P chemical shifts under conditions as similar as

possible to those in the intracellular medium (usually on cellular extract). Then the P_i chemical shift in *in vivo* spectra can be referred to this plot to get the corresponding pH value. In cells, several compartments could be encountered: *i*) intracellular and extracellular; *ii*) different intracellular – typically cytoplasmic and vacuolar in fungi and yeasts. P_i signals due to these compartments found at different chemical shifts give an access to trans-membrane pH gradient measurements. Figure 14 above clearly shows two P_i signals corresponding to the intracellular and extracellular P_i in *E. coli*.

2.3.4 ^{15}N NMR

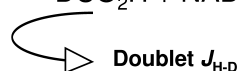
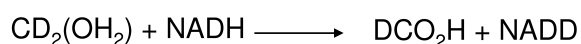
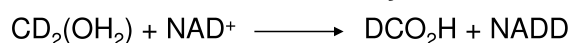
In general, the major drawback in observing ^{15}N nucleus is the insensitivity inherent with a natural abundance of 0.4%. Thus, ^{15}N NMR studies are mostly limited to ^{15}N enriched substrates utilisations. On the other hand, such a low natural abundance background makes it easy to detect an incorporation of a ^{15}N -enriched source and depletion of this isotope if it is diluted and replaced by an excess of ^{14}N fixed into the same species. However, the ^{15}N NMR spectra analysis is complicated by negative NOE factors and sometimes long T_1 values. Moreover, they can have a marked dependence on their physico-chemical environment (pH, ionic charge, viscosity, *etc.*) making difficult the quantitative analysis of cell extracts from experiments using labelling. Some of these dependences were studied by Nassima Houyou *et al.* (2006) in order to find conditions, in which quantitative analysis of some major metabolites (amino acids, ammonium, nitrate, and urea) could be performed reliably and with sufficient signal intensity. In methanogens, the free amino acids turnover was studied using labelled substrates (Roberts *et al.*, 1990).

2.3.5 ^2H NMR

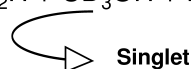
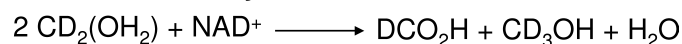
Due to the relatively low magnetogyric ratio and the magnetic properties of the quadrupole nucleus, the detection of ^2H by NMR suffers from its low sensitivity. The considerable line broadening, associated with its quadrupolar nature can be reduced to some extent by measuring at higher temperatures. Nevertheless, ^2H NMR spectroscopy of deuterated samples has frequently been used for biosynthetic investigations, in cases where an analyte is available in sufficient amounts. Taking advantage of the strength of NMR for stereochemical analysis, ^2H NMR can be used to determine the steric course of biosynthetic reactions. Chemical shifts of ^2H resonances are similar to its corresponding ^1H signals and thus the assignment can be performed on non labelled compound and further extrapolated to ^2H labelled one. Another advantage, especially for labelling purposes, is the low natural abundance of ^2H (less than 0.012%). Besides a precursor/product relationships establishment, ^2H NMR is especially useful for studying cyclisations, rearrangements, and the mechanistic aspects of metabolic reactions (Schneider, 2007).

An elegant application of *in vivo* ^2H NMR concerns the biotransformation of ^2H formaldehyde by bacterial strains (Mason and Sanders, 1989). As it was already shown in Chapter 2.2.2, formaldehyde can be reduced to methanol by two ways: *via* an NAD-oxidoreductase activity, or *via* a dismutase activity (see below):

NAD-oxido-reductase activity



Dismutase activity



In the first case (oxido-reductase), the methanol obtained contains both

^2H and ^1H atoms, the resulting CHD_2 signal is thus a doublet in ^2H NMR spectra due to the $J_{\text{H-D}}$ coupling. In the second case (dismutase), methanol is totally ^2H labelled and thus the ^2H NMR signal of the methyl group is a singlet. Figure 15 shows the identification of the mechanisms of enzymatic reaction directly in *in vivo* experiments with various bacterial strains.

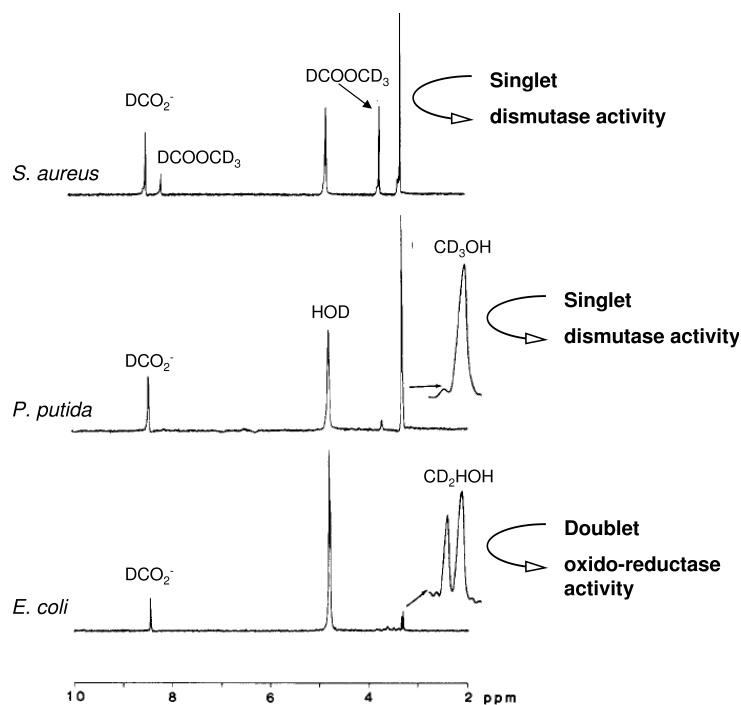


Figure 15. *In vivo* ^2H NMR spectra of incubations of *Staphylococcus aureus*, *Pseudomonas putida*, and *E. coli* cells with deuterated formaldehyde (Mason and Sanders, 1989).

2.3.6 NMR Experiments Used for Structure Elucidations

The resolution in 1D NMR is often inadequate for structure identification of complex mixtures. Enhancement of the resolution can be achieved by spreading of desired data into a second or more dimension(s). Depending

on the type of experiment, needed information can be thus extracted more easily. A drawback of 2D NMR measurements is that they typically require much longer experimental times than 1D spectra acquisition depending on the sensitivity of the observed nucleus, metabolite concentration, and the resolution desired.

2.3.6.1 Homonuclear 2D Experiments

Two common types of ^1H - ^1H homonuclear 2D methods utilise scalar coupling between protons to display the covalent bonding network. The ^1H - ^1H homonuclear COSY (COrrrelation SpectroscopY) and TOCSY (TOtal Correlation SpectroscopY) experiments reveal covalent correlations (manifested as cross-peaks). COSY and its phase sensitive variant DQF-COSY (Double Quantum Filtered COSY) experiments detect only scalar ^1H - ^1H couplings primarily between protons that are 2–4 bonds apart. They use a single step transfer of magnetisation for an identification of neighbouring protons. In the TOCSY experiment, depending on the length of the mixing time, cross-peaks due to the magnetisation transfer to distant protons (showing 3-bond, 4- to 5-bond) are observed. Specific patterns of cross-peaks and their characteristic chemical shifts make thus possible to assign a large number of metabolites directly in crude extracts with high reliability. For metabolite identifications, TOCSY can be superior to COSY, due to its ability to provide a complete covalent network. However, for making individual ^1H -assignments COSY is mostly always needed.

For the structure assigning of complex molecules or those ones, which contain fragment(s) with small scalar coupling not detectable by COSY or TOCSY, dipolar correlations may be necessary. Typical example of such molecule is galactose in which the magnetisation transfer from H1 stops at H4 due to a low value of scalar coupling between H4 and H5 protons.

Dipole-dipole couplings are observed between spins, which are close to one another in space and which undergo cross-relaxation. The aim of NOESY (Nuclear Overhauser Effect Spectroscopy) experiment is to identify nuclear spins undergoing cross-relaxation and also to measure its rates. Correlations between nuclear spins are established in NOESY during a mixing period. Obtained spectrum is similar to COSY, with diagonal peaks and cross-peaks, however, cross-peaks connect resonances from nuclei that are close in space. Most commonly, due to a sensitivity reason, NOESY is used as a ^1H - ^1H homonuclear technique showing cross-peaks for proton nuclei close to each other in space. However, the intensity of build up NOE signals in the spectrum depends on many factors. One of them is a tumbling rate dependence on the molecular mass of the molecule. Thus for certain molecules, whose rotational correlation time falls in range where the NOE effect is too weak or might become zero, it is not detectable in NOESY experiment. This disadvantage is overcome in ROESY (Rotating frame nuclear Overhauser Effect Spectroscopy) experiment in which ROE is evolving in the weak magnetic field of rotating frame and it is not dependent on the molecular mass of the compound. However, as the spin lock is used in ROESY TOCSY-type cross-peak appear in the spectra making their interpretation more difficult.

Both TOCSY and NOESY exist as a 1D variant. In the case when signals in ^1H NMR spectra are sufficiently resolved enabling their selective irradiation and more precise information could be obtained in reasonable shorter times.

2.3.6.2 Heteronuclear Correlations

COSY and TOCSY spectra are used for the assignment of proton spin systems. Although, many common metabolites can be reliably identified

with them, many others remain ambiguous, particularly in strongly overlapped regions of the ^1H spectrum, such as in the 3–5 ppm region occupied by various sugars. For their assignment correlations to heteroatoms (carbon, nitrogen) may be needed. HSQC (Heteronuclear Single Quantum Coherence) and HMBC (Heteronuclear Multiple-Bond Correlation) are the most frequently used heteronuclear correlated experiments. They correlate in most cases ^1H with ^{13}C *via* scalar coupling interaction between the both nuclei. The efficiency of the magnetisation transfer depend on the value of coupling constants, value of which is variable. For example, the one bond coupling constant $^1J_{\text{CH}}$ of the methyl group in alanine and lactate is about 127 Hz, whereas for the methine group it is *ca.* 145 Hz. Anomeric sugar CH have $^1J_{\text{CH}}$ of around 160–170 Hz depending on the α or β configuration, and aromatic CH protons typically have $^1J_{\text{CH}}$ in the range of 180 Hz and higher. Thus, in a single HSQC experiment, the transfer efficiency cannot be optimised for all groups simultaneously. Thus, two separate experiments, each with a $^1J_{\text{CH}}$ setting optimised for aliphatic or aromatic/anomeric protons, are usually needed. In addition, a spectral editing in HSQC spectra is possible on the basis of the different evolution of $^1J_{\text{CH}}$, $^1J_{\text{CH}_2}$, a $^1J_{\text{CH}_3}$ couplings which allow a discrimination between CH/CH₃ (positive) and CH₂ (negative) signals. In the HMBC experiment cross-peaks due to long range interactions between protons and hetero nuclei are detected.

Many more information dealing with the best strategy for structure elucidation is available in numerous textbooks and reviews. As an example Fan and Lane (2007) should be mentioned.

2.4 *Bacillus*, a Bacterial Genus Present in Clouds

2.4.1 Generalities

2.4.1.1 *Bacillus* Genus

As shown in Amato *et al.* (2005), *Bacillus* strains have been frequently isolated from cloud water. This chapter will give thus some information about properties and metabolism of this genus.

The term “aerobic endospore-forming bacteria” is used to embrace *Bacillus* species and related genera, for which the production of resistant endospores in the presence of oxygen remains the defining feature. They are also expected to possess Gram-positive cell wall structures (but staining reactions, even in young cultures, may be Gram-variable or frankly Gram-negative), and may be aerobic or facultative anaerobic. These features formed part of the definition of this group for many years, but some exceptions have emerged. *Bacillus infernus* and *Bacillus arseniciselenatis* are strictly anaerobic, and spores have not been detected in *Bacillus infernus*, *Bacillus subterraneus*, and *Bacillus thermoamylovorans*.

Representatives of this genus are widely distributed in the soil, air, and water where they are involved in a range of chemical biotransformations. Although primarily saprophytes, at least one species (*Bacillus schlegelii*), show facultatively chemolithotrophic activity in O₂/CO₂/H₂ or O₂/CO₂ atmosphere (Schenk and Aragno, 1979), and two species (*Bacillus macerans* and *Bacillus polymyxa*) are able to fix nitrogen (Hino and Wilson, 1958; Witz *et al.*, 1967). The ability of certain strains to tolerate high or low temperature and pH has made them a particularly important

source of commercial enzymes (Norris *et al.*, 1981). The metabolic diversity of *Bacillus* sp., together with reported low incidence of pathogenicity, has led to representatives of this group being used in wide range of industrial processes. Nowadays, strains of *Bacillus* are used for the production of four main types of product: *i*) enzymes, *ii*) antibiotics, *iii*) fine biochemicals including flavor enhancers and food supplements, *iv*) insecticides (Schallmey *et al.*, 2004).

2.4.1.2 Carbon Catabolism in *Bacillus* Species

Limitation of the carbon source in clouds and other extreme environments is a potential reason why in bacteria efficient and competitive ways of its utilisation has been developed. Pathways of carbon source utilisation in *Bacilli* and the different aspects of their regulation have been the subjects of several reviews (Sauer and Eihmanns, 2005; Fisher and Sonenshein, 1991; Holmberg *et al.*, 1990; Priest, 1977). An exhaustive description of the mechanisms of carbon catabolism and its regulation in *Bacillus* species was made by Stülke and Hillen (2000).

2.4.2 Microbial Exopolymeric Substances

2.4.2.1 Generalities

Initially, the term EPS was used to describe extracellular polysaccharides (PS), but recent studies showed that polymeric matrices have complex structures (lipopolysaccharides, glycolipids, lipids, proteins, nucleic acids) (Decho, 2000). Microorganisms convert very efficiently nutrients in exopolymeric substances (EPS) to create a stock of carbon and energy. They can optimise their utilisation also in the case when xenobiotic compounds are provided as a sole carbon source (Wolfaardt *et al.*, 1994).

The ability to synthesise and excrete exopolysaccharides (PS) through rigid cell wall or membrane is widespread among microorganisms (Sutherland, 2004). In some cases, it is a feature of all isolates of a given genus and PS are synthesised virtually under all conditions. In spite of very few data reporting the PS production by *Bacillus* species, large number of microorganisms is able to produce them (Dedonder, 1966; Han and Clarke, 1990). Production of PS results from specific nutritional conditions or as a response on the stress. In some prokaryote it may also result from development or morphological changes. In Gram-positive genera of *Bacillus* and *Clostridium* it may be due to the sporulation. PS may be firmly attached to a microbial surface or it might be excreted into the incubation medium as an amorphous mass of slime, which can be separated from bacteria by centrifugation. Many of bacterial strains might have the capacity to produce simultaneously more than one type of PS. The chemical composition of PS depends on the genetics of the microbial cells and the physico-chemical environment, in which a biofilm matrix develops (Sutherland, 2001). Generally, monosaccharide composition and structure of PS are independent on the carbon substrate available. Some bacterial genera seldom yield PS producing species. Only few Gram-positive *Bacillus* species produce PS.

Microbial PS are multifunctional and can be divided into intracellular polysaccharides, structural and extracellular PS. They are produced by both prokaryotes (eubacteria and archaeobacteria) and eukaryotes (phytoplankton, fungi, and algae). The bacterial PS represent a wide range of chemical structures. Chemically, they may have homo- or heteropolymeric composition of high molecular weight (10 to 30 kDa). They have newfangled applications due to the unique properties they possess. Owing to this, exopolysaccharides have found various applications in the food, pharmaceutical, and other industries (Kumar *et al.*, 2007). They also play a role in the environmental field as biofloculants for instance (Suh *et al.*, 1997)

2.4.2.2 Polysaccharides

The production of PS by many *Bacillus* strains was described, however, in most cases their structural analysis is based on chemical analyses (sugar compositional analysis and methylation analysis) with simple or no NMR description. The following paragraph is focused on some examples of EPS structure elucidation employing various analytical techniques, including NMR structural analysis.

Bacillus circulans belongs to bacteria which yield a number of EPS synthesised isolates:

- Fontaine *et al.* (1991) reported the isolation and structure determination of PS produced by *Bacillus circulans* with a backbone composed of 1,4-linked galactose (Gal) alternated by two 1,4-linked glucose (Glc) units, all in α -configuration. The lateral chain, linked to galactose unit of the backbone at C3, was composed of β -D-Glcp(1 \rightarrow 4) β -D-Man(1 \rightarrow 4) β -D-Galp- with pyruvate linked to terminal Glc unit.

- PS produced by *Bacillus circulans* reported by Isobe *et al.* (1997) was composed of β -L-rhamnopyranose (Rha), α -D-mannopyranose (Man), α -D-Gal, and α -D-glucopyranuronic acid (GlcA).
- *Bacillus circulans* st. AM7 isolated from soil produced two novel cyclic oligosaccharides during the incubation on amylose: cyclomaltopenta- and cyclomaltohexaose, in which 1,4-linked α -Glc were closed to the ring by α -1,6-linkage (Watanabe *et al.*, 2006).

Lee *et al.* (1997) optimised the fermentation condition for production of exopolysaccharide by *Bacillus polymyxa*. Its monomer composition made by TLC and HPLC after hydrolysis of the polymer, showed the presence of Glc, Gal, Man, GlcA, and fucose (Fuc). Pfiffner *et al.* (1986) isolated PS produced on sucrose under anaerobic conditions by encapsulated Gram-positive microorganism similar to *Bacillus* species. Purified polymer contained Man, Glc, arabinose (Ara), ribose (Rib), allose (All) and glucosamine (Glc-NH₂). Muhammadi (2007, 2008) showed that in two strains of *Bacillus* (strain CMG1447 and CMG1403) produced acidic hetero-PS slightly differing in sugar: Gal and Man (~92%) and GlcA (~8%). The only difference between them was the presence of a small quantity of Rha in PS produced by CGM1447 strain. Optimal conditions (C/N ratio of the growth medium, pH, and temperature) for EPS production by *Bacillus megaterium*, isolated from the infected plant leaf of *Aralia* species, were determined by Gandhi *et al.* (1997).

Many *Bacillus* strains have been described to produce bioflocculants, a specific class of EPS produced by microorganisms during their growth. Synthetic flocculants are widely used in industrial fields (*e.g.* for water purification) but they have been shown to be harmful to the environment as a dangerous source of pollution. On the contrary, bioflocculants produced by microorganisms are expected to be eco-friendly because of their biodegradability.

Main *Bacillus* bioflocculant structural features are summarised here and

in Table 7

- Bioflocculant DP-152 (molecular weight of $>2 \times 10^3$ kDa) produced by *Bacillus* sp. DP-152 contained Glc, Man, Gal, and Fuc in a molar ratio of 8:4:2:1 (Suh *et al.*, 1997).
- *Bacillus* sp. F19 produced the bioflocculant named MBFF19. Its chemical analyses of the indicated that it was a sugar-protein derivative, composed of neutral sugar (3.6%, w/w), uronic acid (37.0%, w/w), amino sugars (0.5%, w/w), and protein (16.4%, w/w). The two neutral sugar components were Man and Glc in the molar ratio 6:5 (Zheng *et al.*, 2008).
- *Bacillus megaterium* TF10 isolated from soil, produced bioflocculant, study of which was performed by Yuan *et al.* (2011). The relationship between structure constituents and their flocculation capacity have been shown. Their results suggested that the bridging mechanism is responsible for the flocculation. GC-MS and NMR analyses demonstrated that the PS had long chain composed of Rha, Glc, and Gal with the side chain containing uronic acids, acetyl amino sugars, and the proteins.
- *Bacillus thermantarcticus* produced two PSs with typical mucous character. PS1 repeating unit was constituted of four differently linked α -Man and three β -Glc units with a structure close to some xantan polymers. PS2 with molecular weight 3 kDa was found to be a mannan with traces of pyruvic acid with repeating unit composed of four differently linked α -Man units (Nicolaus *et al.*, 2004).
- *Bacillus licheniformis* ATCC 9945 was studied by NMR in combination with chemical modification and degradation of the polymer. Results showed that the backbone consists of a pyruvylated pentasaccharide repeating units with the structure $\{\rightarrow 3\}\text{-}[(S)\text{Py}\text{-}(3,4)\text{-}\beta\text{-D}\text{-Galp}\text{-}(1\rightarrow 6)]\text{-}\alpha\text{-D}\text{-Glc}\text{pNAc}\text{-}(1\rightarrow 3)\text{-}\beta\text{-D}\text{-Bacp}2\text{N}4\text{NAc}\text{-}(1\rightarrow 3)\text{-}[(S)\text{Py}\text{-}(3,4)\text{-}\beta\text{-D}\text{-Galp}\text{-}(1\rightarrow 6)]\text{-}\beta\text{-D}\text{-GalpNAc}\text{-}(1\rightarrow)\text{n}$,

where Bacp2N4NAc means *N*-acetylbacillosamine (4-acetamido-2-amino-2,4,6-trideoxy-*D*-glucose), Py (pyruvate) (Schäffer *et al.*, 2001).

- *Bacillus licheniformis* of marine origin produced PS which was characterised by Singh *et al.* (2011). Advanced analytical methods such as MALDI-TOF-TOF MS, FT-IR, UV-VIS, and NMR spectroscopy revealed its complex and heterogeneous nature of PS with high thermal stability (330 °C) with the monosaccharidic composition Gal, Glc, Man, and Ara.
- *Bacillus licheniformis* isolated from bottled spoiled cider produced in Normandy, was found to be composed of Man (80% of total polysaccharide) and Glc (6.5%) (based on HPLC and NMR analysis) (Larpin *et al.*, 2002).
- *Bacillus licheniformis* from a shallow marine hot spring of Vulcano Island, Italy was isolated by Arena *et al.* (2006) showed antiviral and immunomodulatory effects. A tetrasaccharide composed of Man units was found as a repeating unit of this PS.
- Curdlan is a water-insoluble homopolysaccharide composed exclusively of β -1,3-linked glucose residues. Most of the reports on curdlan production are on *Agrobacterium* and *Alcaligenes* sp. However, Gummadi and Kumar (2005) reported for the first time that Gram-positive bacterium *Bacillus* sp. SNC 107 is also capable to produce extracellular curdlan in appreciable amounts.
- Corsaro *et al.* (1999) studied a PS produced by an alkaliphilic bacterium closely related to *Bacillus* sp. by NMR, MS, and chemical analysis. The PS contained *D*-galacturonic acid (GalA), 2,4-diacetamido-2,4,6-trideoxy-*D*-glucose (QuiNAc4NAc), 2-acetamido-2-deoxy-*D*-mannuronic acid (ManNAcA) and one uncommon unit of *D*-GalA with the carboxyl group amide-linked to glycine [GalA(Gly)]. The following linear structure of the repeating unit with

saccharides in pyranose form was established: [\rightarrow 3)- α -D-GalA(Gly)-
(1 \rightarrow 4)- β -D-ManNAcA-(1 \rightarrow 4)- α -D-GalA-(1 \rightarrow 3)- α -D-QuiNAc4NAc-(1 \rightarrow)]_n.

- *Bacillus subtilis* bioflocculant PS was described recently by Patil *et al.* (2009). Bacterium was incubated in the media composed of different carbohydrates. The best bioflocculant production was observed on Glc and sucrose (Suc). The PS was found to be composed of Glc, Gal, and fructose (Fru). Chemical analysis showed that sugar/protein ratio was 94:6.
- Production of bioflocculants composed of γ -glutamic acid by a strain of *Bacillus subtilis* has been described (Yokoi *et al.*, 1996).

Table 7. The summary of the published EPS structure studies.

Bacterium studied	Medium / carbon source	Analytical methods used	Structure of EPS / structure of repeating unit	References
<i>Bacillus circulans</i>	Medium for fermentor user (crushed potatoes, CaCO ₃ , bio-soyase)	Methylation analysis, MS, ¹ H and ¹³ C NMR	1,4-linked α -Gal unit alternated by two 1,4-linked α -Glc units	Fontaine <i>et al.</i> , 1991
<i>Bacillus circulans</i>	Glucose , asparagin, yeast extract	Sugar analysis, methylation analysis	β -L-Rhap, α -D-Manp, α -D-Galp, α -D-GlcA	Isobe <i>et al.</i> , 1997
<i>Bacillus circulans</i> st AM7	Amylose	TLC, ¹³ C NMR, MS	cyclomaltopenta- and cyclomaltohexaose, with 1,4-linked α -Glc closed to the ring by α -1,6-linkage	Watanabe <i>et al.</i> , 2006
<i>Bacillus megaterium</i>	Glucose, fructose, sucrose, lactose, maltose		No data about structure; only optimisation of EPS production	Gandhi <i>et al.</i> , 1997
<i>Bacillus polymyxa</i> 8648P	KCTC Sucrose , yeast peptone extract,	TLC, HPLC	Gal, GlcA, Man, Fuc	Lee <i>et al.</i> , 1997
Genus similar to <i>Bacillus</i>	Various monosaccharides (glucose, fructose, mannose, cellobiose, maltose, sucrose, arabinose, mannitol, starch, galactose, xylose)	FT-IR, GLC	Man, Glc, Ara, Rib, All, GlcNH ₂	Pfiffner <i>et al.</i> , 1986
<i>Bacillus</i> CMG1447	No data	TLC	91.83% of hexoses as neutral sugars and 8.16% uronic acids	Muhammadi <i>et al.</i> , 2007

Table 7. cont`d

Bacterium studied	Medium / carbon source	Analytical methods used	Structure of EPS / structure of repeating unit	References
<i>Bacillus</i> CMG1403	No data	No data	92.13% hexoses as neutral sugars and 7.86% uronic acids	Muhammadi <i>et al.</i> , 2008
<i>Bacillus</i> sp DP-152	Glucose , yeast extract, soytone, tryptone	FT-IR, GPC	Glc:Man:Gal:Fuc = 8:4:2:1	Suh <i>et al.</i> , 1997
<i>Bacillus</i> sp. F19	Glucose , yeast extract	Chemical analysis, FT-IR	Man:Glc = 6:5, amino sugars, proteins	Zheng <i>et al.</i> , 2008
<i>Bacillus megaterium</i> TF10	No data	GC-MS, NMR	Rha, Glc, Gal, uronic acids, acetyl aminosugars, proteins	Yuan <i>et al.</i> , 2011
<i>Bacillus thermantarcticus</i>	Glucose , yeast extract	GC-MS, NMR	EPS1) α -D-Man and three β -D-Glc structure of which is close to some xantan polymers EPS2) mannan with traces of pyruvic acid with repeating unit composed of four differently linked α -D-Man	Nicolaus <i>et al.</i> , 2004
<i>Bacillus licheniformis</i>	Glucose	Antiviral activity determination and immunomodulatory tests	tetrasaccharide with manno-pyranosidic configuration	Arena <i>et al.</i> , 2006
Bacterium closely related to <i>Bacillus</i> spp	No data	NMR , MS, chemical analysis	\rightarrow 3)- α -D-GalpA(Gly)-(1 \rightarrow 4)- β -D-ManpNAcA-(1 \rightarrow 4)- α -D-GalpA-(1 \rightarrow 3)- α -D-QuipNAc4NAc-(1 \rightarrow	Corsaro <i>et al.</i> , 1999

Table 7. cont`d

Bacterium studied	Medium / carbon source	Analytical method used	Structure of EPS / structure of repeating unit	References
<i>Bacillus licheniformis</i>	Man, Rogosa, and Sharpe medium (MRS medium)	TLC, HPLC, ¹³ C NMR	Heteropolymer: 80.5% Man, 6.5% Glc, 13% unidentified	Larpin <i>et al.</i> , 2002
<i>Bacillus licheniformis</i> ATCC 9945	Sauton's medium	Chemical analysis, electron microscopy, NMR	{→3}-[(S)Py-(3,4)-β-D-Galp-(1→6)]-α-D-GlcpNAc-(1→3)-β-D-Bacp2N4NAc-(1→3)-[(S)Py-(3,4)-β-D-Galp-(1→6)]-β-D-GalpNAc-(1→)} _n	Schäffer <i>et al.</i> , 2001
<i>Bacillus</i> sp. SNC07	Sucrose, peptone, yeast extract	Chemical analysis, HPLC	Curdlan (or β-1,3-glucan)	Gummadi and Kumar, 2005

Gal - galactose; Glc - glucose; GlcA - glucuronic acid; Fuc - fucose; Ara - arabinose; Rib - ribose; All - allose; GlcNH₂ - glucosamine; Galp - galactopyranose; Rhap - rhamnopyranose; Manp - mannopyranose; QuipNAc4NAc - 2,4-diacetamido-2,4,6-trideoxy-D-glucopyranose; Bacp2N4NAc - N-acetylbaicillosamine; Py - pyruvate

2.4.2.3 Polyhydroxyalkanoates

Exopolymeric substances produced by microorganisms are not limited only to polysaccharides. Many bacterial strains produce polyhydroxyalkanoates (PHA). PHA are polyesters of 3-, 4-, 5-, and 6-hydroxyalkanoic acids produced by a variety of bacterial species when their growth is restricted by an essential nutrient (as nitrogen, phosphorus, sulphur, magnesium, potassium, or iron) and if a carbon source is available in excess (Anderson and Dawes, 1990; Oeding and Schlegel, 1973; Steinbüchel and Schlegel, 1989). These water insoluble storage polymers are biodegradable, exhibit thermoplastic properties and can be produced from renewable sources. PHAs serve as energy and they are stored as granular inclusion bodies in the cytoplasm of many bacteria and archaea (Lemoigne, 1926; Anderson and Dawes, 1990; Steinbüchel, 1991). Accumulation of intracellular storage polymers has been considered as a strategy used by bacteria to increase survival in a changing environment. PHA comprises a vast number of structures with repeating subunits of a general formula $-\text{[O-CH(R)-(CH}_2\text{)}_x\text{-C(O)]-}$ and molecular masses 0.1 to 30 kDa.

The first identified PHA was polyhydroxybutyrate (PHB) from *Bacillus megaterium* (Lemoigne, 1926). Until now a great number of many other different constituents have been described (Steinbüchel and Valentin, 1995), however, PHB occurs as an inclusion surrounded by a phospholipid monolayer. Proteins, referred as a “granulate-associated proteins”, are associated with the membrane and located only at the surface of the PHB granules (Hocking *et al.*, 1994; Huisman *et al.*, 1991; Steinbüchel *et al.*, 1995). Recent studies have shown that the polymer is in fluid state inside the cell and crystallises to form granules when is released from the cells (Kawaguchi and Doi, 1990).

In a screening study made by Valappil *et al.* (2007), *Bacillus cereus* was identified as the best PHAs producing strain using various carbohydrate

substrates (Glc, Fru, Suc, various fatty acids, and gluconate).

2.4.2.4 Putative Importance of EPS for Atmospheric Environment

The production of EPS has a specific importance for a microorganism living in the atmospheric environment because it is closely related to the ability of microorganisms to form biofilms. Biofilms are generally formed by bacteria, which are intimately attached to some surface and aggregate into a heterogeneous hydrated polymeric matrix themselves synthesised. Formation of biofilms seems to be the typical way how bacterial cells grow in nature. Each microcolony in the biofilm is surrounded by extracellular polymeric substances (EPS) (predominantly exopolysaccharides, DNA, and proteins). EPS layer exhibits secondary, sometimes even tertiary structure (Chapell and Evangelou, 2002). There are more reasons for biofilm formation: First, biofilms are important for bacterial self-defence and survival of cells (chance to resist to physical and chemical external stresses are higher for cells in biofilms). Second, biofilm formation allows cells to remain in a favourable niche (bacterial cells are fixed in location where nutrients are more abundant or are constantly replenished). Third, biofilms allows bacteria to live in close association with other ones and thus their survival chances are increased. Moreover, when cells are in close proximity to each other, opportunities for genetic exchanges are more available (Madigan *et al.*, 2009). Also, the presence of a gelled PS layer around the cell may have paramount effects on the diffusion properties, both into and out of the cell (Dudman, 1977). For instance, cells buried within a polymer matrix would be inaccessible to antibiotics. The ability of a microorganism to surround itself with a highly hydrated exopolysaccharide layer may provide it with protection against desiccation and predation by protozoa. The environmental conditions are the most important factor influencing the physico-chemical

properties of biofilms (such as porosity, density, water content, charge, sorption and ion exchange properties, hydrophobicity, and mechanical stability).

If we now consider the atmosphere, the production of EPS by microorganisms present in this environment could be of special interest. First, this structure surrounding the cells could protect them against desiccation, UV exposure, $\cdot\text{OH}$ and $\text{NO}_3\cdot$ radicals, *etc.* and could help them to survive in this extreme environment. Second, such functionalised and very hydroscopic structures could influence the formation of CCN by modification of the bacterial cells surface bioaerosols. Finally, EPS could be released in the atmosphere and could be thus considered as SOAs.

3

THESIS OBJECTIVES

The objective of this thesis is to continue and deepen the knowledge about the metabolism of bacteria isolated from clouds. We will try to understand their possible role in atmospheric processes and their ability to survive in such stressing environment.

The work will be mainly focused on the strain *Bacillus* sp. 3B6 as it was shown to be very active and also because *Bacillus* genus is frequently present in cloud water.

In order to achieve this goal the following strategy was chosen:

1. Study of the carbohydrate metabolism of *Bacillus* sp. 3B6

In situ NMR spectroscopy was chosen as a main tool to monitor the biodegradation kinetics of various saccharides and to identify the major metabolites.

A large range of carbohydrates have been chosen for the study: Different saccharides (aldoses, ketoses) covering penta- and hexasaccharides in acyclic and cyclic forms as well as oligo- and polysaccharides. Obtained

results will give on one side an extensive overview of the metabolic abilities of this strain, which is unknown. On the other side, they will have an impact on the knowledge about cloud chemistry because some of these compounds are present in the atmosphere.

A special attention will be paid to the synthesis of EPSs as it is well known that these polymers are essential for bacteria living in a changing environment. In the specific context of atmospheric environment, where this strain has been isolated, the synthesis of such substances could have consequences on the survival of the strain and on precipitation formation.

2. Study of the metabolism of formaldehyde and methanol by *Bacillus* sp. 3B6 and other typical strains present in cloud waters

Formaldehyde and methanol have been chosen as they represent major atmospheric VOCs and because virtually no results are reported about their biodegradability in clouds.

Their biodegradation rates will be measured by *in situ* and *in vivo* NMR using both unlabelled and ^{13}C labelled compounds. Obtained data on their microbiological transformations will be then compared with those due to radical chemistry processes.

Different scenarios will be used by taking into account the temperature changes (winter/summer), the degree of pollution, and the day/night alternance. The idea is to evaluate quantitatively the relative importance of biodegradation processes compared to radical processes, even though the chosen experimental conditions here are still too far from real cloud environment.

The long term goal, which is beyond the scope of the proposed project, is the utilisation of knowledge about metabolic pathways and kinetic constants in atmospheric chemistry numeric models (collaboration with

physicists of the atmosphere in Clermont-Ferrand, France). These models will be the only way to simulate realistic clouds and thus really know whether microorganisms play a role in atmospheric chemistry. Also any data concerning the formation of compounds that would impact on the microphysics of clouds will be taken into account by our colleagues from atmospheric sciences.

4 RESULTS

4.1 NMR Study of Carbohydrate Metabolism

4.1.1 Introduction

Bacillus genus is frequently present in the atmosphere. For *Bacillus* strains isolated from cloud water no detailed study of their carbohydrate metabolism have been reported to date. We focussed our attention on the strain *Bacillus* sp. 3B6 as it has been shown to be very active towards the degradation of pollutants (Durand *et al.*, 2006; Amato *et al.*, 2007) and also because its behaviour during cultivation suggested the synthesis of polysaccharides. The understanding of its metabolism on carbohydrate is important to explain how it can survive in the atmosphere where such substrates might be present, and the identification of metabolites might be of interest for atmospheric chemistry and microphysics. In addition, some metabolites can be of interest for industrial purposes as it is well known that these bacterial species can produce various polysaccharides for food, cosmetic, nutraceutical, and environmental applications. The study of carbohydrates utilisation as a carbon source by this bacterium is

divided into two parts:

- The first part deals with an extensive study of sucrose metabolism that gave rise to a publication in *Carbohydrate Research* (see Chapter 4.1.2) focussed on the polysaccharide (PS) production: This study describes a compositional analysis of the incubation medium and individual fractions issued from its separation by GPC.
- The second part is an extended investigation of the metabolism of carbohydrates focused on their ability to produce exopolymeric substances (EPSs) mainly polysaccharides (PS). The manuscript is planned to be submitted to *Carbohydrate Polymers* (see Chapter 4.1.3). This study starts with a screening of the ability of this bacterium to metabolise different saccharides. Then the production of EPSs is examined when the bacterium is incubated with low concentration of substrates. Finally, the case study of the incubation with high concentration of glucose is reported.

4.1.2 NMR Structural Study of Fructans Produced by *Bacillus* sp. 3B6, Bacterium Isolated in Cloud Water.



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NMR structural study of fructans produced by *Bacillus* sp. 3B6, bacterium isolated in cloud water

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ABSTRACT

Bacillus sp. 3B6, bacterium isolated from cloud water, was incubated on sucrose for exopolysaccharide production. Dialysis of the obtained mixture (MWCO 500) afforded dialyzate (DIM) and retentate (RIM). Both were separated by size exclusion chromatography. RIM afforded eight fractions: levan exopolysaccharide (EPS), fructooligosaccharides (FOSs) of levan and inulin types with different degrees of polymerization (dp 2–7) and monosaccharides fructose:glucose = 9:1. Levan was composed of two components with molecular mass ~3500 and ~100 kDa in the ratio 2.3:1. Disaccharide fraction contained difructose anhydride DFA IV. 1-Kestose, 6-kestose, and neokestose were identified as trisaccharides in the ratio 2:1:3. Fractions with dp 4–7 were mixtures of FOSs of levan (2,6-βFruf) and inulin (1,2-βFruf) type. DIM separation afforded two dominant fractions: monosaccharides with fructose:glucose ratio 1:3; disaccharide fraction contained sucrose only. DIM trisaccharide fraction contained 1-kestose, 6-kestose, and neokestose in the ratio 1.5:1:2, penta and hexasaccharide fractions contained FOSs of levan type (2,6-βFruf) containing α-glucose. In the pentasaccharide fraction also the presence of a homopentasaccharide composed of 2,6-linked βFruf units only was identified. Nystose, inulin (1,2-βFruf) type, was identified as DIM tetrasaccharide. Identification of levan 2,6-βFruf and inulin 1,2-βFruf type oligosaccharides in the incubation medium suggests both levansucrase and inulosucrase enzymes activity in *Bacillus* sp. 3B6.

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1. Introduction

Microbial polysaccharides offer a potential new source of functional biopolymers for food, industrial, cosmetic or medical applications. Depending on the carbon source used they could be homopolymers (composed of one type of monosaccharide) or heteropolymers. Sucrose is the substrate for bacterial production of fructans. Levan and inulin are fructans composed of 2,6-linked β-fructofuranoses (2,6-βFruf) and 1,2-linked β-fructofuranoses (1,2-βFruf), respectively. Strains producing fructans can be found in many genera of bacteria.¹ Fructans produced by bacteria are mostly of levan type and they are synthesized from sucrose by levansucrase^{2,3} and only few bacterial species are equipped by inulosucrase and excrete inulin.^{4,5} In *Lactobacillus reuteri* 121 both, levansucrase and inulosucrase were identified.⁶ Levan producing capacity can be limited to a few strains of a particular bacterial species.⁷ Besides levan polymer some bacterial levansucrases are able produce also fructooligosaccharides (FOSs) with only few βFruf units linked to the glucose molecule.^{8–11} Depending on the

type of linkage between βFruf units FOSs of levan type (2,6-βFOSs), inulin type (1,2-βFOSs) or mixed type may be formed. Low molecular mass FOSs have a sweet taste, while those of higher molecular weight have a neutral one. They form emulsions, and due to their properties to increase foam stability and a fat-like texture which improves organoleptic characteristic, they are designated for their application in food industry.^{12,13} Due to the absence of enzymes that are able to degrade fructans in the upper human digestive tract, fructans and FOSs make a part of non digestible fibers and they act as prebiotics. In the digestive system they serve as a carbon source for colon bacteria, such as lactobacilli and bifidobacteria, which are beneficial to human health. In this way fructans help to modify the composition of the colon flora by stimulating bacterial growth and/or activity. Besides their function as dietary fibers, many other properties are ascribed to fructans. For example, prevention of colon cancer due to stimulation of mineral absorption from the colon or immune system stimulation.^{14,15} Linkage type and molecular mass of fructan polysaccharide and/or FOSs depends on enzymes involved in their synthesis, sucrose concentration, and composition of the incubation medium. Diffructose anhydrides (DFAs), cyclic disaccharides composed of two fructose residues, also belong to FOSs. Depending on the type of linkage between two βFruf units four types of DFA were described: DFA I, α-D-Fruf

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- β -D-Fruf-2',1:2,1'-dianhydride; DFA III, α -D-Fruf- β -D-Fruf-2',1:2,3-dianhydride; DFA IV, β -D-Fruf- β -D-Fruf-2',6:2,6'-dianhydride and DFA V, α -D-Fruf- β -D-Fruf-2',6:2,1'-dianhydride. Production of DFA has been observed for different strains of *Arthrobacter*, *Pseudomonas*, *Aspergillus fumigatus*, *Streptomyces* sp. MCI-2524 and *Bacillus* sp. Snu-7 when they were grown on inulin or levan as substrates. Enzymes involved in their synthesis are levan/inulin depolymerising fructosyltransferases.^{16–19} DFA due to their unique structures and properties are expected to have novel physiological functions. It was already shown that they increase mineral absorption (iron, calcium),^{20–23} They have beneficial effect on colon microbiota.¹² DFA has half of the sucrose sweetness, they have a stable structure and the fact that they are not hydrolyzed by invertase and inulinase strongly suggests its use in the food industry for dietary purposes as a low calorie sweetener.^{19,24}

Besides the importance of oligo- and polysaccharides produced by the microorganisms for their different applications in biotechnologies (particularly in nutraceutical, pharmaceutical and food industry), these molecules are well known to play a role in ecological niches. The precise role of EPS in EPS-producing bacteria is dependent on the natural environment of the microorganism. Most of functions ascribed to EPS are of a protective nature. Fructans were already shown to protect microbial cells against an abiotic and biotic stress, such as desiccation, freezing, antibiotics, or toxic compounds, and attacks of parasites and predators.^{13,25}

In this paper, we describe the production, purification, and detailed NMR structural identification of the exopolysaccharide levan and FOSs produced by *Bacillus* sp. 3B6 when it was grown on sucrose. As *Bacillus* sp. 3B6 has been isolated in cloud water²⁶ a discussion is given about the potential implication of EPS production in such an atmospheric environment.

2. Material and methods

2.1. Isolation and identification of the bacterial strain

Cloud samples were taken at Puy de Dôme summit (1465 m) in the Massif Central Region (France) as described by Amato et al.²⁶ Briefly, cloud droplets were collected with single-stage cloud collectors with a cut-off droplet diameter of 7 μ m. All material needed for sampling was previously sterilized by autoclaving. A sterile mask and gloves were worn during sample collection to avoid any contamination. Samples were kept in cold (4 °C) for a few hours until laboratory experiments. Microorganism cultures were made on solid nonselective media for bacteria already described²⁶ by application of 0.1 mL of 1 to 10⁻³ dilutions of cloud water. From these mixed cultures, pure strains were isolated from individual colonies on Petri dishes.

First, the bacterial strain was cultivated in Tryp-case-Soy broth medium, 27 °C, 200 rpm. Cells were collected after 24 h of incubation (centrifugation, pellet re-suspended in phosphate-buffered saline) for DNA extraction using Easy-DNA kit (InvitrogeneTM; Invitrogen, Cergy Pontoise, France). Amplification of the 16S rRNA gene was then performed by polymerase chain reaction (PCR), with about 100 ng of genomic DNA for 0.3 U of Taq DNA Polymerase (1.5 U; Qbiogene, Illkirch, France), and eubacterial universal primers (F8 5'-AGA GTT TGA TCM TGG CTC-3' and 1492r 5'-GNT ACC TTG TTA CGA CTT-3', where M corresponds to A or C and N to any of the four nucleotides). The PCR conditions were as follows: 94 °C for 5 min; 25 cycles of: 30 s at 94 °C (denaturation), 30 s at 55 °C (hybridization), and 15 min at 72 °C (elongation). It was terminated by a 7 min step at 72 °C. The PCR products were purified using Strataprep Purification kit (StratageneTM, Amsterdam, Netherlands) and finally freeze-dried. The entire 16S rRNA gene sequence was obtained by capillary electrophoresis using the previously described primers.

2.2. Incubation conditions

Growth conditions: *Bacillus* sp. 3B6 strain was grown in 100 mL portions of Tryp-case-Soy broth (bioMérieux, France) in 500 mL Erlenmeyer flasks incubated at 27 °C and 200 rpm for 24 h. Culture (500 mL) were centrifuged at 8000 \times g and 4 °C for 5 min. The bacterial pellet was twice washed with NaCl (ACROS) (8‰) and thereafter re-suspended in the medium for incubation with sucrose (ACROS).

2.3. Incubation with sucrose

2.3.1. Incubation procedure No. 1

Media for metabolism study were prepared by dissolution of sucrose in Volvic® mineral water. The final concentration 20 mmol L⁻¹ of saccharides and 10⁹ cell mL⁻¹ of microorganisms was used. *Bacillus* sp. 3B6 was grown in this medium for 5 days. Samples were taken in regular intervals (0, 15, 19, 24, 39, 48, 63, 72, 96, 120, 144 h). Extracellular incubation media were separated from cells by centrifugation (3 min, 12,000 \times g).

2.3.2. Incubation procedure No. 2

For EPS production a medium proposed by Gandhi et al.²⁷ was used. Its composition was as follows: sucrose (30 g L⁻¹) (ACROS), ammonium nitrate (0.4 g L⁻¹) (ACROS), potassium sulfate (1 g L⁻¹) (ACROS), sodium chloride (1 g L⁻¹) (ACROS), potassium dihydrogen phosphate (3 g L⁻¹) (FLUKA), calcium chloride (0.02 g L⁻¹) (SIGMA A.), magnesium sulfate (0.001 g L⁻¹) (SIGMA A.). Medium was autoclaved for 15 min at 121 °C. Old inoculum (24-h) was used to seed the sterile medium. The culture was incubated in a rotary shaker 200 rpm at 32 °C. After 8 days of incubation, the medium was centrifuged (20,000 \times g, 25 °C, 30 min) and *Bacillus* sp. 3B6 cells were separated from the supernatant.

2.4. Isolation of carbohydrates from culture medium and gel filtration chromatography

Total volume (1000 mL) of the incubation medium was reduced by rotatory evaporator (~200 mL) and subjected to dialysis for 3 days against distilled water (MWCO 500). Dialyzate (DIM) containing salts, low molecular mass saccharides and amino acids was treated by cation and anion exchangers and further subjected to size

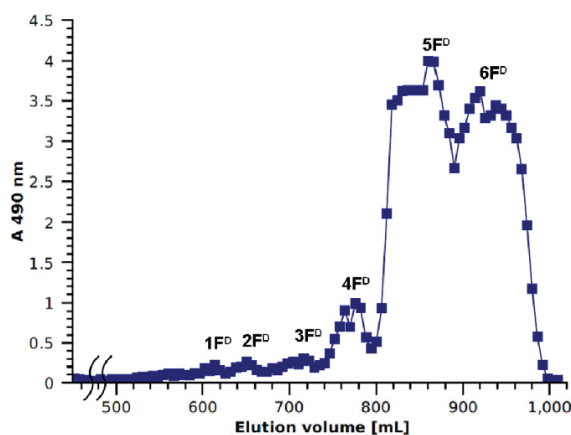


Figure 1. Elution profile on Bio-Gel P2 of the dialyzate (DIM) obtained after a dialysis of a complex incubation medium (incubation procedure No. 2) after 3 days dialysis against water (8 days of incubation of *Bacillus* sp. 3B6 on sucrose).

exclusion chromatography (SEC). SEC of DIM afforded major fractions: 6F^D containing fructose and glucose (1:10) and 5F^D (sucrose); fractions 4–1F^D contained traces of oligosaccharides (Fig. 1). Retentate represented retained incubation medium (RIM). Carbohydrates in RIM were recovered by precipitation with five volumes of acidified 96% ethanol and left overnight in a cold room. The precipitate was removed by centrifugation (5000×g, 25 °C, 10 min), dissolved in water and dialyzed another three days against distilled water. It was concentrated and freeze-dried to give light brown material of a crude carbohydrates mixture, which was separated into two parts. One part was directly analyzed by NMR and the second one was, after dissolving in water, separated on Bio-Gel P2 column (200 × 2.5 cm). Fractions (5 mL) were collected in which the carbohydrate content was checked according the procedure of Dubois et al., 1956.²⁸ Separation of RIM afforded eight fractions: monosaccharides 8F^R, oligosaccharides 7–2F^R and a polymeric residue 1F^R (Fig. 2).

2.5. NMR spectroscopy

NMR spectra were measured on 600 MHz VNMR5 Varian equipped with HCN ¹³C enhanced salt tolerant cold probe in D₂O at 25 °C using acetone as an external standard. Samples of oligosaccharides were freeze-dried from D₂O before dissolution in 99.98% D₂O. Shigemi NMR tubes or micro-capillaries were used in the case of small quantity of FOS samples. Advanced techniques from Varian pulse sequence library of 2D homo- and hetero-correlated spectroscopy (gCOSY, gTOCSY, gHSQCAD, gHMBCAD, gH2BC) including 1D sequences with selective excitations (1DNOESY, 1DTCOSY) were used for the signal assignments.

3. Results

3.1. Monitoring of sucrose metabolism by in situ ¹H NMR spectroscopy

Sucrose degradation by *Bacillus* sp. 3B6 was monitored by in situ ¹H NMR spectroscopy (spectra not shown) directly in samples of incubation media (Incubation procedure No. 1) taken at regular intervals without any treatment.²⁹ At time zero only sucrose ¹H NMR signals were present. After 15 h of incubation the first signals due to released metabolites appeared. Anomeric signals due to free glucose were observed at δ 5.234 ppm for αGlc and δ 4.647 ppm for βGlc (its H2 is present at δ 3.246 ppm) and a doublet

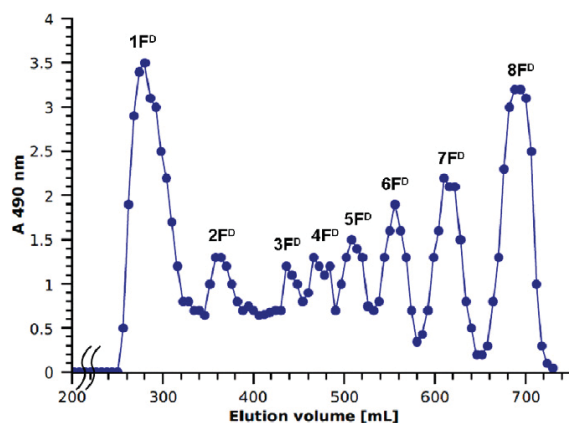


Figure 2. Elution profile on Bio-Gel P2 of the retentate (RIM) obtained after a dialysis of a complex incubation medium (incubation procedure No. 2) retained after 3 days dialysis against water (8 days of incubation of *Bacillus* sp. 3B6 on sucrose).

at δ 4.118 ppm with ³J_{H,H} coupling constant 3.5 Hz revealed the presence of Fru. In the course of the incubation, Glc signal intensities decreased and they disappeared after 24 h. ¹H NMR spectra of samples taken between 63 to 120 h suggested a complex mixtures of metabolites.

3.2. EPS production by *Bacillus* sp. 3B6

The complexity of the in situ ¹H NMR spectrum collected at the end of the incubation of *Bacillus* sp. 3B6 on sucrose (not shown) highly suggested a mixture of metabolites in which an EPS could be present. In order to produce the medium with higher concentration of metabolites for structural analyses, *Bacillus* sp. 3B6 was incubated during 8 days using the incubation procedure No. 2. After 8 days a complex incubation medium of very high viscosity was obtained. After dialysis, a retentate of the incubation medium (RIM) and dialyzate (DIM) were obtained. Analysis of ¹H, ¹³C, COSY and HSQC spectra (not shown) showed that RIM was a mixture of various compounds. In the anomeric region of the ¹H NMR spectrum the presence of three signals at δ 5.41, 5.39, and 5.17 ppm with small ³J_{H1,H2} coupling constants indicated α configuration of these sugars, most probably of glucose (Glc) origin. Their intensity was not in accordance with signal intensities of skeletal protons in the region δ 4.3–3.3. Comparison of RIM ¹H and HSQC NMR spectra with those due to sucrose and fructose showed the presence of a small quantity of sucrose, not used by the bacterium, fructose released after sucrose hydrolysis and other not identified signals of low intensities. However, dominant signals due to a new metabolite were broader indicating its high molecular mass. The HSQC spectrum revealed a huge amount of CH₂ signals suggesting the presence of a metabolite composed of fructose or fructose similar sugar units (not shown).

3.3. RIM separation into oligomeric fractions on Bio-Gel P2

RIM separation by size exclusion chromatography on Bio-Gel P2 afforded eight fractions that is, monosaccharides 8F^R, oligosaccharides fractions 7–2F^R and polymeric residue 1F^R. NMR analysis of the lowest molecular mass fraction 8F^R confirmed the presence of fructose and glucose in the ratio 9:1. The comparison of spectral patterns of ¹H NMR spectra indicated that fractions 1–6F^R belong to a coherent series of oligo- and poly- saccharides, while the one due to 7F^R was different. Figure 3 shows ¹H NMR spectra of RIM fractions 1F^R, 4–6F^R, and sucrose used as a standard.

3.4. FOSs identification in RIM

The ¹H NMR spectrum (Fig. 3) of high molecular mass fraction 1F^R showed in the region of skeletal protons (δ 4.25–3.10) seven signals due to βFruf units, which were easily assigned by COSY and HSQC spectra (Table 1). The ketoanomeric signal C2 due to βFruf appeared at δ 105.06 ppm, C1 and C6 signals were detected at δ 60.74 and 64.25 ppm, respectively. 2,6-Linkages between βFruf units were confirmed by C2/H6 cross peaks δ 64.25 of βFruf in the HMBC spectrum (not shown) and confirmed the structure of levan. The absence of the H1 signal due to α-glucose avoided a polymer molecular mass estimation from signal intensities in the ¹H NMR spectrum. By HPLC it was found that 1F^R has two components, one of molecular mass ~3500 and the other of ~100 kDa in the ratio 2.3:1, respectively. In the ¹H NMR spectra of penta- (4F^R) and tetrasaccharides (5F^R) (Fig. 3) some signals remained similar to those observed in the 1F^R suggesting thus the presence of oligomers of levan type (2,6-βFOSs). However, besides them, in ¹³C NMR spectra also diagnostic signals of inulin type FOSs with 1,2-linked βFruf could be detected; particularly the ketoanomeric C2 at δ 104.1, further C5 at δ 82.2, C6 at δ 63.0, and C1 at δ 61.5.^{30,31} Because the detailed analysis of 4F^R–6F^R

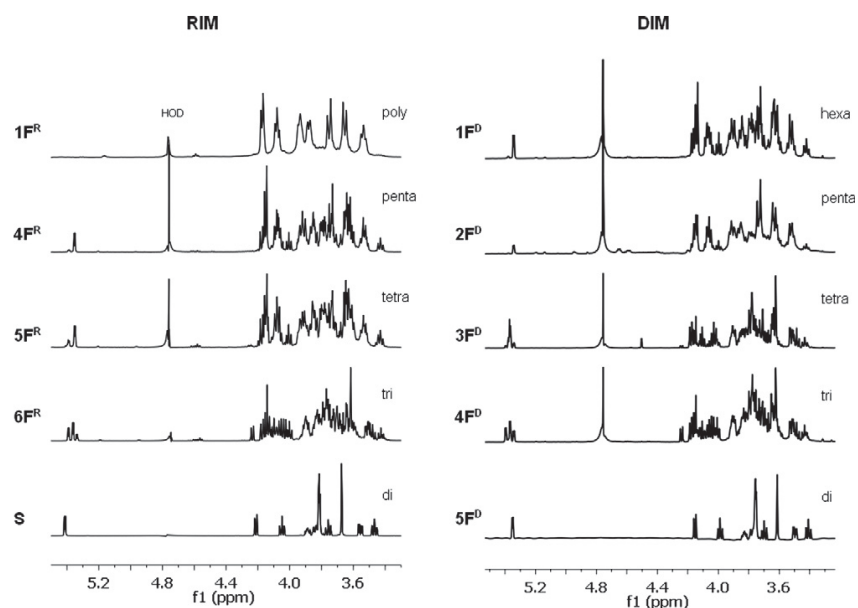


Figure 3. Series of ^1H NMR spectra of fractions obtained by separation on Bio-Gel P2 of retentate (RIM) and dialyzate (DIM). Fractions 1F^{R} and $4\text{--}6\text{F}^{\text{R}}$ from RIM, fractions $1\text{--}5\text{F}^{\text{D}}$ from DIM, di—disaccharide, tri—trisaccharide, tetra—tetrasaccharide, penta—pentasaccharide, hexa—hexasaccharide, poly—polymer, HOD—residual signal of water.

Table 1
 ^1H and ^{13}C NMR data of levan, inulin, DFA IV compared with α , β Fruf, and sucrose as standards

Compound	Fraction	Sugar unit		^1H and/or ^{13}C chemical shifts/ δ					
				1,1'	2	3	4	5	6,6'
Fructose	Standard	β Fruf	C	63.09	102.2	76.1	75.17	81.39	63.38
			C	61.81	105.2	82.02	76.6	82.68	63.63
Sucrose	Standard	α Glc(1 \rightarrow 2) β Fruf	H	5.403	3.547	3.748	3.459	3.831	3.847
			C	92.89	71.78	73.29	69.94	73.12	60.84
			H	3.666	—	4.205	4.039	3.877	3.805
			C	62.07	104.4	77.13	74.72	82.09	63.08
Levan	1F^{R}	2,6- β Fruf	H	3.77, 3.670	—	4.178	4.096	3.958	3.898, 3.549
			C	60.75	105.00	77.15	76.00	81.07	64.20
Inulin	^a	1,2- β Fruf	H	3.79, 3.69	—	4.19	4.030	3.85	3.80, 3.635
			C	60.57	104.10	76.68	74.04	81.39	62.36
DFA IV	7F^{R}	[-6] β Fruf(2-) ₂	H	3.747, 3.639	—	4.182	4.597	3.842	3.947, 3.640
			C	60.038	103.9	77.58	72.56	81.62	61.09

^a Taken from HSQC spectrum of the 2F^{D} fraction (Fig. 4).

proton spectra was not straightforward their 2D hetero-correlated HSQC spectra (Fig. 4) were examined. Characteristic levan type cross peaks were well separated in the spectrum of the levan polymer (1F^{R}). H5/C5 at δ 3.958/81.07, and H6,H6'/C6 at δ 3.898, 3.545/64.2 were used as levan type diagnostic cross peaks. Thus in the RIM spectra of lower molecular mass samples $4\text{--}6\text{F}^{\text{R}}$ (Fig. 4) cross peaks due to inulin type 1,2- β FOSS H5/C5 3.85/81.39, H6,H6'/C6 at δ ~3.80, 3.635/62.36, and H1,H1'/C1 at δ ~3.79, 3.69/60.57 could be easily recognized. They suggested the presence of nystose (α Glc-1,2- β Fruf-1,2- β Fruf-1,2- β Fruf) in the fraction 5F^{R} and in 4F^{R} a pentasaccharide (α Glc-1,2- β Fruf-[1,2- β Fruf]₂-1,2- β Fruf) of inulin type. Sucrose building block was confirmed in all oligosaccharides by the presence of α -Glc cross peaks. Very high molecular weight of the polymer (1F^{R}) may be the reason of their absence in the HSQC spectrum. Spectral pattern of the ^1H NMR spectrum of trisaccharide fraction 6F^{R} was the most complex (Fig. 3). It showed the presence of α -glucose unit signals at δ 5.415; 5.386, and 5.362 suggesting at least three different

trisaccharides in 6F^{R} . The spectral pattern was in strong accordance with the one published by Ammar et al.³² for a mixture of 1-kestose, 6-kestose, and neokestose (Fig. 5) in which proton signals of identical chemical shifts were assigned as follows: 5.415 to 1-kestose, 5.386 to neokestose, and 5.362 to 6-kestose. Chemical shifts of C2 ketoanomeric and C1 anomeric signals in the 6F^{R} in the ^{13}C NMR spectrum were found to be in accordance with the literature data for these trisaccharides.^{33,34} The ratio of 1-kestose, 6-kestose, and neokestose in the 6F^{R} fraction was 2:1:3, respectively. Ascending TLC afforded a qualitative proof of their presence in 6F^{R} .³⁵ 6F^{R} HSQC spectrum is shown in Figure 4.

3.5. FOSS identification in DIM

SEC of dialyzate (DIM) afforded two dominant fractions, 6F^{D} containing monosaccharides glucose and fructose (3:1) and a disaccharide fraction (5F^{D}) containing sucrose only. Fraction of trisaccharides

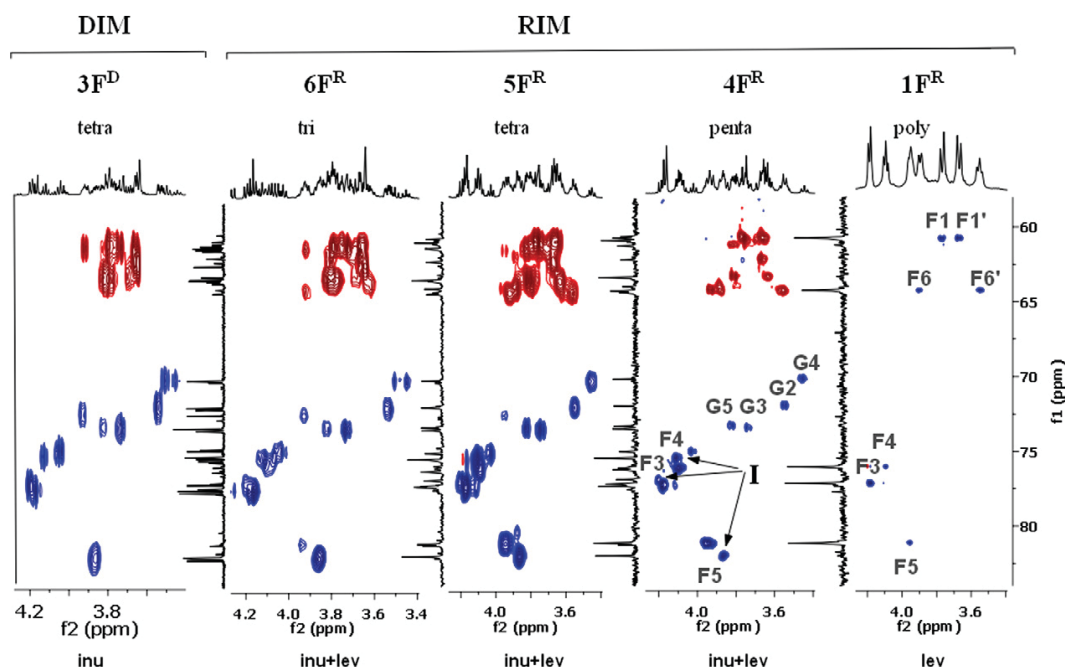


Figure 4. Part of HSQC NMR spectra (cross peaks of skeletal protons and carbons) of chosen fractions obtained by separation on Bio-Gel P2 of retentate (RIM) and dialyrate (DIM). $3F^D$ tetrasaccharide DIM fraction, $1F^R$ and $4-6F^R$ —polymer and tri-, tetra-, and pentasaccharides of RIM, inu—inulin type 1, 2- β FOs, lev—levan type 2,6- β FOs, S—sucrose, G—glucose, F— β -fructofuranose, I—cross peaks due to inulin type 1,2- β FOs.

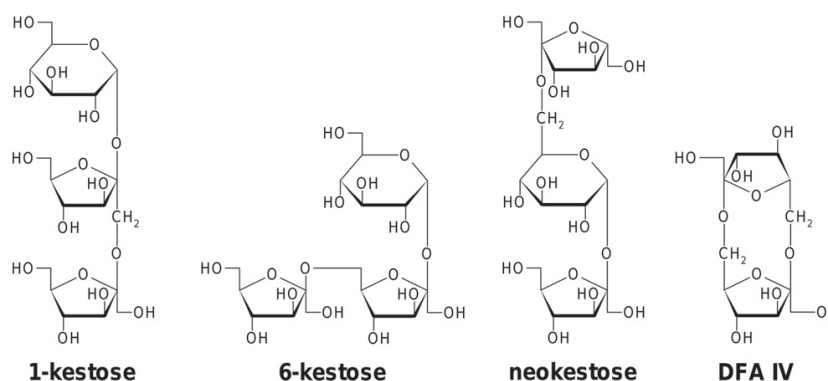


Figure 5. Structures of 1-kestose, 6-kestose, neokestose, and DFA IV.

$4F^D$ was identified as a mixture of 1-kestose, 6-kestose, and neokestose. In fractions 1- $3F^D$ mixtures of higher molecular mass oligosaccharides (dp 4–6) were present in trace amounts. DIM Elution profile on the Bio-Gel P2 column and 1H NMR spectra of individual fractions are shown in Figures 1 and 3, respectively.

Spectral pattern of 1H NMR spectra due to the levan polymer in $1F^R$ RIM was similar to those of $1F^D$ and $2F^D$ DIM fractions (Fig. 3) suggesting the levan type FOs with 2,6-linked Fruf residues in $1F^D$ and $2F^D$. However, in $2F^D$ the integral ratio of the H3 β Fruf signals at δ 4.20 and α -Glc was 9:1 suggesting a decasaccharide in this fraction. However, this conclusion was not in accordance with the molecular mass of this fraction suggested by elution profile (pentasaccharide) and the ratio of abovementioned signals in $1F^D$ fraction showing the presence of hexasaccharides there. These facts indicated that in $2F^D$ two types of pentasaccharides are present. In the first one four β Fruf units are linked to α -Glc (α Glc-[2,6-

β Fruf] $_4$), while the other is a homopentasaccharide composed of five 2,6-linked β Fruf units ([2,6- β Fruf] $_5$).

When signals in the 1H NMR spectra of $4F^D$ and $3F^D$ fractions were compared with NMR data of inulin type 1,2- β FOs published by Fukushi et al. (2000)³⁶ it was found that chemical shift of signals in the $3F^D$ spectrum are consistent with those of nystose. Spectral pattern of its HSQC spectrum (Fig. 4) confirmed inulin type structure of this tetrasaccharide showing characteristic chemical shift H5/C5 due to 1,2-linked β Fruf units, while the cross peak due to H5/C5 of 2,6-linked β Fruf units was missing in the spectrum.

3.6. Identification of difructose anhydride DFA IV

NMR spectra (Fig. 6) of the disaccharide fraction $7F^R$ showed that dominant signals belong to only one β Fruf unit in which H4/C4 was unusually downfield shifted to δ 4.597/72.56 (Table 1).

The HMBC of 7F^R revealed cross peaks due to long range interactions between ketoanomeric C2 signal present at δ 103.90 and all protons of both CH₂ groups (H6, H6', H1, and H1') of β Fruf ring. This fact confirmed that in this compound two molecules of β Fruf are linked by two 2,6-linkages forming thus a cyclic symmetric disaccharide molecule—difructose anhydride type DFA IV (Fig. 6). Its ¹³C chemical shifts agreed with literature data.^{17,18,37}

In the ¹H NMR spectrum of 7F^R besides signals of DFA IV other signals could be detected indicating the presence of low amounts of further compound in this fraction. Previous NMR analysis of complex RIM showed the presence of sucrose not metabolized by the bacterium. Degree of polymerization of 7F^R was dp = 2 and thus it should contain sucrose. The signal at δ 5.40 was identified as H1 due to α -Glc in sucrose molecule. The ratio of sucrose and DFA IV in 7F^R was found to be 1:5. The origin of other very low intensities signals present in the spectra was not identified.

NMR spectra of 3F and 2F fractions were very complex showing the presence of diagnostic signals of both, levan 2,6- β FOSs and inulin 1,2- β FOSs together with signals due to DFA IV. Further studies will be necessary to precise structures of compounds present in these mixtures.

4. Discussion

Monitoring the metabolism of sucrose at rather low concentrations by *Bacillus* sp. 3B6 using in situ ¹H NMR showed that this bacterium started to degrade sucrose to free glucose and fructose. While glucose was used by the bacterium as a source of energy, fructose remained in the medium. At higher sucrose concentration, fructose accumulated in the medium and it served for the EPS synthesis. Dialysis of complex incubation medium afforded retentate (RIM) and dialyate (DIM). Both, monosaccharide fractions of DIM and RIM contained glucose and fructose. In the disaccharide fraction of RIM the ratio of difructose dianhydride DFA IV and sucrose was 5:1, while in that one of DIM only sucrose was found. In RIM trisaccharides frac-

tion 1-kestose, 6-kestose, and neokestose were present in the ratio 2:1:3. In DIM their ratio was 1.5:2:1. DIM higher molecular mass fractions contained only traces of tetra, penta, and hexasaccharides. In penta and hexasaccharides 2,6-linked β Fruf units were linked to α -glucose. However, DIM pentasaccharide fraction contained also homopentasaccharide composed of 2,6-linked β Fruf units only. In DIM tetrasaccharide fraction nystose was identified. 1-Kestose is a precursor for its synthesis. Polymeric levan (RIM) was found to be composed of two components, one of molecular mass ~3500 and the other of ~100 kDa with the ratio 2.3:1. Identification of levan 2,6- β Fruf and inulin 1,2- β Fruf type oligosaccharides in the incubation medium suggests both levansucrase and inulosucrase enzymes activity in *Bacillus* sp. 3B6 similarly to *L. reuteri* 121.⁶

Depolymerising levan/inulin fructosyltransferase was found to produce DFA when bacteria were grown on the polymer, inulin, or levan.^{16–18} The high quantity of DFA IV identified in the incubation medium suggests that the activity of this enzyme in *Bacillus* sp. 3B6 is very high. Further studies will be necessary to elucidate enzyme activities in this bacterium.

Identification of fructans production by *Bacillus* sp. 3B6, isolated by Amato et al.²⁶ from cloud water, raises questions about their potential role in the natural environment where this strain was first isolated. Clouds represent an aqueous environment of low temperature (–10 till 15 °C) which transports material and energies to long distances between ecosystems. It was admitted, that liquid and super-cooled water represents better temporary environment for living organisms transported by air than dry air. Thus droplets in clouds can be considered as an environment where cells can reproduce themselves.^{38,39} Transformation of sucrose by *Bacillus* sp. 3B6 into EPS shown in this work might have some implications considering cloud environment, because the presence of various saccharides in atmosphere has been already proven. From identified saccharides, glucose and sucrose were the most abundant.^{40,41} It is known that EPS, and especially fructans, can protect microorganisms against abiotic and biotic stress, such as desiccation and

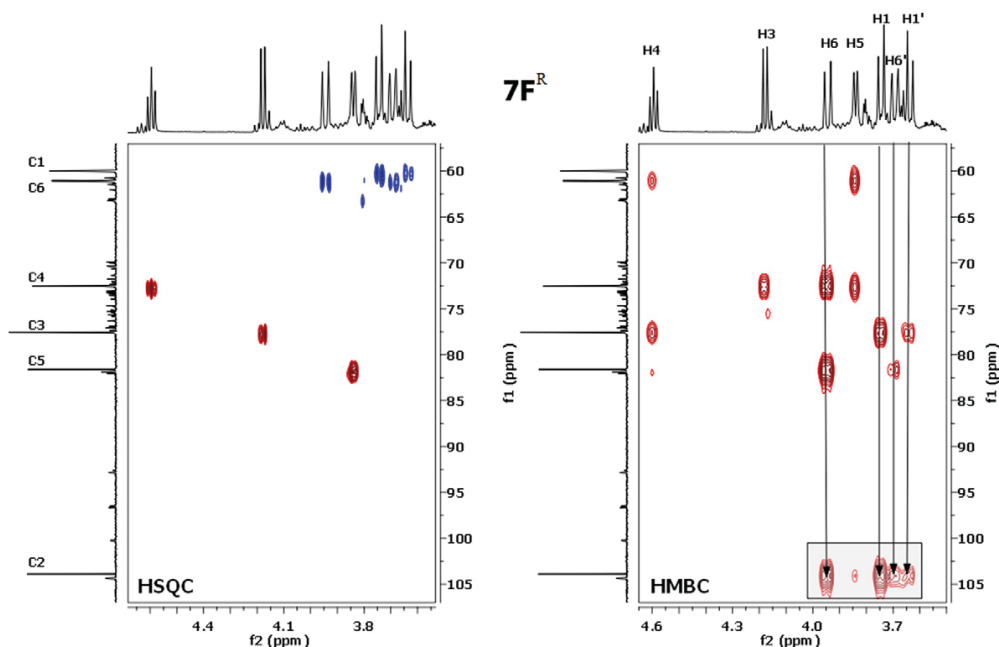


Figure 6. HSQC and HMBC spectra of the disaccharide difructose anhydride DFA IV present in the 7F^R fraction obtained after the separation of the dialyzed complex incubation medium RIM (incubation procedure No. 2) on Bio-Gel P2.

freezing.¹² Production of EPS could be an efficient way for this strain to survive in the extreme environment of clouds, stay metabolically active, and to contribute to atmospheric chemistry and nucleation processes.^{42–44}

5. Conclusions

Obtained results clearly show the potential interest of using this new strain, *Bacillus* sp. 3B6 in biotechnology processes in order to obtain levan polysaccharide, levan and inulin type FOSS, and difructose anhydride DFA IV. These compounds are particularly relevant for applications in nutraceutical, pharmaceutical, and food industry. However, these results also indicate a new direction of the research about the potential role of bacteria producing EPS in the atmosphere.

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4.1.3 Production of EPS by *Bacillus* sp. 3B6 on Various Saccharides

Production of exopolysaccharides by *Bacillus* sp. 3B6 on various saccharide sources

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Abstract

The ability of *Bacillus* sp. 3B6 to metabolise different types of carbohydrates and to produce exopolymeric substances (EPSs) was evaluated by using *in situ* NMR spectroscopy. Different saccharides (aldoses, ketoses) were chosen covering penta- and hexasaccharides in acyclic and cyclic forms as well as oligo- and polysaccharides. Under low concentration of substrates, it was shown that when carbohydrate carbon source is nearly depleted, this bacterium starts to excrete a mixture of EPSs. One EPS component was identified as 1,6-linked α -galactan. It was detected in incubation media on turanose, glucose, cellotetraose, cellulose, fructose, sucrose, and starch. Other EPSs present in the medium did not contain saccharide molecules. Their structure was not identified precisely. However, NMR data indicate that they belong to polyhydroxyalkanoate polymers (PHA). As 3-hydroxybutyrate was found in the medium it is likely that these PHAs have the structure very close to polyhydroxybutyrate (PHB). When this strain was incubated with high concentration of glucose, non soluble EPSs precipitated gradually form in the incubation medium. Sugar compositional and elemental analyses as well as infrared spectra suggest a glycoprotein structure of the main precipitate. Other components of incubation medium have been also identified: butyric acid, glycerol, and esters of glycerol. Finally, as *Bacillus* sp. 3B6 has been isolated from cloud water a discussion is given about the potential implication of EPSs in such an atmospheric environment.

Keywords: exopolymeric substances, EPS, structure, NMR, *Bacillus*, clouds

1 Introduction

Microorganisms can convert nutrients in exopolymeric substances (EPS) very effectively and thus create a stock of carbon and energy. These EPSs can be polysaccharides (PS) but also other polymers such as proteic structures or polyhydroxyalkanoates (PHA) for instance.

Many microorganisms are able to synthesise and excrete exopolysaccharides (PS) through rigid cell wall or membrane (Sutherland, 2004; Dedonder, 1966; Han & Clarke, 1990). In some cases it is a feature of all isolates of a given genus and PSs are synthesised virtually under all conditions. Production of PS results from specific nutritional conditions or as a response on the stress. In some prokaryote it may also result from development or morphological changes. In Gram-positive genera of *Bacillus* and *Clostridium* it may be due to the sporulation. PS may be firmly attached to a microbial surface or it might be excreted into the incubation medium as an amorphous mass of slime, which can be separated from bacteria by centrifugation. Many of bacterial strain might have the capacity to produce simultaneously more than one type of PS. The chemical composition of PS depends on the genetics of the microbial cells and the physicochemical environment, in which a biofilm matrix develops (Sutherland, 2001). Generally, monosaccharide composition and structure of PS are independent of the carbon substrate available. Some bacterial genera seldom yield PS producing species. Only few Gram-positive *Bacillus* species produce PSs.

Microbial PSs are multifunctional and can be divided into intracellular polysaccharides, structural and extracellular PS. They are produced by both prokaryotes (eubacteria and archaeobacteria) and eukaryotes (phytoplankton, fungi, and algae). The bacterial PSs represent a wide range of chemical structures. Chemically, they may have homo- or heteropolymeric composition of high molecular weight (10 to 30 kDa).

They have newfangled applications due to the unique properties they possess. Owing to this, exopolysaccharides have found various applications in the food, pharmaceutical, and other industries (Kumar *et al.*, 2007). They also play a role in the environmental field as biofloculants for instance (Suh *et al.*, 1997).

Some bacterial strains produce polyhydroxyalkanoates, polyesters of 3-, 4-, 5-, and 6-hydroxyalkanoic acids under nutrient-limiting conditions such as nitrogen, phosphorus, sulphur, magnesium, potassium, or iron with excess of carbon (Anderson & Dawes, 1990; Oeding & Schlegel, 1973; Steinbüchel & Schlegel, 1989). These water insoluble storage polymers are biodegradable, exhibit thermoplastic properties and can be produced from renewable sources. PHAs serve as energy and they are stored as granular inclusion bodies in the cytoplasm of many bacteria and archaea (Lemoigne, 1926; Anderson & Dawes, 1990; Steinbüchel, 1991). Accumulation of intracellular storage polymers has been considered as a strategy used by bacteria to increase survival in a changing environment. PHA comprises a vast number of structures with repeating subunits of a general formula $-[O-CH(R)-(CH_2)_x-C(O)]-$ and molecular masses 0.1–30 kDa.

In a recent paper, we had shown that *Bacillus* sp. 3B6 was able to produce exopolysaccharide levan and FOSs (fructooligosaccharides) of levan and inulin type when incubated at high concentration of sucrose as carbon source (Matulová *et al.*, 2011). These interesting results prompted us to extend our study to a wide range of carbohydrates (alditols, mono-, di-, tetra-, and polysaccharides) to evaluate the potential of this strain to produce EPSs. First, the ability of this bacterium to use these various carbohydrates as carbon sources was investigated using *in situ* 1H NMR. Second, the structure of exopolymeric substances excreted by *Bacillus* sp. 3B6 was analysed by *in situ* multidimensional NMR. Finally, a more detailed study concerned the case of EPSs production under high concentration of glucose.

As *Bacillus* sp. 3B6 has been isolated from cloud water (Amato *et al.*, 2007) a discussion is given about the potential implication of EPS in such atmospheric environment.

2 Material and methods

2.1 Microbial incubations

Bacterial strain

Bacillus sp. 3B6 was isolated from cloud water sampled at Puy de Dôme summit (1465 m) in the Massif Central Region (France) and was identified by 16S rRNA gene sequencing as described by Amato *et al.* (2007).

General incubation conditions

Liquid pure pre-culture was incubated in 100 mL portion of TSA (Trypcase Soy broth, Biomerieux, Marcy l'Etoile, France) in 500 mL Erlenmeyer flask at 27 °C and 200 rpm. Cells were harvested by centrifugation ($8\,000 \times g$ and 4 °C for 5 min) after 24 hours of growth and rinsed twice in NaCl 0.8% and thereafter re-suspended in the medium for carbohydrate metabolism study.

Incubation procedure № 1 (Low carbohydrate concentration)

Media for metabolism study were prepared by dissolution of saccharides in Volvic® mineral water. Each tested saccharide was used as a unique carbon source. The final concentration of saccharides in incubation medium was 20 mM for mono- and disaccharides, and 10 mM for oligo-

and polysaccharides. The concentration of used microorganisms was 10^9 cell mL^{-1} . 24-Hours old inoculum was used to seed the sterile medium. *Bacillus* sp. 3B6 was grown in this medium for 6 days under aerobic conditions (27 °C, 200 rpm). Samples were taken in regular intervals (0, 15, 19, 24, 39, 48, 63, 72, 96, 120, 144 h). Extracellular incubation media were separated from cells by centrifugation (3 min, $12\,000 \times g$) and supernatants were kept frozen (-40 °C) until measurement. Inulin, levan, and maltose were ordered from Sigma-Aldrich. All the other tested saccharides were from the production of the Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia.

Incubation procedure № 2 (High glucose concentration)

For EPS production under high glucose concentration a medium proposed by Gandhi *et al.* (1997) was used. Its compositions was as follows: glucose (30 g L^{-1}) (Acros), ammonium nitrate (0.4 g L^{-1}) (Acros), potassium sulphate (1 g L^{-1}) (Acros), sodium chloride (1 g L^{-1}) (Acros), potassium dihydrogen phosphate (3 g L^{-1}) (Fluka), calcium chloride (0.02 g L^{-1}) (Sigma-Aldrich), magnesium sulphate (0.001 g L^{-1}) (Sigma-Aldrich). Medium was autoclaved for 15 min at 121 °C. 24-Hours old inoculum was used to seed the sterile medium. The culture was incubated in a rotary shaker at 200 rpm and 32 °C. After 8 days of incubation, the medium was centrifuged ($20\,000 \times g$, 25 °C, 30 min) and *Bacillus* sp. 3B6 cells were separated from supernatant.

2.2 NMR spectroscopy

Structural analyses

NMR spectra were measured on 600 MHz VNMRS Varian equipped with HCN ^{13}C enhanced salt tolerant cold probe in D_2O at 25 °C using TSPd₄ as

an external standard. Chosen samples containing metabolites were freeze-dried from D₂O before dissolution in 99.98% D₂O. Shigemi NMR tubes or micro-capillaries were used in the case of small quantity of metabolites in the samples. Advanced techniques from Varian pulse library of 2D homo- and hetero-correlated spectroscopy (gCOSY, gTOCSY, gHSQCAD, gHMBCAD, gH2BC) including 1D sequences with selective excitations (1DNOESY, 1DTCOSY) were used for the signal assignments.

Measurement of metabolite concentrations

Supernatants from biodegradation test media were prepared for ¹H NMR by mixing volume of 575 μL of the sample with 25 μL of 20 mM sodium tetra deuterated trimethylsilyl propionate (TSPd₄, Eurisotop) in D₂O. D₂O was used for shimming and locking, while TSPd₄ constituted a reference for chemical shifts (0 ppm) and quantification. Final volume of 600 μL of prepared samples was placed in the 5 mm-diameter NMR tubes. Acquisition was performed at 500 MHz on Bruker Avance NMR spectrometer or on 600 MHz VNMRS Varian equipped with HCN ¹³C enhanced salt tolerant cold probe. Water signal was eliminated by presaturation and by Pulse field gradient spin echo (GPFSE). The concentration of metabolites was calculated as follows:

$$[m] = \frac{9}{b} \frac{A_0}{A_{ref}} [TSPd_4]$$

where $[m]$ is the concentration of compound to be quantify; A_0 is the area of m resonance; A_{ref} is the area of TSPd₄ resonance; 9 and b are the numbers of protons in TSPd₄ and in the metabolite m , respectively.

Calculation of biodegradation rates

The biodegradation rates k_c of the compound C have been determined by linear regression fits as follows:

$$k_c = k \frac{[C]_0}{N_{cells}}$$

with $[C]_0$ the initial concentration of selected compound C [mol L^{-1}]; k the pseudo-first order decays [s^{-1}]; N_{cells} the concentration of cells participating to the biodegradation and determined by CFU counts [cells L^{-1}].

2.3 Infrared spectroscopy

Fourier-transform infra red (FT-IR) spectra were measured with a Nicolet 6700 (Thermo Fisher Scientific, USA) spectrometer equipped with DTGS detector and Omnic 8.0 software. The spectra were collected in the middle region from 4000 to 400 cm^{-1} at a resolution of 4 cm^{-1} , the number of scans was 128. Diamond Smart Orbit ATR accessory was applied for measurement in solid state.

2.4 Chemolytic analyses

The carbohydrate content in EPSs-2ns, EPSs-3ns, and EPSs-4ns was determined by the phenol-sulphuric acid assay (Dubois *et al.*, 1956). Samples were hydrolysed with 2 M trifluoroacetic acid TFA for 1 hour at 120 °C. The quantitative determination of neutral sugars was carried out in the form of their trifluoroacetates by gas chromatography on a Hewlett-Packard Model 5890 Series II chromatograph equipped with a PAS-1701 column (0.32 mm \times 25 m), the temperature program of 110–125 (2 °C min^{-1}), 125–165 °C (20 °C min^{-1}), and a flow rate of hydrogen 2 $\text{cm}^3 \text{min}^{-1}$ (Shapira, 1969).

2.5 Elemental analysis

Elemental analysis was performed with EA 1108 apparatus (FISONS Instruments, East Grinstead, UK). Protein was calculated from the nitrogen content ($\%N \times 6.25$).

3 Results

3.1 Ability of *Bacillus* sp. 3B6 to metabolise carbohydrates

The ability of *Bacillus* sp. 3B6 to metabolise different types of carbohydrates was monitored by *in situ* ^1H NMR spectroscopy under low concentrations of carbohydrate (incubation procedure № 1). Different saccharides (aldoses, ketoses) were chosen covering penta- and hexasaccharides in acyclic and cyclic forms as well as oligo- and polysaccharides (Table 1). Samples were taken at chosen intervals (0, 24, 39, 48, 63, 72, 96, 120 hours) from the incubation media. ^1H NMR spectra were measured directly without any further sample treatment. At the beginning of the incubation ($t = 0$ h) only signals of the substrate and TSPd₄ standard added at known concentration were present in the spectra. Signal intensity changes due to carbohydrate used in relation to that of standard was monitored. Samples, in which the formation of metabolites with carbohydrate structure was observed, were subjected to a detailed structural analysis after freeze-drying and dissolving in D₂O.

Table 1 summarises the results concerning the screening for

carbohydrates used as a carbon sources by *Bacillus* sp. 3B6 as well as their degradation rates.

Alditols

Alditols are acyclic carbohydrates formed by reduction of their corresponding aldoses. In the screening, five chosen alditols were tested: D-mannitol, D-glucitol, D-galactitol, D-arabitol, and L-arabitol. ¹H NMR Spectra of the incubation media (IM) showed that D-mannitol and D-glucitol were completely metabolised within 24–48 hours. The degradation of L-arabitol started after 48 hours of the incubation and its quantity decreased from 96% at 48 h to 55% at 72 h and it completely disappeared at 96 h. However, D-arabitol and D-galactitol were not degraded. In the case of all alditols no formation of metabolites or intermediates was observed.

Hexoses

A comparison of biodegradation rates of tested aldoses and ketoses (Table 1) showed that the fastest degraded saccharides were D-glucose (Glc) together with D-galactose (Gal) and D-mannose. Their signals disappeared completely from the ¹H NMR spectra after 48 hours. The degradation of D-fructose (Fru) and L-arabinose was slightly slower and their rates of the degradation were comparable. In both cases, substrate signals disappeared after 48 hours. In the case of arabinose and L-rhamnose, the production of some metabolites was observe. However, their structures were not studied in details.

Pentoses

The intensity of fructose (Fru) signals in the course of the incubation decreased very fast; after 25 hours less than 25% of Fru were present and after 48 hours they disappeared completely. The degradation rate of L-arabinose was only slightly lower than that of Fru followed by those of D-ribose. The biodegradation rate of D-ribose was low; all substrate was

degraded after 96 hours of the incubation. The degradation rates of L-ribose and D-xylose were comparable and NMR spectra of their IM showed that after 96 hours almost 60% of substrate was still present there.

Disaccharides

Maltose ($\alpha\text{Glc}(1-4)\text{Glc}$) was the fastest degraded disaccharide; it disappeared after 48 hours. The degradation rates of sucrose ($\alpha\text{Glc}(1-2)\beta\text{Fruf}$), trehalose ($\alpha\text{Glc}(1-1)\alpha\text{Glc}$), turanose ($\alpha\text{Glc}(1-3)\alpha\text{Fruf}$), and cellobiose ($\beta\text{Glc}(1-4)\text{Glc}$) were similar; in their ^1H NMR spectra all signals were absent after 72 hours. Signals of lactose ($\alpha\text{Glc}(1-4)\text{Glc}$) disappeared only after 96 hours indicating slower metabolism of this sugar.

Polysaccharides (PS)

No degradation of levan polymer (2,6-linked Fruf) was observed contrary to inulin (1,2-linked Fruf), which degradation rate was high. After 24 hours already 30% of the polymer was already used and its signals disappeared from the spectrum after 72 hours. The degradation rate of cellotetraose was the same as that of cellobiose. Contrary to cellotetraose, cellulose was not soluble and thus its signals were not present in ^1H NMR spectrum at $t = 0$ h. The ^1H NMR spectrum of the incubation medium taken at $t = 15$ h contained signals of amino acids and first signals indicating the presence of some sugar metabolites appeared after 27 hours.

Table 1. *Bacillus* sp. 3B6 ability to degrade different types of saccharides.

Tested saccharides	Incubation time [h]					Biodegradation rate [mol cell ⁻¹ s ⁻¹]
	0	24	48	72	96	
Alditols						
Mannitol	+++	++	–	–	–	5.4×10^{-7}
D-Glucitol	+++	+++	+	–	–	5.38×10^{-7}
D-Galactitol	+++	+++	+++	+++	+++	0
L-Arabitol	+++	+++	+++	++	–	2.47×10^{-7}
D-Arabitol	+++	+++	+++	+++	+++	0
Monosaccharides						
L-Arabinose	+++	++	–	–	–	4.08×10^{-7}
D-Xylose	+++	+++	+++	++	++	1.04×10^{-7}
D-Ribose	+++	++	+	+	–	2.33×10^{-7}
L-Ribose	+++	+++	+++	++	++	1.12×10^{-7}
D-Fructose	+++	+	EPS	EPS	EPS	6.04×10^{-7}
D-Glucose	+++	+	EPS	EPS	EPS	7.14×10^{-7}
D-Galactose	+++	+	–	–	–	7.4×10^{-7}
D-Mannose	+++	+	–	–	–	6.25×10^{-7}
L-Rhamnose	+++	++	–	–	–	6.84×10^{-7}
Glucuronic acid	+++	+++	+++	+++	+++	0
Disaccharides						
Lactose	+++	++	++	+	–	5.72×10^{-7}
Sucrose	+++	++	+	–	EPS	5.76×10^{-7}
Maltose	+++	++	–	–	–	5.14×10^{-7}
Trehalose	+++	++	+	–	–	5.32×10^{-7}
Turanose	+++	+++	+	EPS	EPS	5.67×10^{-7}
Cellobiose	+++	++	+	–	–	5.41×10^{-7}
Tetrasaccharides						
Cellotetraose	+++	++	+	EPS	EPS	5.21×10^{-7}
Polysaccharides						
Cellulose	+++	+++	EPS	EPS	EPS	NA
Arabinogalactan	+++	+++	+++	++	+	1.14×10^{-7}
Glucuronoxylan	+++	++	+	–	–	5.52×10^{-7}
Inulin	+++	++	+	–	–	5.73×10^{-7}
Levan	+++	+++	+++	+++	+++	0
Starch	+++	++	EPS	EPS	EPS	5.28×10^{-7}
CMC	+++	+++	+++	+++	+++	0
α-D-Cyclodextrin	+++	+++	+++	+++	+++	0
β-D-Cyclodextrin	+++	+++	+++	+++	+++	0

CMC – carboxymethyl cellulose, EPS – mixture of exopolymeric substances, NA – not available

+++ 100% of substrate present in IM

++ cca 60% of substrate present in IM

+ cca 30% of the substrate present in IM

– substrate consumed completely

3.2 EPSs production on different carbon sources

Analysis of *in situ* ^1H NMR spectra of samples taken at different times and different substrates revealed similar features of spectral patterns, especially at the end of the incubations when substrate was already depleted. Spectral pattern in all spectra shown in Figure 1 suggest the presence of high molecular mass exopolymeric substances (EPSs).

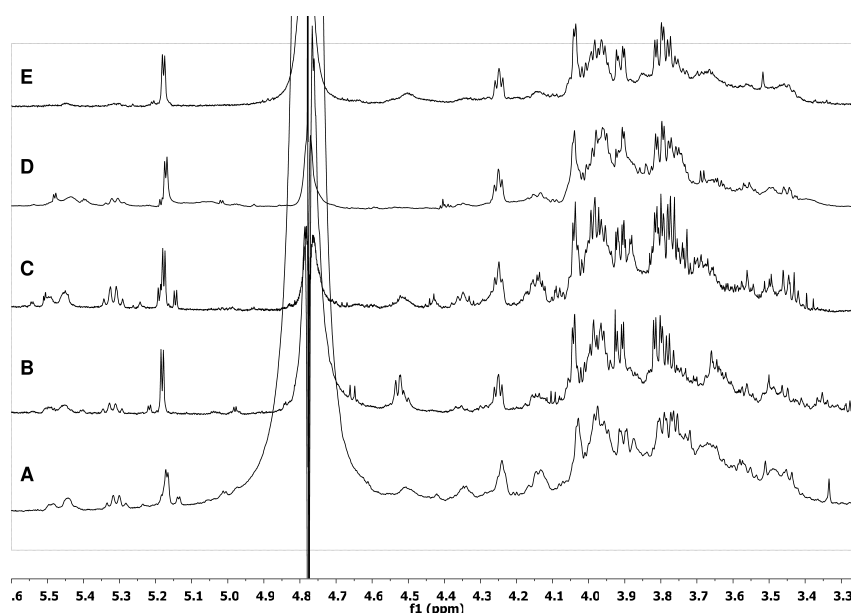


Figure 1. ^1H NMR spectra of incubation media (IM) of *Bacillus* sp. 3B6, in which the carbohydrate substrate was already depleted: A ($t = 63$ h) on glucose; B ($t = 43$ h) on cellotetraose; C ($t = 63$ h) on cellulose; D ($t = 72$ h) on turanose; and E ($t = 63$ h) on starch. In B ($t = 43$ h) on cellotetraose still some substrate was present. Similar spectral pattern was observed also in the case of fructose and sucrose at the end of the incubation.

This observation was very interesting due to the fact that different substrates were used as a carbon source (fructose, sucrose, glucose, turanose, cellotetraose, cellulose, and starch; Table 1). However, the complexity of ^1H NMR spectra suggested rich mixtures of metabolites. In most cases the concentration of metabolites was very low due to the conditions used for screening (Incubation procedure № 1) preventing a

very detailed analysis of EPS structure. However, in the sample of IM on turanose ($t = 72$ h) the quantity of individual components was differed to such level that one of them was more or less dominant in the mixture. This fact allowed its partial structural determination by a combination of selective 1D and 2D NMR techniques. Valuable information in form of characteristic 2D COSY and HSQC spectral patterns due to individual components was also used in the analysis of the complex IM.

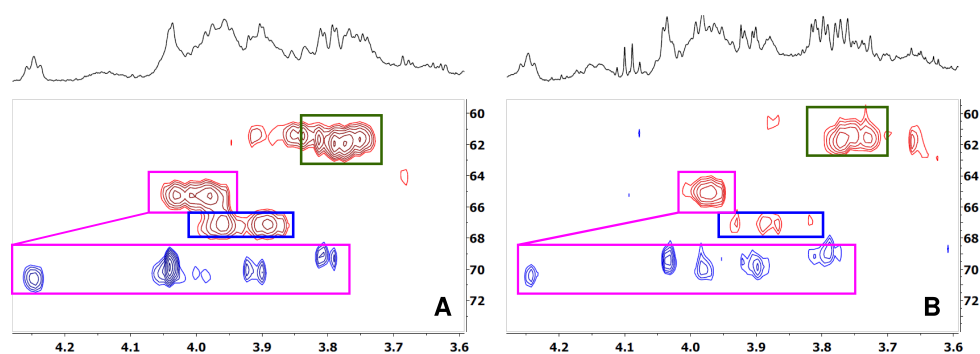


Figure 2. EPSs spectral patterns in HSQC spectra of IM media of *Bacillus* sp. 3B6 on: A – turanose after 72 h and B – cellulose after 63h. Attribution of colours: i) spectrum cross-peak: red – CH₂; blue – CH; ii) marks: pink 1,6-linked α -galactan; green and blue – non identified –O–CH₂– due to PHA.

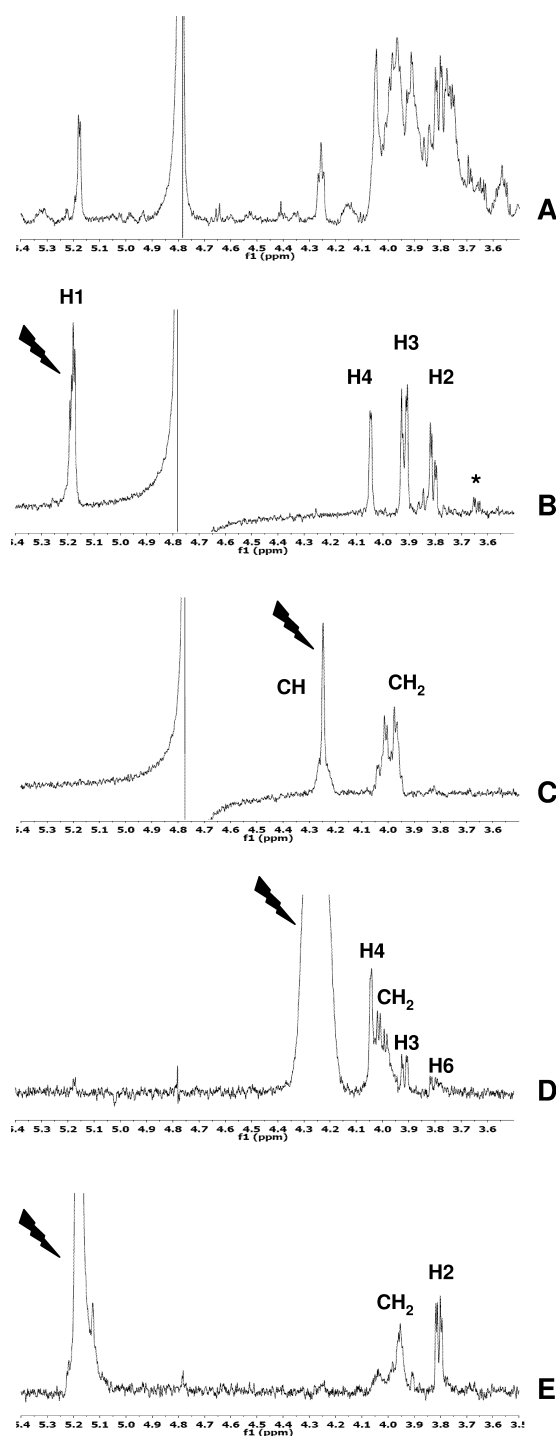


Figure 3. Selective 1D NMR spectra of the incubation medium of *Bacillus* sp. 3B6 on turanose: ^1H NMR spectrum of turanose $t = 72$ h (A); selective 1D TOCSY with mixing time 150 ms spectra with irradiation at δ 5.176 ppm (B) and δ 4.245 ppm (C); selective 1D NOESY spectra with mixing time 200 ms with irradiation of the signal at δ 4.245 (D) and δ 5.176 (E); * unknown.

3.2.1 EPSs produced on turanose

Comparison of ^1H NMR spectra on different substrates (Figure 1) showed that the spectrum of the medium collected after 72 h of incubation with turanose (Figure 1D) showed the simplest spectral pattern and the quantity of metabolites in this sample was higher than in IM on starch (Figure 1E). Therefore, this sample was chosen for a detailed NMR analysis. Comparison of ^1H NMR spectra suggested that the metabolism of turanose ($\alpha\text{Glc}(1-3)\beta\text{Fru}f$) under screening conditions (Incubation procedure № 1) was similar to that one at higher concentration on sucrose ($\alpha\text{Glc}(1-2)\beta\text{Fru}f$) (Incubation procedure № 2) described in our previous paper in which *Bacillus* sp. 3B6 hydrolysed sucrose to Glc and Fru f . Glc was further used as a source of energy and Fru f for the synthesis of inulin and levan type fructooligosaccharides (FOSs) and levan polysaccharide (Matulová *et al.*, 2011). The same behaviour was observed on turanose, however, here under these screening conditions, the concentration of the substrate was much lower and thus synthesised microbial FOSs were finally used as a source of carbon by the bacterium. The HSQC spectrum collected after 72 hours of incubation is shown in Figure 2A.

Anomeric H1/C1 signals at δ 5.178/99.45 ppm indicated some saccharide unit. A series of 1D TOCSY and 1D NOESY spectra obtained by selective irradiation of chosen H signals of this sample are shown in the Figure 3. In the 1D TOCSY experiment by selective irradiation of anomeric H1 at δ 5.178 the magnetisation was transferred to: H2 (δ 3.801/ δ 69.18), H3 (δ 3.912/ δ 70.12) and it has stopped at H4 (δ 4.03/ δ 70.00) suggesting α -Gal unit in the EPS backbone.

Irradiation of the triplet at δ 4.245 (CH group, C δ 70.56) revealed in 1D TOCSY and COSY CH_2 group signals in its proximity at δ 4.03 and 3.980 (C δ 65.38). NOE response observed in 1D NOESY from this CH group (δ 4.245) showed the previously assigned H4 (δ 4.03/ δ 70.00) of α -Gal and

thus it was attributed to H5 of the same sugar unit. Selective irradiation of H1 signal at δ 5.178 in 1D NOESY showed its neighbour in the sugar ring, H2 signal at δ 3.801; further two H6 and H6' signals at δ 4.03 and 3.980 (C δ 65.38), respectively (Figure 3E). All signals of sugar ring were thus assigned (Table 2) indicating 1,6-linkage between α -Gal units. Chemical shifts of α -Gal were in a very good accordance with data published for α -1,6-linked galactan by Carbonero *et al.* (2008). In the HSQC spectrum of turanose IM $t = 72$ h in the Figure 2A signals of 1,6-linked α -Gal EPS are highlighted by a pink colour. A positive intensity of NOE signals in 1D NOESY was indicated a high molecular mass of the compound. Any further details about the substitution of the backbone could not be obtained due to the complexity of the spectrum and a low concentration of the sample.

Table 2. NMR chemical shifts of various metabolites synthesised by *Bacillus* sp. 3B6.

Compound	Solvent	Chemical shift δ H/C [ppm]				
		CH	CH ₂	CH ₃	COOH	
PHA	D ₂ O	4.050/70.21	3.967; 3.908/67.14	2.023/22.95	177.52	
3HB	D ₂ O	4.135/66.77	2.391; 2.297/47.20	1.179/22.67	181.28	
3HB	CDCl ₃ :MeOH= 7:3	5.253/67.97	2.614; 2.492/40,95	1.271/19.86	168.95	
Butyric acid	D ₂ O		3.761/62.25 3.63; 3.541/63.40	1.473/17.06	178.5	
Glycerol	CDCl ₃ :MeOH= 7:3	3.877/70.47	3.640, 3.55/63.49 4.124/65.38			
1,6-linked α -galactan						
Chemical shift δ [ppm]	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6;H6'/C6
α -Galactose	5.176/99.47	3.803/69.19	3.917/70.10	4.036/69.76	4.245/70.56	4.026, 3.98/65.21

PHA - polyhydroxyalkanoate; 3HB - 3-hydroxybutyrate

3.2.2 EPSs produced on other sugars

1,6-Linked α -galactan

Figure 4 shows an example of COSY NMR spectra recorded on samples of incubations with glucose ($t = 48$ h and $t = 63$ h) and cellulose ($t = 63$ h). In the IM spectrum on glucose after 48 h (Figure 4A) traces of glucose were present and between many metabolites present there cross-peaks due to 1,6-linked α -galactan could be identified. In the COSY spectrum of the IM on glucose after $t = 63$ h (Figure 4B), neither glucose nor low molecular metabolites cross peaks could be detected, only those of EPSs with higher molecular mass were present there. The 1,6-linked α -galactan was one of the main components of the mixture. As cellulose is not soluble, no signal was detected at the beginning of the experiment; first metabolites appeared in the spectrum after 27 hours corresponding to amino acids signals in the region δ 2.4–0.7 ppm. Well resolved H1 anomeric signal at δ 5.179 ppm, indicating the presence of 1,6-linked α -galactan, appeared only after 48 h of the incubation. In the spectrum of IM on cellulose after $t = 63$ h cross-peaks due to 1,6-linked α -galactan were dominant (Figure 4C).

Polyhydroxyalkanoates (PHA)

In the HSQC spectrum of IM $t = 72$ h on turanose (Figure 2A) besides cross-peaks due to 1,6-linked α -galactan those due to CH_2 group of high intensity were present: δ 3.97–3.84/67.5, 3.98/65.70, 3.84–3.72/62.14. Cross-peak of CH_3 group was observed at δ 2.03/22.84 ppm. However, HMBC spectrum did not reveal any cross-peak due to a long range interaction from CH_2 to carboxyl group, which could be present in the molecule. The only cross-peak with carboxyl group was detected from CH_3 group. Moreover, ^{13}C chemical shifts values of these CH_2 group suggested a close vicinity of oxygen similar to a $-\text{CH}_2-\text{O}-$ structure. The complexity of the spectra prevented more precise attribution of signals to

some structure. However, on the basis of these data we could deduce the presence of some polyhydroxyalkanoate (PHA) in the incubation medium but its structure remained unknown. Further studies will be necessary for its elucidation.

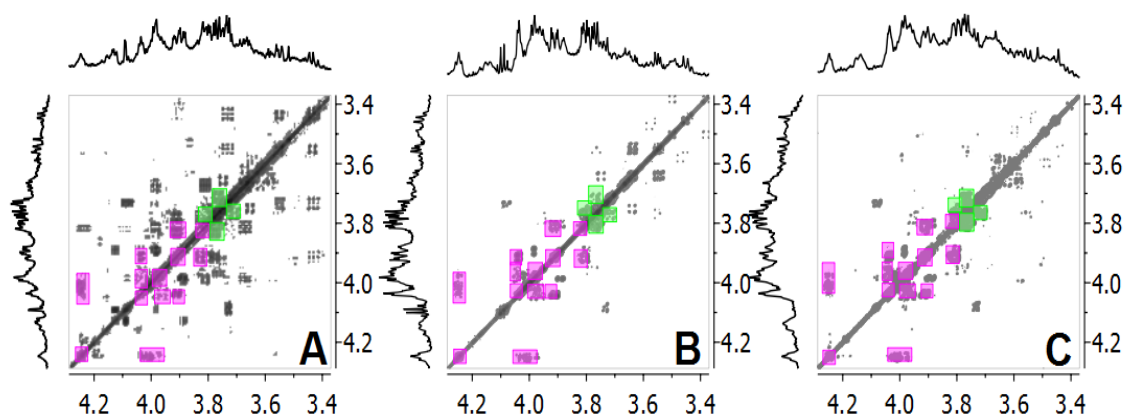


Figure 4. EPSs spectral patterns in COSY spectra of IM media of *Bacillus* sp. 3B6 on: A – glucose after 48 h; B – glucose after 63 h and C – cellulose after 63 h. Cross-peaks marked by: pink colour – due to dextran type of polysaccharide with α -1,6-linked galactose in the backbone; green colour – non identified isolated $-O-CH_2-$ group.

3.3 The case study of incubation with high concentrations of glucose

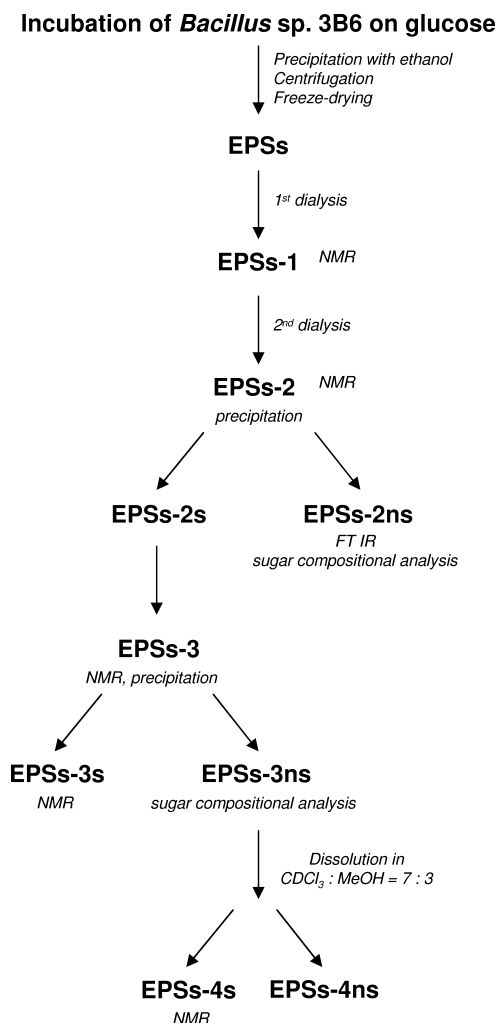
The production and structure of microbial EPSs depend on specific nutritional conditions as a component of the stress response. In the previous section, the ability of *Bacillus* sp. 3B6 to produce EPSs has been investigated using relatively low concentration of the substrate. In particular, incubation on glucose gave rise to the production of 1,6-linked α -galactan and PHAs. To test the possible change in the glucose metabolism from the point of view of EPSs production, *Bacillus* sp. 3B6 was incubated in rich medium with an excess of glucose (Ghandi *et al.*, 1997) (Incubation procedure № 2).

3.3.1 Description of EPS fractions obtained from IM on glucose

Structural analysis of the incubation medium obtained after 8 days of incubation was complicated by gradual precipitation of water non soluble EPSs formed by the bacterium. Thus, more steps of dialysis and separations were necessary and they are summarised in the Scheme 1.

A crude IM was subjected to 3 days dialysis against water, the volume was reduced by evaporation and a freeze-drying afforded EPSs-1. ^1H NMR spectrum of EPSs-1 showed that glucose, amino acids and salts were still present in the sample and thus EPSs-1 was dialysed again to give EPSs-2. After freeze-drying, EPS-2 was dissolved in D_2O and during the NMR measurement a non soluble fraction EPSs-2ns precipitated while some dissolved compounds remained in the solution and they were marked as EPSs-2s. Separated EPSs-2ns was analysed by sugar compositional analysis, elemental analysis, and infrared spectroscopy. EPSs-2s was further analysed by NMR in spite of the presence of small amount of the non soluble components, which slowly precipitated from the solution. Because of EPSs-2s (a mixture of dissolved and precipitated compounds) still contained low molecular mass compounds (amino acids), a third dialysis has been done affording EPSs-3. The non soluble components were removed by centrifugation affording non soluble EPSs-3ns fraction and a supernatant EPSs-3s containing dissolved compounds. Composition of EPSs-3s was checked by NMR. Separated EPSs-3ns was analysed by sugar compositional analysis and infrared spectroscopy. Its quantity was not sufficient for the elemental analysis. Attempts for EPSs-3ns dissolution were also made; it was non soluble in DMSO. In a mixture of solvents $\text{CDCl}_3:\text{MeOH} = 7:3$ its dissolution was partial affording a soluble fraction EPSs-4s and the non soluble part EPSs-4ns, which remained stuck on the NMR sample tube. EPSs-4s was separated and further analysed by 1D and 2D NMR. ^1H NMR spectra of

EPSs soluble fractions are presented in the Figure 5.



Scheme 1. Origin of fractions containing exopolymeric structures EPSs issued by gradual precipitation of non soluble EPSs from the incubation medium obtained after 8 days of the incubation of *Bacillus* sp. 3B6 on glucose.

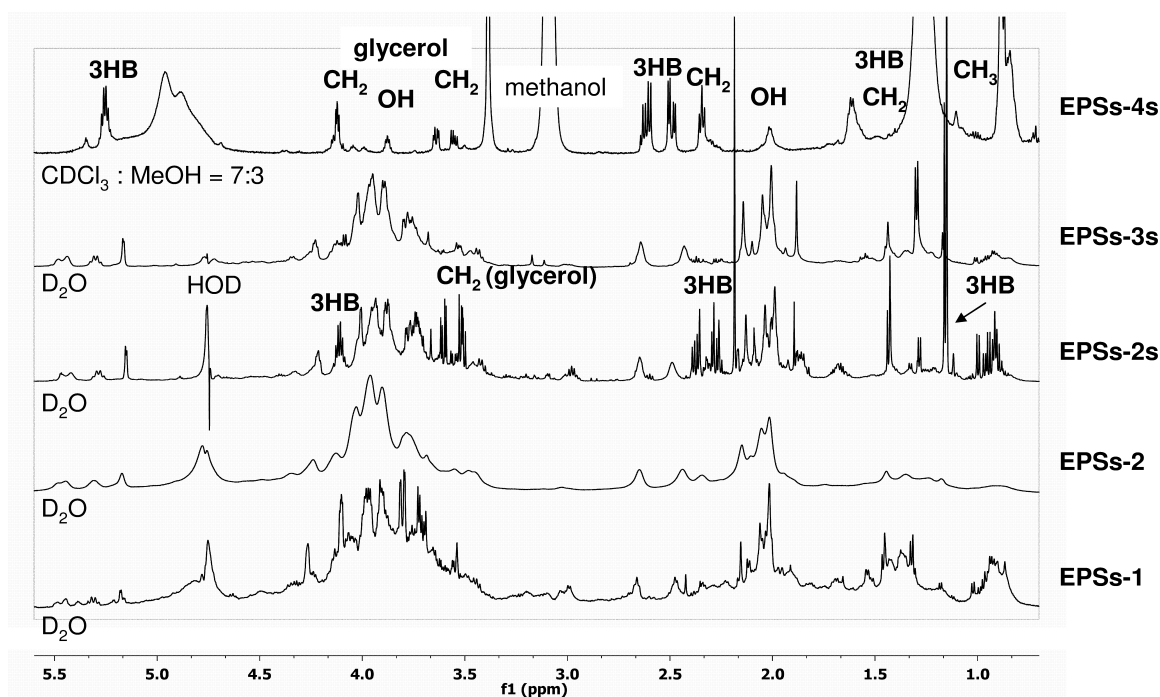


Figure 5. ^1H NMR spectra of soluble EPSs fractions obtained from IM on glucose (Incubation procedure № 2) by gradual precipitation of non soluble substances: EPSs-1 – IM after first dialysis; EPSs-2 – IM after second dialysis before the precipitation; EPSs-2s – IM after the precipitation; EPSs-3s – after EPSs-2s dialysis; EPSs-4s – soluble part of EPSs-3ns in a mixture of $\text{CDCl}_3:\text{MeOH} = 7:3$. See also Scheme 1; 3HB – 3-hydroxybutyrate.

3.3.2 Analysis of soluble fractions EPS-1, EPSs-2, EPSs-2s, EPSs-3s

All soluble fractions obtained from the IM after 8 days of incubation on glucose were analysed by NMR; the identified metabolites are listed in Table 3.

Table 3. Identified soluble and insoluble compounds in individual fractions formed by gradual precipitation from the incubation medium (IM) on glucose (Incubation procedure № 2) after 8 days of incubation.

Identified Compounds	Fractions
1,6-Linked α -galactan	EPSs-2, EPSs-2, EPSs-3s
PHA	EPSs-2, EPSs-2s, EPSs-3s
3HB	EPSs-2s, EPSs-4s
Butyric acid	EPSs-2s
Glycerol	EPSs-2s, EPSs-4s
Esters of glycerol	EPSs-4s
Proteoglycan	EPSs-2ns

In the ^1H NMR spectrum of EPSs-2 sample obtained after a second dialysis broad lines indicated the presence of high molecular mass EPSs. In the HSQC spectrum of the incubation mixture one component was identified as 1,6-linked α -galactan polymer on the basis of its previously characterised spectral pattern. Chemical shifts of its cross-peaks were in accordance with those already assigned for this polymer (see Table 2). In addition, cross-peaks of high intensity due to another polymer present in the mixture could be recognised: CH_3 at δ 2.023/ δ 23.00, two CH_2 groups at δ 3.757/ δ 61.84 and δ 3.976–3.854/ δ 67.74 and CH at δ 4.011/ δ 70.61. Their spectral pattern and chemical shifts were similar to PHA observed in the media obtained at the end of incubations at low concentration of the substrate. They suggested the PHA presence also in this IM.

During the measurement of EPS-2 NMR spectra, a spontaneous precipitation of some non soluble components was observed. The EPSs-2ns precipitate was separated and the supernatant was marked as EPSs-2s. Characteristic broad lines pattern due to low quantity of EPSs were still present in its NMR spectra; however, their quantity was low. HSQC spectrum indicated a mixture of lower molecular mass compounds. One of them was identified as 3-hydroxybutyrate. Its structure was confirmed by COSY, HSQC, and HMBC spectra and the chemical shifts data are presented in the Table 2. Another one was glycerol.

During NMR measurements further precipitation occurred in the sample tube in small quantities. New sharp signals, which were not observed before, appeared in the spectra. Analysis of NMR spectra confirmed the presence of butyric acid (Table 2). Moreover, the characteristic spectral pattern due to 1,6-linked α -galactan indicated the presence of its oligomers.

3.3.3 Analysis of water non soluble fractions EPSs-2ns, and EPSs-4ns

Table 3 summarises the results obtained for different non soluble fractions.

EPSs-2ns was subjected to sugar compositional and elemental analyses and infrared spectroscopy. The main carbohydrates identified by sugar compositional analysis were galactose (35%), glucose (33%), and mannose (28%). Only traces of xylose (3%) and arabinose (1%) were found. Elemental analysis confirmed the presence of proteins (~18%). In the FT-IR spectrum (Figure 6) the presence of bands typical for carbohydrate and protein moieties could be identified. In the region 1200–1000 cm^{-1} characteristic for polysaccharide moiety, significant band maxima were present at 1093, 1072, and 1021 cm^{-1} derived from hexosans (galactose, glucose, and mannose) based polysaccharide structures. However, due to the EPSs-2ns complex structure and an overlap of vibration bands it was not possible to identify the heteropolysaccharide backbone. Bands found at 2960, 2935, and 2862 cm^{-1} could derive from $\nu(\text{C-H})$ stretching vibrations of CH groups and that one at 3394 cm^{-1} from stretching vibrations of OH groups in sugar moiety. The band at 887 cm^{-1} indicated β -glycosidic linkages of carbohydrates. The presence of carbohydrates linked by α -configuration was deduced on the basis of observed signals at 706 and 775 cm^{-1}

characteristic of α -linkage. Two additional signals characteristic of the α -linkages (expected to appear at 930 and 850 cm^{-1}) were hidden by other signals. In addition, IR bands at 1641 and 1566 cm^{-1} relate to the $\nu(\text{C}=\text{O})$ and $\delta(\text{NH}_2)$ vibrations of the peptide bonds amide I and amide II, respectively. Bands at 3278 and 3095 cm^{-1} $\nu(\text{NH}_2)$ originate from the vibrations of primary amide groups. Obtained data suggest that the main component in the precipitate EPS2-ns has a glycoprotein structure.

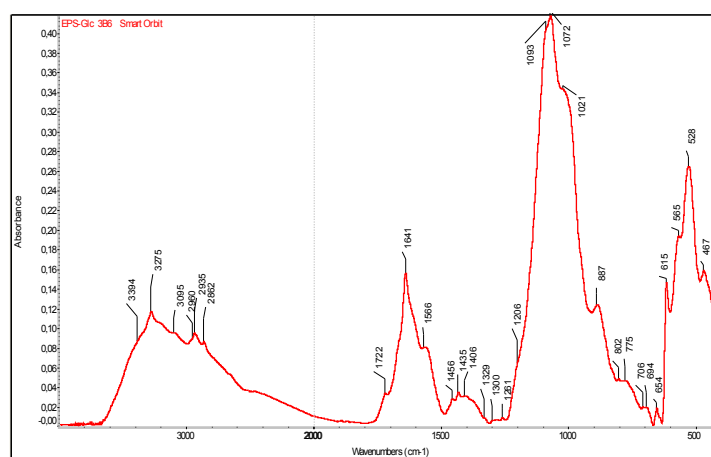


Figure 6. FT-IR spectrum of EPSs-2ns.

The precipitated polymer EPSs-2ns and EPSs-3ns was not soluble in DMSO. EPSs-3ns was partially soluble in the mixture of chloroform and methanol ($\text{CDCl}_3:\text{MeOH} = 7:3$). Non soluble material (EPSs-4ns) was removed and dissolved compounds remained in the EPSs-4s. Carbohydrate compositional analysis did not prove the presence of carbohydrates in EPSs-3ns and EPSs-4ns. NMR analysis of the EPSs-4s fraction confirmed the presence of 3-hydroxybutyrate, glycerol (Table 2) and esters of glycerol. Esters of glycerol showed H/C signals in COSY and HSQC spectra due to characteristic signals of long chains composed of $-(\text{CH}_2)_n-\text{CH}_3$ of glycerol esters: CH_3 at δ 0.882/ δ 14.24, 0.834/ δ 20.40 and 0.808/ δ 19.95; CH_2 groups at δ 1.256/ δ 29.78, 1.621/ δ 24.70, 1.61/ δ 24.92, 2.34/ δ 34.44, 1.29/ δ 29.16, 1.255/ δ 29.78. In the HMBC spectrum some of long range interactions could be detected from CH_3 at δ 0.88 to CH_2 at δ 21.63, 34.86 and from CH_2 at δ 2.34/ δ 34.44 to CH_2 at δ 24.19

and carboxyl at δ 174.64. Identified chemical shifts of glycerol esters were in accordance with published data (Lie Ken Jie & Lam, 1995).

4 Discussion and conclusion

Although *Bacillus* genus is highly present in the environment, relatively few papers deal with the structural analysis of EPSs by this type of bacterial strains. We show here that the strain *Bacillus* sp. 3B6, isolated from atmospheric water, can produce many EPSs on various carbohydrates as carbon sources including fructose, glucose, sucrose, turanose, cellotetraose, cellulose, and starch. *In situ* NMR allowed identification some of these structures.

Analysis of IM obtained during the incubation of *Bacillus* sp. 3B6 with different saccharides at low concentration showed that bacterium starts to excrete a mixture of exopolymeric substances (EPS) when this source of the carbon is nearly depleted. First incubation on turanose produced some FOSs. FOSs were previously found in incubation medium of the same strain with sucrose (Matulová *et al.*, 2011). Another EPS component was identified as 1,6-linked α -galactan, it was produced in the presence of turanose, glucose, cellotetraose, and cellulose. To our knowledge, this is the first report of such a structure produced by a *Bacillus* strain. Other EPSs present in the medium did not contain saccharide molecules. Their structure was not identified precisely; however, NMR data indicate that they belong to polyhydroxyalkanoic polymers (PHA). As 3-hydroxybutyrate was found in the medium, it is likely that these PHAs have the structure very close to polyhydroxybutyrate (PHB). In the literature other strains of *Bacillus* have been reported as PHA producers. The first identified PHA was polyhydroxybutyrate (PHB) from *Bacillus megaterium* (Lemoigne, 1926). In screening for the best PHA producer, *Bacillus cereus* was identified as a strain using various carbohydrate

substrates (glucose, fructose, sucrose, various fatty acids, and gluconate) for PHA production (Valappil *et al.*, 2007). In this study, *Bacillus* sp. 3B6 produced PHA when incubated with turanose, glucose, and cellulose. Interestingly, when the concentration of glucose was increased in the incubation medium other components with a non saccharidic composition, such as 3-hydroxybutyrate, butyric acid, glycerol, and esters of glycerol, have been identified. The presence of 3-hydroxybutyrate is consistent with the synthesis of PHB. Steinbüchel & Valentin (1995) report that a great number of many other different constituents are present in PHB producers and that PHB occurs as an inclusion surrounded by a phospholipid monolayer. In addition, it has been shown that proteins, referred as “granulate-associated proteins”, are associated with the membrane and located only at the surface of the PHB granules (Hocking *et al.*, 1994; Huisman *et al.*, 1991; Steinbüchel *et al.*, 1995). The polymer is in fluid state inside the cell and crystallises to form granules when is released from the cells (Kawaguchi & Doi, 1990). In the case of *Bacillus* sp. 3B6, the detection of glycerol and esters of glycerol, metabolites which are related to phospholipid metabolism, could be involved in the membrane synthesis surrounding PHB granules. Also the presence of proteoglycan identified in the incubation medium could be related to “granulate-associated proteins”. These last points are only hypotheses but they are consistent with the actual knowledge about PHB granules in microorganisms.

As already specified, *Bacillus* genus is highly present in the environment, and thus the production of EPSs has a specific importance for these microorganisms as it is closely related to the ability to form biofilms. Formation of biofilms seems to be the typical way how bacterial cells grow in nature. These biofilms are important for bacterial cells as *i*) they serve for the self-defence and survival of cells (chance to resist to physical and chemical external stresses are higher for cells in biofilms), *ii*) they allow cells to remain in a favourable niche (bacterial cells are fixed in location where nutrients are more abundant or are constantly replenished), *iii*) they benefit to bacteria as they can live in close

association with other ones increasing their survival chances and their opportunities for genetic exchanges (Madigant *et al.*, 2009). The ability of a microorganism to surround itself with a highly hydrated exopolysaccharide layer may provide it with protection against desiccation and predation by protozoa.

If we now consider the case of the atmospheric environment where *Bacillus* sp. 3B6 has been isolated, the production of EPSs could be of special interest. First, this structure surrounding the cells could protect them against desiccation, freezing, UV exposure, $\cdot\text{OH}$ and $\text{NO}_3\cdot$ radicals, *etc.* and could help them to survive in this extreme environment (Delort *et al.*, 2010). Second, such functionalised and very hydroscopic structures could influence the formation of Cloud Condensation Nuclei (CCN) and thus the formation of precipitations (Möhler *et al.*, 2007). Finally, EPS could be released in the atmosphere and could be thus considered as Secondary Organic Aerosols (SOAs). This last point is of importance as it shows that SOAs could be produced by microorganisms as an alternative to radical reactions (Hallquist *et al.*, 2009; Blando & Turpin, 2000).

It is also worth noting that some carbohydrates used as carbon sources in this study are present in the atmosphere, it is the case of mannitol, glucitol, arabitol, glucose, sucrose, and trehalose (Jia *et al.*, 2010a, 2010b; Wang *et al.*, 2011). We have shown that *Bacillus* sp. 3B6 could efficiently metabolise them. On the other hand, glycerol, which is also found in airborne particles, can be produced by this strain.

In conclusion, carbohydrate metabolism and the EPS production could be an efficient way for *Bacillus* sp. 3B6 to survive in the extreme environment of clouds, stay metabolically active and contribute to atmospheric chemistry and nucleation processes.

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4.1.4 Conclusion on Carbohydrate Metabolism

The first important result of our study is the fact that the strain *Bacillus* sp. 3B6 was able to metabolise many carbohydrates. The use of *in situ* ^1H NMR allowed to measure precise biodegradation rates and thus to divide the tested substrates into 3 groups: *i*) substrates completely degraded within 24–48 hours; (mannitol, glucitol, L-arabinose, glucose, fructose, mannose, rhamnose, sucrose, maltose, trehalose, turanose, cellobiose, cellotetraose, cellulose, glucuronoxylan, and inulin) *ii*) slowly degraded substrates – their concentration decreased only slowly and at the end of incubation (96–120 hours) only traces of substrates were present (arabitol, ribose, xylose, ribose, lactose, arabinogalactan); *iii*) not degraded substrates (galactitol, arabitol, glucuronic acid, levan, CMC, α -D-cyclodextrin, β -D-cyclodextrin).

The second important point is that *Bacillus* sp. 3B6 produced different EPSs on various carbohydrates as carbon sources. This production started when the carbon source was depleted. The 2D NMR spectral patterns of these EPS suggested similar structures.

Incubations with an excess of glucose and sucrose could be analysed in more details thanks to multidimensional NMR experiments performed on fractionated samples. The following metabolites could be evidenced:

- On Sucrose: levan exopolysaccharide (EPS), fructooligosaccharides (FOSs) of levan and inulin types and difructose anhydride DFA IV.
- On Glucose: glycoprotein, polyhydroxyalkanoate polymers (PHA), polysaccharide having 1,6-linked α -galactan backbone, 3-hydroxybutyrate, glycerol, esters of glycerol, and butyric acid.

These types of EPS were also found in the incubation with the other saccharides (fructose, sucrose, glucose, turanose, cellotetraose, cellulose, and starch) at lower concentrations.

Such an extensive study of carbohydrate metabolism by *Bacillus* species and particularly of EPS structural elucidation by NMR is rather rare in the literature. These data are the first ones reported for a strain isolated from the atmospheric compartment.

Our results show that this strain could be of interest for biotechnological applications in the domain of pharmacy, nutraceutic and food industry. Mixture of FOSs of levan and inulin type produced by this bacterium on sucrose could find its application in food industry as a prebiotic. Bacteria only seldom produce inulin FOSs and polysaccharides and inulin FOSs are mostly prepared from chicory inulin. DFA IV, β -D-Fru α - β -D-Fru α -2',6:2,6'-dianhydride, could find its application in food industry as a low calorie sweetener but also as a promising drug for cancer treatment. On glucose, the production of PHA of unknown structure was observed. PHA reveal several properties that lend them to various technical, medical, pharmaceutical and other applications (termoplasticity, biodegradability...). Therefore, PHA have attracted the interest of the chemical industry.

In addition, these results also indicate a new direction of research concerning the potential role of bacteria producing EPS in the atmosphere. Indeed some of the saccharides tested were found in the atmosphere in rather high amounts.

4.2 NMR Study of C1 Compounds

4.2.1 Introduction

The role of microorganisms in atmospheric chemistry is still an emerging question. Recent data have shown the potential of microorganisms, as biocatalysts, to transform carboxylic acids present in cloud water. A

comparison between biocatalysis and radical chemistry showed that microorganisms could be a major sink of organic acids at night (Väitilingom *et al.*, 2010, 2011).

The biotransformation of methanol and formaldehyde, major VOCs in the atmosphere, has not been investigated yet. One of the limitations and difficulties is to measure the concentration of these compounds, especially methanol, and few data on this later substrate are available in the literature.

In this paper we used *in situ* ^1H and ^{13}C NMR spectroscopy to monitor specifically the biodegradation kinetics of these compounds and measure precisely the biodegradation rates by various bacteria. Four bacterial strains isolated from cloud water have been chosen, they belong to genera frequently found in the atmosphere (*Bacillus*, *Pseudomonas*, and *Frigobacterium*).

We also used *in vivo* ^{13}C NMR to investigate in more details the metabolism of ^{13}C formaldehyde by *Bacillus* sp. 3B6, which is the bacterium of major interest in presented PhD work.

After the description of the metabolic routes used for formaldehyde and methanol in the various strains, a comparison of the biodegradation rates with those calculated from the reactivity of $\cdot\text{OH}$ and $\text{NO}_3\cdot$ radicals has been made. The objective was to quantify the relative contribution of biocatalysis versus photochemistry under various environmental scenarios, including the temperature of the clouds (5 or 17 °C), the origin of the clouds (urban and remote) and the diurnal cycle (day- and night-time).

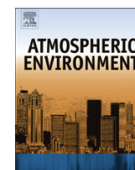
4.2.2 Biotransformation of Methanol and Formaldehyde by Bacteria Isolated from Clouds. Comparison with Radical Chemistry



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Biotransformation of methanol and formaldehyde by bacteria isolated from clouds. Comparison with radical chemistry

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ABSTRACT

The kinetics of biodegradation of methanol and formaldehyde in phosphate buffer at pH 7 by 4 bacterial strains (*Pseudomonas* spp., *Bacillus* sp. and *Frigoribacterium* sp.) isolated from cloud water at the puy de Dôme mountain have been investigated using ¹H and ¹³C NMR spectroscopy. We showed that biodegradation occurred at 5 °C and 17 °C, respectively average and summertime temperature considered within the cloud system at this site. They ranged from 10⁻¹⁹ to 10⁻²¹ mol cell⁻¹ s⁻¹ both at 5 and 17 °C for formaldehyde, and from 10⁻²¹ to 10⁻²³ mol cell⁻¹ s⁻¹ at 5 and 17 °C for methanol. Metabolic intermediates were identified, with notably production of C3 compounds (glycerol, 1,2- and 1,3-propanediol) from formaldehyde by the strain *Bacillus* sp. In order to evaluate to which extent microbiological oxidation of organic compounds has to be considered as an alternative route to radical chemistry in cloud water, the biodegradation rates measured were compared with rates related to the reactivity of organic species with free radicals ·OH (daytime chemistry) and NO₃ (nighttime chemistry) under two cloud situations (urban and remote cases). Clearly, measured biological and chemical reaction rates were in the same range of magnitude and their relative contribution varies according to the scenarios we tested, including the temperature of the clouds (5 or 17 °C), the category of the clouds (urban and remote) and the diurnal cycle (day and nighttime). Except for the degradation of methanol at 5 °C in remote clouds, our results show that biotransformation processes could be the main sink for C1 compounds in liquid clouds ($T \geq 5$ °C ≡ “warm cloud”) during the night and both in polluted and non polluted clouds.

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1. Introduction

Large quantities of Volatile Organic Compounds (VOCs) are emitted into the atmosphere by natural and/or anthropogenic sources. Oxidation of VOCs leads to the formation of gaseous and particulate secondary products which, in turn, have significant impact on the atmospheric composition (Atkinson et al., 2006; Kawamura et al., 2005). In this context, secondary organic aerosols (SOA) are formed by gas-to-particle conversion of these low volatile organic products. These particles account for a significant fraction of ambient tropospheric aerosols and impact on

atmospheric processes, climate and human health (Finlayson-Pitts and Pitts, 2000; Hallquist et al., 2009).

Oxidation of VOCs occurs both in the atmospheric gas and liquid phases (cloud, rain). In the gas phase, VOCs are primarily transformed by photolysis and/or radical chemistry and reactions with other oxidants such as O₃. In the presence of clouds and depending on their solubility, several VOCs (Van Pinxteren et al., 2005) are significantly transferred into the atmospheric liquid phase where they can be oxidized. Methanol (CH₃OH) is a significant component of the volatile organic carbon in the atmosphere (Heikes et al., 2002) and is the predominant oxygenated compound in the mid to the upper troposphere (Singh et al., 2000, 2001); its life time in the atmosphere has been evaluated to 1–2 weeks (Dufour et al., 2007). The anthropogenic primary source and oxidation taking place in the atmosphere represent 11–20% of the methanol while the biogenic contribution mainly issued from plant metabolism is the most significant part (80–90%) (Galbally and Kirstine, 2002;

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Tie et al., 2003; Jacob et al., 2005; Brunner et al., 2007). Methanol plays an important role in atmospheric chemistry as it influences the oxidizing capacity of the atmosphere by reacting with hydroxyl radicals ($\cdot\text{OH}$) or by producing peroxy radical (RO_2) and formaldehyde (HCHO). Formaldehyde is a key atmospheric VOC since it is an important tracer of Non-Methane Volatile Organic Compound (NMVOC) emissions and photochemical activity. HCHO is a primary emission product from biomass burning (Carlier et al., 1986) and fossil fuel combustion (Anderson et al., 1996; Holzinger et al., 1999). However, its major source in the atmosphere is the photochemical oxidation of methane and non-methane hydrocarbons (Altshuller, 1993).

Methanol and formaldehyde are soluble and efficiently transferred into atmospheric waters (Henry's law constants respectively equal to $2.2 \times 10^2 \text{ M atm}^{-1}$ and 10^3 M atm^{-1}) (Sander, 1999). Very few papers report methanol concentrations in atmospheric waters; it was found at concentrations of $0.7 \mu\text{M}$ in rain (Snider and Dawson, 1985). Formaldehyde concentration varies in a very wide range of values (see Table 1); from $0.001 \mu\text{M}$ in rainwater in rural areas (Kieber et al., 1999) to $710 \mu\text{M}$ in highly polluted fog (Jacob et al., 1984). The average concentration measured in cloud water at the puy de Dôme station is $2.9 \mu\text{M}$ (Parazols, 2007) and depends on the air mass origins ($6.5 \mu\text{M}$ for "polluted" category and $2.0 \mu\text{M}$ for "remote" category).

It is generally admitted that the reactivity of organic acids in clouds is driven by the presence of free radicals ($\cdot\text{OH}$, NO_3) or oxidants such as O_3 or H_2O_2 (Herrmann et al., 2005; Tilgner and Herrmann, 2010). However, recent studies raised the idea that microbial activity can play a significant role in the liquid phase transformation of some organic compounds (Ariya and Amyot, 2004; Deguillaume et al., 2008; Delort et al., 2010). It is now well established that living microorganisms are present in clouds (Ahern et al., 2007; Amato et al., 2005, 2007a,c; Bauer et al., 2002; Fuzzi et al., 1997; Sattler et al., 2001). They are active in clouds as shown by ATP (Adenine Tri Phosphate) measurements (Amato et al., 2007c) and CTC (5-Cyano-2,3-ditotyl Tetrazolium Chloride) staining (Hill et al., 2007), these two techniques describe the

metabolic activity and the energetic state of the cells. This implies the uptake of nutrients from the liquid phase by cells and suggests that they can develop within cloud water. One key question for cloud chemistry now is to quantify biological oxidation as respect to chemical and photochemical processes. In the recent paper, from Vaitilingom et al. (2010), it was shown that cloudborne microorganisms could indeed contribute to the transformation of atmospheric organic compounds (carboxylic acids: acetate, succinate and formate), particularly during the night.

The aim of this study is to extend this work to the transformation processes of two important atmospheric organic compounds in clouds: methanol and formaldehyde, considering both biological activity and photochemical reactivity. It is known that the metabolic pathway involving C1 compounds results in very similar intermediates as radical chemistry (Fig. 1). However, contrarily to radical chemistry, only microorganisms can either reduce or oxidize compounds such as formaldehyde.

14 cloud samples have been collected between March 2003 and October 2004 at the puy de Dôme station (1465 m.a.s.l., $45^\circ 46'$ North, $2^\circ 57'$ West, France, see for more information: <http://www.obs.univ-bpclermont.fr/SO/beam>) (Marinoni et al., 2004; Amato et al., 2005, 2007a). From these samples, we isolated by cultivation more than one hundred strains of bacteria, fungi and yeasts. These included a large proportion of non sporing bacteria able to grow at low temperature. Amato et al. (2005, 2007b) demonstrated their abilities to degrade methanol and formaldehyde at 27°C . Here, we examined the effect of temperature on biological activity by determining the rates of biodegradation at 5°C and 17°C ; these temperatures correspond to the mean annual temperature and to the maximal temperature observed at the puy de Dôme summit when clouds form, respectively (see the database at: <http://www.obs.univ-bpclermont.fr/opgc/index.php>).

The results obtained with 4 bacterial strains belonging to the genera *Pseudomonas*, *Bacillus* and *Frigoribacterium*, the most current microorganisms isolated from clouds, are presented. The metabolism of these strains was investigated using ^1H and/or ^{13}C NMR spectroscopy. This provided indication about the pathways

Table 1

Methanol and formaldehyde concentrations measured in atmospheric waters (rain, fog and cloud) at different sampling sites under various influence (continental, marine, urban, polluted).

Compound	Sites	Samples	Concentrations (μM) Min–Max or (average)	References
HCHO	Wilmington, Rural with maritime influence (NC, US)	Rain	<0.001–13	Kieber et al., 1999
	San Rita, Rural (Arizona, US)	Rain	(7.33)	Snider and Dawson, 1985
	Florence, Urban (Italy)	Rain	0.16–14.8	Largiuni et al., 2002
	Heraklion, Coastal Urban (Crete)	Rain	0.4–11.1	Economou and Mihalopoulos, 2002
	Gdansk Wrzeszcz, Urban (Poland)	Rain	1.7–44	Polkowska et al., 2006
	Los Angeles, Urban (California, US)	Rain	0.85–45	Kawamura et al., 2001
	Los Angeles, Urban (California, US)	Rain	0–20	Grosjean and Wright, 1983
	Galicia, rural with anthropogenic influence from a thermal power plant (Northwest Spain)	Rain	0.2–2.1	Peña et al., 2002
	Sierra Nevada, Rural (US)	Cloud	8–14	Collett, Jr. et al., 1990
	5 sites, Virginia, Rural (US)	Cloud	0.8–228	Munger et al., 1995
	Los Angeles, Urban (US)	Cloud	6–43	Richards, 1995
	Henninger flat, Rural (California, US)	Cloud	0.3–36	Grosjean and Wright, 1983
	San Pedro Hill, Coastal (California, US)	Cloud	13.6–37.3	Igawa et al., 1989
	Henninger flat, Inland Mountainous site (California, US)	Cloud	45.9–61.5	Igawa et al., 1989
	puy de Dôme mountain (air masses from various origins) (Fr)	Cloud	0.1–14.3 (2.0–remote) (6.5–polluted)	Parazols, 2007
	San Joaquin, Polluted (California, US)	Fog	53–710	Jacob et al., 1984
	San Joaquin, Polluted (California, US)	Fog	2.3–410.2	Collett, Jr. et al., 1999
	5 sites, Urban/remote/marine (California, US)	Fog	0.3–76.7	Grosjean and Wright, 1983
	Fairbanks, Urban (Alaska, US)	Ice fog	16.7–38.7	Grosjean and Wright, 1983
	Riverside campus, Polluted (California, US)	Fog	4.1–228	Igawa et al., 1989
CH ₃ OH	San Rita, Rural (US)	Rain	0.7	Snider and Dawson, 1985

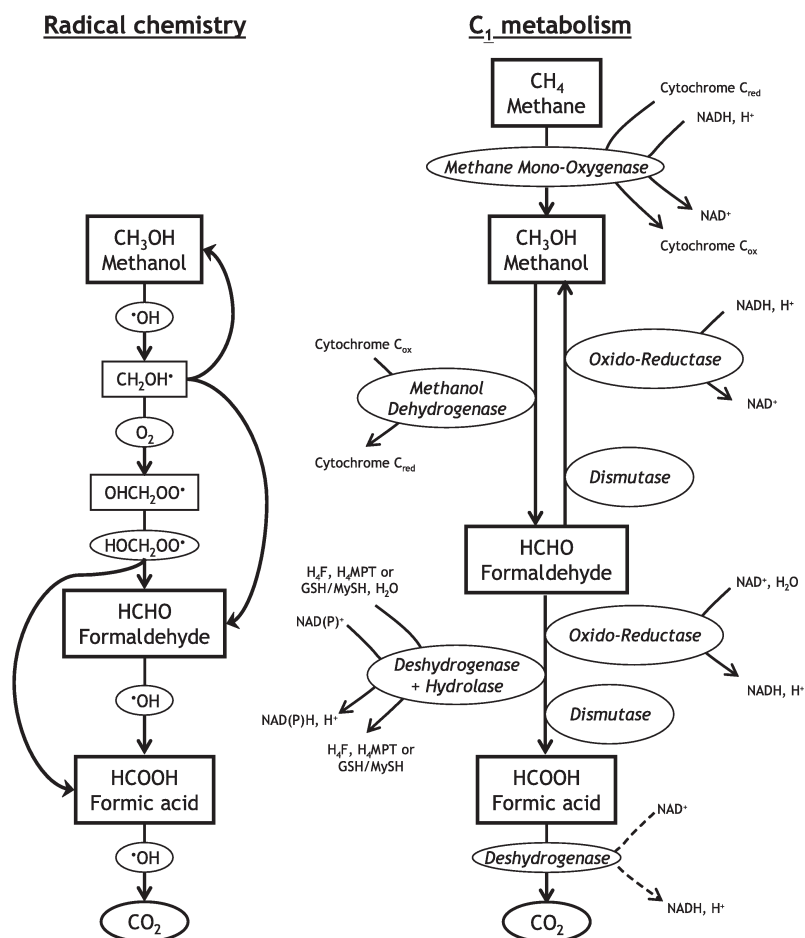


Fig. 1. Non-exhaustive schemes of the ways of degradation of C_1 compounds by radical chemistry and by metabolism. The scheme corresponding to radical chemistry has been drawn from reactions given in Monod et al. (2000) and references therein. C_1 metabolism scheme was reconstructed from Hanson and Hanson (1996), and Delort (2006) and references therein. Known enzymes are described for each reaction in which they can be involved, as well as cofactors when required (the dot line in the case of Formate, dehydrogenase signifies the existence of several types of this enzyme, with and without a cofactor). NAD: Nicotinamide adenine dinucleotide; H_4F : Tetrahydrofolate; H_4MPT : Dephospho-tetrahydromethanopterin; GSH: Glutathione; MySH: Mycothiol.

involved and allowed determining the rates of biotransformation under experimentally controlled conditions. Extrapolating to the conditions existing in the liquid phase of clouds issued from urban and remote air masses during daytime (high concentration of radicals, mainly $\cdot OH$) and during nighttime (lower level of radicals, mainly $\cdot NO_3$), the contribution of microbial activity is compared to the degradation of methanol and formaldehyde with that from radical chemistry. The objective is to evaluate the extent to which microbiological processes of organic compounds can be considered as an “alternative” route to radical chemistry in cloud water.

2. Materials and methods

2.1. Conditions of incubation for the biodegradation tests

For each isolated strain, liquid pure pre-culture was incubated at 17 °C or 27 °C in either TSA (Trypase Soy broth, Biomerieux, Marcy l'Etoile, France) or R2A broth culture media (Reasoner and Geldreich, 1985). A large volume (about 400 mL) of culture was

then incubated under similar conditions and cells were harvested by centrifugation (4000 g, 15 min, 4 °C) after 24 or 48 h of growth and rinsed twice in NaCl 0.8%. The cell pellet was then resuspended in the test medium composed of 25 mL of 0.1 M phosphate buffer at pH 7.0, supplemented with 20 mM ^{13}C -formaldehyde ($H^{13}CHO$) (Eurisotop, solution at 20% v/v in water) or methanol (CH_3OH) (Aldrich). The isotopic form of formaldehyde was used because the unlabeled commercial product contains methanol for stabilizing the solution. In addition, 1H NMR could not detect the hydrated form of formaldehyde (see for more details Amato et al., 2007b) and ^{13}C NMR allowed to monitor the fate of ^{13}C and to identify metabolites. Incubations were carried out for 24–64 h at 5 °C or 17 °C in the dark under aerobic conditions (200 rpm). Samples for analysis were taken at regular intervals of time. One millilitre of the incubation medium was centrifuged (12,000 g, 3 min) to pellet cells; and supernatants were kept frozen (−40 °C) until measurement. The experimental blank was made in parallel for each strain and consisted in cells suspended in phosphate buffer in the absence of carbon source.

In the case of *Bacillus* sp 3B6, incubations with ^{13}C -formaldehyde were performed using a perfusion system (see section 2.4) instead of agitation in flasks. ^{13}C NMR spectra were recorded *in vivo* on the sample without separation of the cells.

Cell concentration in the test media was adjusted based on the optical density ($\text{OD}_{575\text{nm}}$) to match the cell/substrate ratio existing in cloud water. In order to express the rates of degradation as $[\text{mol}]/[\text{cell}]^{-1} [\text{s}]^{-1}$, the exact cell concentration was determined by standard dilution plating of the incubation medium on R2A or TSA and CFU (Colony Forming Units) counts after 2 days incubation at 17°C or 27°C .

2.2. Measurements by ^1H NMR

Supernatants from biodegradation test media were prepared for ^1H NMR by mixing a volume of $450\ \mu\text{L}$ of the sample with $50\ \mu\text{L}$ of sodium tetra deuterated trimethylsilyl propionate (TSPd₄, Euriso-top) in solution in $^2\text{H}_2\text{O}$. $^2\text{H}_2\text{O}$ was used for locking and shimming, while TSPd₄ constituted a reference for chemical shifts (0 ppm) and quantification. Final volumes of $500\ \mu\text{L}$ of prepared samples were put in 5 mm-diameter NMR tubes. Acquisition of spectra was made at 400.13 MHz, 21°C , on a Bruker Avance 400 spectrometer, by collection of 32 scans (45° pulse, 4789.27 Hz SW, 65,536 data points, 6.84 min total acquisition time). Water signal was eliminated by presaturation, and no filter was applied before Fourier transformation. Using Bruker software (X-Win NMR), baseline was corrected before integration for quantification. The concentration of metabolites was calculated as following:

$$[m] = \frac{(9 \times A_0 \times [\text{TSPd}_4])}{(b \times A_{\text{ref}})} \quad (\text{E1})$$

where $[m]$ is the concentration of the compound to quantify, A_0 is the area of m resonance; A_{ref} is the area of TSPd₄ resonance; 9 and b respectively are the numbers of protons of TSPd₄, resonating at 0 ppm, and of metabolite m .

2.3. Formaldehyde assay

Formaldehyde resonates at about 4.5 ppm in ^1H NMR, and so it was masked by the residual signal of water, making it undetectable by this technique at 21°C . Because of this, formaldehyde was assayed by a sensitive automatic analyser (Aerolaser AL4021). The principle is based on the reaction of formaldehyde with acetylacetone and ammonia, resulting in the formation of the detected fluorochrome complex ($\lambda_{\text{excitation}} = 400\ \text{nm}$; $\lambda_{\text{emission}} = 510\ \text{nm}$), for which light emission is directly proportional to formaldehyde concentration.

Alternatively ^{13}C labeled formaldehyde was analyzed by ^{13}C NMR as described previously (Amato et al., 2007b) or by *in vivo* ^{13}C NMR (see section 2.4). ^{13}C NMR analysis allows following specifically the ^{13}C labeled metabolites issued from the transformation of ^{13}C -formaldehyde by the bacterium.

2.4. *In vivo* ^{13}C NMR study

In vivo NMR experiments were performed at 500 MHz on Bruker Avance NMR spectrometer using a perfusion system described in Choraó et al. (2009). *Bacillus* sp. 3B6 cells were perfused with phosphate buffer containing 10% of $^2\text{H}_2\text{O}$ and saturated with air. The perfusion rate was controlled by peristaltic pump (flow rate $20\ \text{mL min}^{-1}$). Bacterial suspension circulated continuously in the system. Benzene was used as an internal reference to calibrate chemical shifts ($128.39\ \text{ppm}$ relative to TMS (0 ppm)) in a sealed

capillary placed in 10 mm sample tube. Spectra were acquired with power-gated ^1H decoupling WALTZ16 sequence. Acquisition parameters were as followed: 1000 scans (corresponding to 15 min acquisition for each spectrum); 30° carbon pulse ($7\ \mu\text{s}$); relaxation delay 1 s; acquisition time 0.14 s; spectral window 30.303 Hz; data points 8000 zero filled to 16000.

2.5. Calculation of degradation rates in cloud water

The biodegradation rates of the compound C (k_c) have been determined by linear regression fits as follows:

$$k_c = (k \times [C]_0) / N_{\text{cells}} \left[\text{mol cells}^{-1} \text{s}^{-1} \right] \quad (\text{E2})$$

with $[C]_0$ the initial concentrations of selected compound C (mol L^{-1}), k the pseudo-first order decays (s^{-1}), N_{cells} the concentration of cells participating to the biodegradation and determined by CFU counts (cells L^{-1}).

The calculations of the estimated degradation rates in cloud water have been determined by:

- for bacteria:

$$R_c = k_c \times N_{\text{cells}(\text{cloud})} \left[\text{mol L}^{-1} \text{s}^{-1} \right] \quad (\text{E3})$$

- for free radicals:

$$R_c^* = k_{c,\text{radical}} \times [\text{Radical}] \times [C]_{\text{cloud}} \left[\text{mol L}^{-1} \text{s}^{-1} \right] \quad (\text{E4})$$

with $[C]_{\text{cloud}}$ the initial concentrations of selected compound C (mol L^{-1}) in the cloud water, k_c the biodegradation rate of compound C ($\text{mol cell}^{-1} \text{s}^{-1}$), $N_{\text{cells}(\text{cloud})}$ the bacterial concentration in cloud water (cells L^{-1}), $[\text{Radical}]$ (mol L^{-1}) the concentration of free radical $\cdot\text{OH}$ or NO_3 from literature and $k_{c,\text{radical}}$ ($\text{L mol}^{-1} \text{s}^{-1}$) is the 2nd order chemical rate constant between compound C and $\cdot\text{OH}$ or NO_3 from the literature.

3. Results and discussion

Four strains of bacteria, *Pseudomonas graminis* (DQ512786), *Sphingomonas* sp. (DQ512776), *Pseudomonas syringae* (DQ512783), *Frigoribacterium* sp (DQ512796) and *Bacillus* sp 3B6 (DQ512741), isolated from cloud water samples collected at the puy de Dôme summit, were studied. Their isolations and identifications were described elsewhere (see Amato et al., 2007a); briefly pure strains were isolated from individual colonies on Petri dishes and were identified by DNA sequencing and comparison with known sequences in genomic data banks (<http://www.ncbi.nlm.nih.gov/BLAST/>). These strains were selected as they belong to genera frequently encountered in atmospheric waters (Amato et al., 2005, 2007a; Fuzzi et al., 1997; Ahern et al., 2007). *Pseudomonas* strains are also usually found on vegetation, *Frigoribacterium* in cold environments and *Bacillus* are more ubiquitous (Amato et al., 2007a).

3.1. Biotransformation of formaldehyde and methanol at 5°C and 17°C

Biotransformation of formaldehyde and methanol by the 4 selected strains was investigated using mainly ^1H and ^{13}C NMR spectroscopy as previously described in Amato et al. (2007b) (see Table S1 in the supplementary data). Our experiments involved formaldehyde and methanol at concentrations higher than those in

cloud water (20 mM instead of a few μM) to allow analysis by NMR. However the substrate to cell ratios used in the incubation media were similar to those found in natural clouds. In a previous work (Vaitilingom et al., 2010) we showed that, at the concentrations used, the biodegradation rates did not depend on cell or substrate concentrations at given substrate to cell ratio.

3.1.1. Biotransformation of methanol

The biodegradation rates of methanol measured from ^1H NMR experiments at both 5°C and 17°C by the four bacterial strains are reported in Table 2. They are expressed as mole of compound degraded per cell and per second. At 17°C , all strains degraded methanol, at rates ranging from 10^{-21} to 10^{-23} mol cell $^{-1}$ s $^{-1}$. At 5°C , the rates of methanol transformation were lower and could be only measured for two strains, *P. syringae* and *Frigoribacterium* sp.; these were 5.8×10^{-22} and 2.5×10^{-23} mol cell $^{-1}$ s $^{-1}$, respectively.

Considering the metabolic pathways involved in the transformation of C1 compounds, no intermediate was detected, suggesting that methanol was directly oxidized into formaldehyde, then formate and CO_2 as described in Fig. 1.

3.1.2. Biotransformation of formaldehyde

The biotransformation of ^{13}C -formaldehyde at 5°C and 17°C was studied by ^1H NMR or ^{13}C NMR spectroscopy. The degradation rates measured are reported in Table 1 for the four bacterial strains. Formaldehyde was efficiently degraded by all four studied bacterial strains, with values ranging between 10^{-19} – 10^{-21} mol cell $^{-1}$ s $^{-1}$ at both temperatures 17°C and 5°C . Thus, contrarily to methanol, low temperature was not a limiting factor for formaldehyde bacterial degradation.

In addition metabolites detected on NMR spectra allowed to confirm the metabolic routes described in Fig. 1. First, the 4 strains were able to produce methanol by reduction of formaldehyde; in all cases methanol remained in the incubation medium and accumulated since its degradation was much slower (see above). Second, the 4 strains were also oxidizing formaldehyde into formate. In the case of *Bacillus* sp, *P. syringae* and *P. graminis*, formate concentration decreased with time as it was further transformed into CO_2 . This result is consistent with our previous observations showing that *P. syringae* and *P. graminis* were good formate degraders (Vaitilingom et al., 2010). In the case of *Bacillus* sp, the formation of $^{13}\text{CO}_2$ was clearly detected (see later Fig. 2). In the case of *Frigoribacterium* sp. formate originating from the oxidation of formaldehyde accumulated in the incubation medium. This later point was verified by incubating this strain in the presence of formate: its degradation was extremely slow and the measured degradation rates were 0.2×10^{-22} and 1.2×10^{-21} mol cell $^{-1}$ s $^{-1}$ at 5°C and 17°C , respectively.

Special attention was paid to the metabolism of *Bacillus* sp 3B6 for different reasons: first, although representatives of this genus are very frequently found in the atmosphere, few studies have

linked their metabolism to atmospheric processes; second, this particular strain was found to degrade herbicides (Durand et al., 2006), to synthesize oligosaccharides (Matulová et al., 2011) and was thought to be a good candidate having specific properties toward C1 compounds. Its metabolism was thus studied in details using a more sophisticated approach, namely *in vivo* ^{13}C -NMR. The monitoring of ^{13}C -formaldehyde biotransformation at 17°C by *Bacillus* sp 3B6 is presented in Fig. 2A. Methanol ($\delta = 49.17$ ppm), formate ($\delta = 170.24$ ppm) and HCO_3^- ($\delta = 160.5$) are the metabolites of formaldehyde expected from the C1 metabolic pathway described in Fig. 1. Fig. 3 shows that the transformation of ^{13}C -formaldehyde was completed after 200 min, that of formate after 300 min while methanol decreased very slowly after 200 min over time after its formation (this is consistent with the very low degradation rate of methanol by this strain, see Table 2). When *in vivo* ^{13}C NMR is performed, $^{13}\text{CO}_2$ is distributed between the aqueous phase ($\text{H}^{13}\text{CO}_3^-$) and the gas phase ($^{13}\text{CO}_{2\text{gas}}$). $\text{H}^{13}\text{CO}_3^-$ is in the measurement area of the spectrometer and can thus be detected by ^{13}C NMR; $^{13}\text{CO}_{2\text{gas}}$ is out of the measurement area and doesn't give any ^{13}C NMR signal. As soon as $\text{H}^{13}\text{CO}_3^-$ is formed, part of it goes to the gas phase ($^{13}\text{CO}_{2\text{gas}}$), as a result the concentration of $\text{H}^{13}\text{CO}_3^-$ seems to remain constant with time. Besides these main metabolites, other ^{13}C NMR signals (Fig. 2B) indicated the presence of glycerol ($\delta = 72.37$; 63.05 ppm), propane-1,2-diol ($\delta = 68.28$, 67.75, 18.78 ppm) and propane-1,3-diol ($\delta = 61.76$, 34.13 ppm). The biotransformation of formaldehyde into propane-1,2- and 1,3-diol has been already described in *Escherichia coli* (Hunter et al., 1984). These 3 metabolites were accumulating with time in the medium (see Fig. 2A). In addition transient signals of low intensity corresponding to the carbons of serine could be identified ($\delta = 60.65$, 62.45 and 174, 0 ppm). The presence of these metabolites demonstrated that formaldehyde can enter serine metabolism, which is connected to the metabolism of glycerophospholipid and then to that of glycerolipid involving glycerol. Glycerol can then be converted into propane-1,3-diol and propane-1,2-diol. (<http://www.genome.jp/kegg/pathway.html>, see Figs. S1–S4 in the supplementary data). This complex metabolism, involving a large number of enzymes, shows that functionalization and condensation to larger molecules (C3 compounds) from formaldehyde (C1 compound) is also possible.

3.2. Comparison with radical chemistry

Oxidation processes in the atmosphere are largely catalyzed by free radicals, of which the principals are $\cdot\text{OH}$ and NO_3 . Radical hydroxyls are generated by photochemical pathways and are implicated in the oxidation processes existing in the atmosphere during the day, while nitrate radicals are the most efficient radical oxidants during the night (Finlayson-Pitts and Pitts, 1997). Hence, in order to compare microbiological with radical chemistry

Table 2
Biodegradation rates (mol cell $^{-1}$ s $^{-1}$) of methanol and formaldehyde at 5°C and 17°C by microbial strains isolated from cloud samples. Given values are results of first approximation calculations, in which degradation rates were assumed to be linear over time.

Compound	Methanol		Formaldehyde	
	Biodegradation rate (mol cell $^{-1}$ s $^{-1}$)			
Strain and temperature	5°C	17°C	5°C	17°C
<i>Pseudomonas graminis</i>	0	5.6×10^{-22}	8.1×10^{-21}	1.9×10^{-20}
<i>Pseudomonas syringae</i>	5.8×10^{-22}	5.7×10^{-21}	8.6×10^{-20}	1.4×10^{-19}
<i>Frigoribacterium</i> sp.	2.5×10^{-23}	3.5×10^{-23}	6.4×10^{-21}	6.4×10^{-21}
<i>Bacillus</i> sp.	0	2.9×10^{-21}	3.1×10^{-21}	2.0×10^{-20}
Average (\pm Standard deviation)	$1.5 (\pm 2.9) \times 10^{-22}$	$2.3 (\pm 2.6) \times 10^{-21}$	$2.6 (\pm 4.0) \times 10^{-20}$	$4.6 (\pm 6.3) \times 10^{-20}$

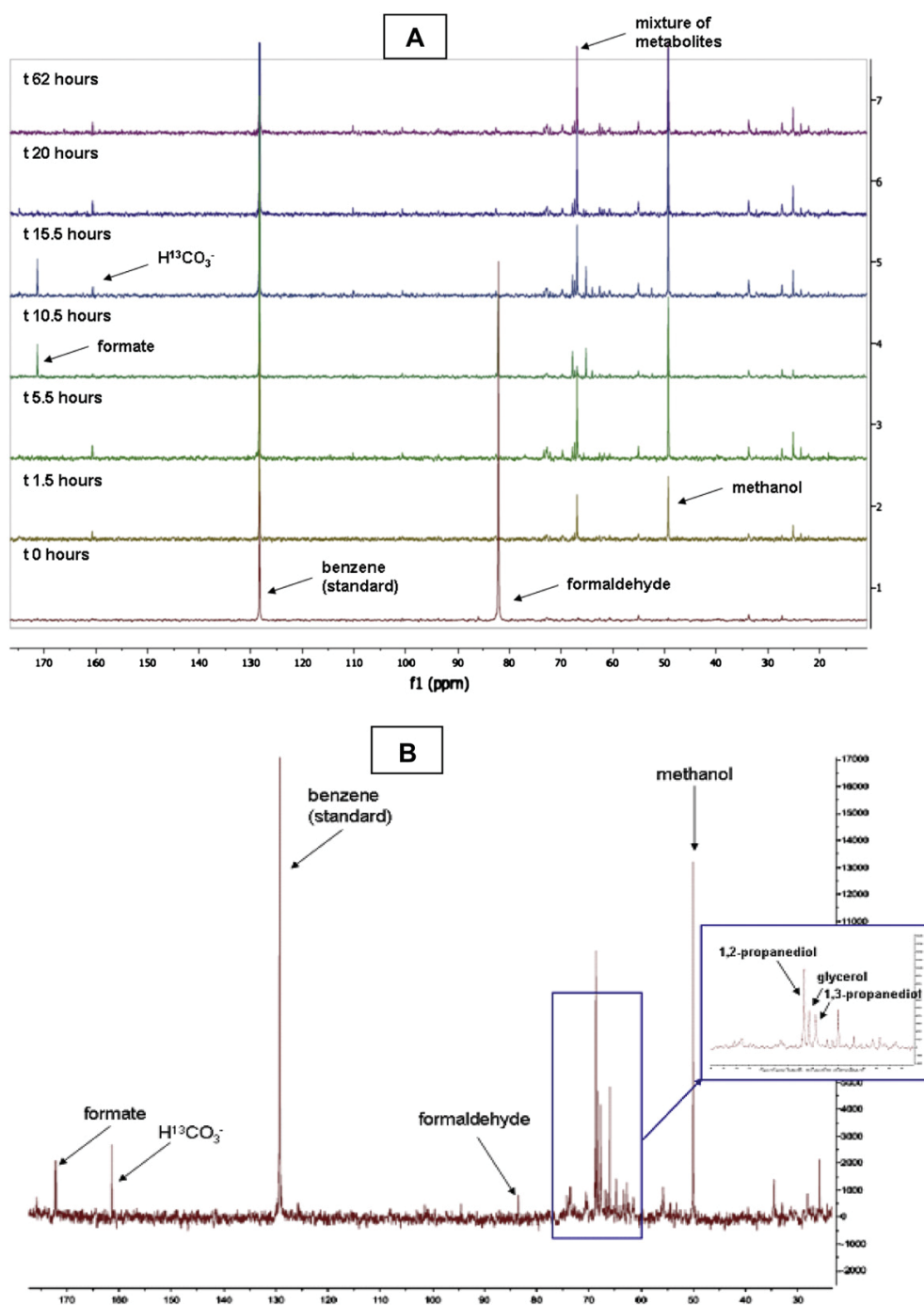


Fig. 2. A) *in vivo* ^{13}C NMR spectra collected during the incubation of *Bacillus* sp 3B6 with ^{13}C formaldehyde; B) Example of *in vivo* ^{13}C NMR spectrum collected after 4 h. Cell concentration was 10^9 cells mL^{-1} and substrate concentration was 20 mM.

processes on the transformation of methanol and formaldehyde, we have considered their reactivity with $\cdot\text{OH}$, NO_3 (Table 3). The rate constants of $\cdot\text{OH}$, NO_3 measured at 25 °C have been extrapolated at 5 °C and 17 °C by using the temperature dependency of the

kinetic constants. For microbial activity the average rate between the four strains was used (Table 2).

We extrapolated our results to typical chemical and microbial concentrations measured in cloud waters at puy de Dôme: i)

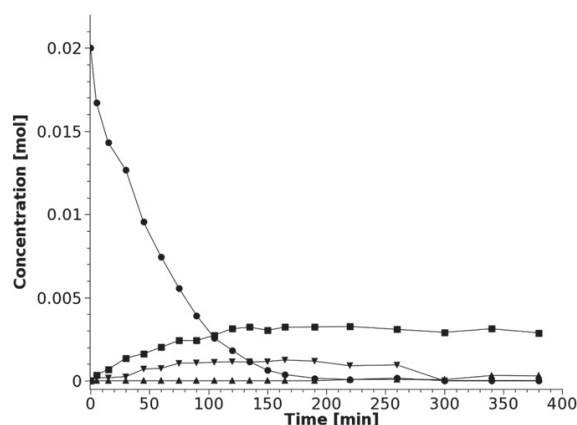


Fig. 3. Time course of the concentrations of ^{13}C -formaldehyde (●), ^{13}C -methanol (■), ^{13}C -formate (▼) and H^{13}CO_3 (▲) measured by *in vivo* ^{13}C NMR during the incubation by *Bacillus* sp 3B6 sp at 17°C .

concentrations of $6.5\ \mu\text{M}$ and $2.0\ \mu\text{M}$ for formaldehyde as mean values for urban and remote clouds respectively (Parazols, 2007); ii) active biomass of 8.1×10^4 bacteria mL^{-1} (Amato et al., 2007c). As no methanol was measured at the puy de Dôme station, we used the value of $0.7\ \mu\text{M}$ given by Snider and Dawson (1985).

For free radical concentrations, we have used the latest values published recently by Tilgner and Herrmann (2010) who considered clouds from urban origin ($[\text{OH}] = 1 \times 10^{-14}$ mol L^{-1} and $[\text{NO}_3] = 2 \times 10^{-13}$ mol L^{-1}); and clouds from remote origin ($[\text{OH}] = 5 \times 10^{-14}$ mol L^{-1} ; $[\text{NO}_3] = 5 \times 10^{-15}$ mol L^{-1}). These values are simulated by a cloud chemistry model that considers explicit multiphase chemistry. TMI (iron, manganese, copper), H_xO_y , nitrogen, sulphur and organic chemistry is taken into account and calculations are performed to simulate concentrations of radicals as

a function of chemical scenarios (remote and polluted environments) and photochemical conditions (night vs. day).

These conditions were used to quantify the relative participation of bacterial activity and free radicals reactivity to the oxidation of methanol and formaldehyde during the day and during the night (Fig. 4).

In clouds from urban origin (Fig. 4A), at 5°C , in the presence of sunlight, OH radicals are present and bacterial activity would account for 0% and 5% of the degradation of methanol and formaldehyde respectively; during the night, biological activity would only compete with NO_3 radicals and its participation to the oxidation of methanol and formaldehyde would reach 32% to 71%. At 17°C , the contribution of bacterial activity to the oxidation of methanol and formaldehyde would slightly increase (3% and 7%, respectively) during daytime and bacteria would become the major actors at night (79% and 90% respectively). In conclusion, in urban clouds, the contribution of bacteria is not negligible during the day under typical summer time conditions (17°C); this is especially true for formaldehyde degradation. At night, bacteria are much more active than NO_3 at both temperatures for formaldehyde degradation, and for methanol at high temperature. However the activity of NO_3 is the most important factor for methanol degradation at low temperature, and remains important at 17°C .

In clouds from remote origin (Fig. 4B), during day time, the influence of bacterial activity is less important than that observed in urban case, its contribution varies from 0% to 1% for methanol degradation (at 5 and 17°C respectively) and from 3% to 5% for formaldehyde degradation (at 5 and 17°C respectively) and is thus very low compared to OH activity. However, the reverse situation is observed during night time, bacterial activity becomes almost the only actor compared to NO_3 it accounts for 95–99% of methanol degradation at 5 and 17°C respectively and for 100% of formaldehyde degradation at both temperatures. Clearly, these results show that NO_3 have a very little influence on methanol and formaldehyde degradation in clouds from remote origin compared to enzymatic activity; as a consequence microbial activity would be driving the

Table 3

Degradation rates of methanol and formaldehyde extrapolated to cloud conditions (urban and remote) at 5°C and 17°C .

Cloud condition	Organism or compound (temp [$^\circ\text{C}$])	Methanol		Formaldehyde	
		Reaction rate or reaction rate constant ^a	Rate in cloud water ($\times 10^{-14}$ $\text{mol L}^{-1} \text{s}^{-1}$)	Reaction rate or reaction rate constant ^a	Rate in cloud water ($\times 10^{-14}$ $\text{mol L}^{-1} \text{s}^{-1}$)
Urban and remote	Bacteria (5°C)	1.5×10^{-22}	1.2	2.6×10^{-20}	209.5
	Bacteria (17°C)	2.3×10^{-21}	18.6	4.6×10^{-20}	376.0
Urban	OH (5°C)	8.7×10^{8b}	607.3	6.1×10^{8c}	3974.5
	OH (17°C)	9.5×10^{8b}	662.9	7.1×10^{8c}	4618.1
	NO_3 (5°C)	1.9×10^{5d}	2.7	3.4×10^{5e}	85.7
	NO_3 (17°C)	3.6×10^{5d}	5.1	6.6×10^{5e}	43.9
Remote	OH (5°C)	8.7×10^{8b}	3036.6	6.1×10^{8c}	6114.6
	OH (17°C)	9.5×10^{8b}	3314.5	7.1×10^{8c}	7104.8
	NO_3 (5°C)	1.9×10^{5d}	0.1	3.4×10^{5e}	0.3
	NO_3 (17°C)	3.6×10^{5d}	0.1	6.6×10^{5e}	0.7

The following values of concentrations have been used for calculations of rate in cloud water:

- [bacteria] = 8.1×10^4 cell mL^{-1} (Amato et al., 2007a–c);

- for urban case $[\text{OH}] = 1 \times 10^{-14}$ mol L^{-1} and $[\text{NO}_3] = 2 \times 10^{-13}$ mol L^{-1} (Tilgner and Herrmann, 2010); for remote case $[\text{OH}] = 5 \times 10^{-14}$ mol L^{-1} ; $[\text{NO}_3] = 5 \times 10^{-15}$ mol L^{-1} (Tilgner and Herrmann, 2010);

- $[\text{CH}_2\text{OH}] = 7 \times 10^{-7}$ mol L^{-1} (for urban and remote case);

- for urban case $[\text{HCHO}] = 6.5 \times 10^{-6}$ mol L^{-1} and for remote case $[\text{HCHO}] = 2 \times 10^{-6}$ mol L^{-1} .

^a Reaction rate values ($\text{mol s}^{-1} \text{cell}^{-1}$) are given for bacteria, and reaction rate constant values ($\text{L mol}^{-1} \text{s}^{-1}$) are given for free radicals.

^b Elliot and McCracken, 1989.

^c Chin and Wine, 1994.

^d Herrmann and Zellner, 1998.

^e Exner et al., 1993.

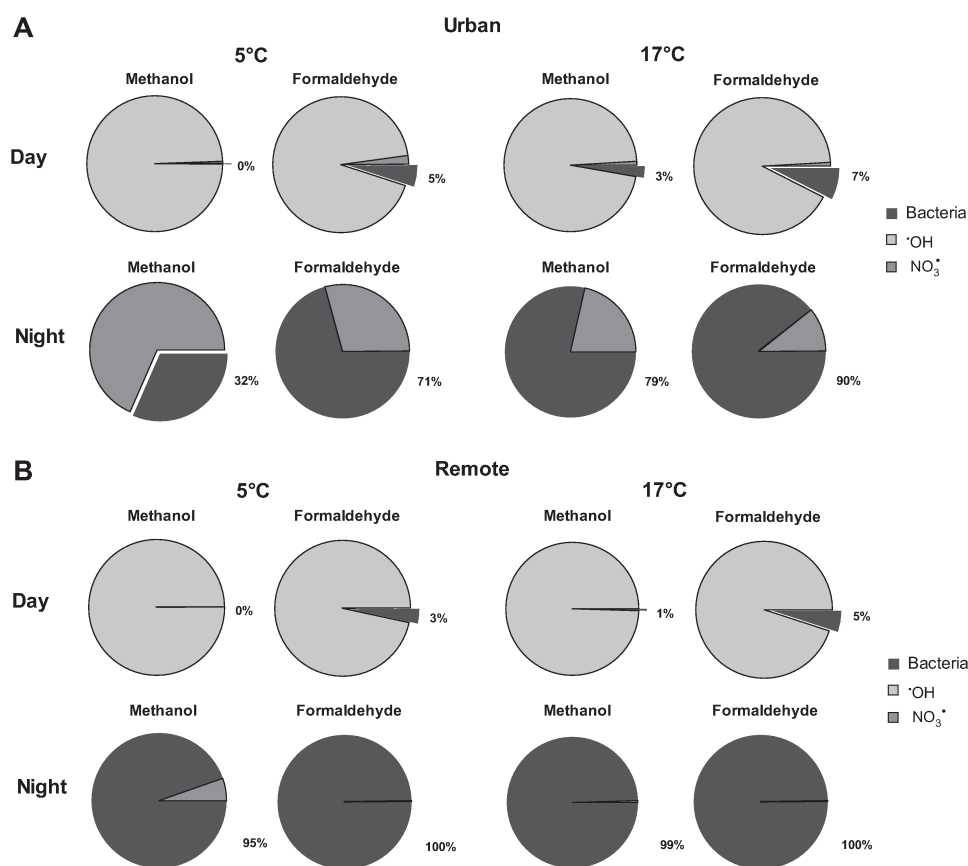


Fig. 4. Estimated relative influence of bacterial activity and free radicals ($\cdot\text{OH}$ and NO_3^-) on the degradation of methanol and formaldehyde in cloud water at 5 °C and 17 °C during the day and the night for both cloud air mass types: urban (A) and remote (B) cases. During nighttime (i.e. in the absence of photochemical reactivity), $\cdot\text{OH}$ radicals are considered not present. The rates of degradation for cloud conditions are those reported in Table 2.

chemical reactivity of C1 compounds in warm clouds ($\geq 5^\circ\text{C}$) during the night.

4. Conclusions

The biodegradation of methanol and formaldehyde by strains from cloud water collected at puy de Dôme were already demonstrated to occur under optimal conditions of temperature (Amato et al., 2005, 2007b). Here we brought new evidences that biodegradation is also possible at *in situ* temperatures: 5 °C and 17 °C, corresponding to the annual average and maximum temperatures of clouds at puy de Dôme. This is consistent with their ability to grow at 5 °C (Amato et al., 2007a), and attests of the presence of cold tolerant enzymes. Also this study allowed the comparison between microbial transformations of methanol and formaldehyde with radical pathways.

The metabolic pathways of photo- and bio-degradation of methanol and formaldehyde presented some similarities. As an example, photochemistry, through the production of radicals, is involved in the progressive oxidation of organic compounds to CO_2 in cloud water. The final step is constituted by the oxidation of formate (Monod et al., 2000; Herrmann et al., 2005), and this reaction can also be catalyzed by three out of the four microbial strains studied here (*P. graminis*, *P. syringae* and *Bacillus* sp). Methanol can be oxidized into formaldehyde by the four strains

tested or by reactivity with free radicals (see Fig. 1). The main difference between radical chemistry and biological activity is the ability of some microbes to catalyze the reduction of formaldehyde into methanol even under very oxidative conditions. In addition, we showed the ability of *Bacillus* sp strain to produce complex compounds such as propane-1,2-diol, propane-1,3-diol and glycerol. This last point is of importance concerning atmospheric chemistry as it suggests that not only successive oxidations that lead to shorten molecules can occur in cloud water but also reactions that can result in compounds of larger molecular size. In our study, C3 molecules have been built from a C1 compound. It is of major importance to be aware that such functionalization processes in the atmosphere, identified as determining for atmospheric sciences (Hallquist et al., 2009; Blando and Turpin, 2000), can be catalyzed by microorganisms.

Second, the relative contributions of microbial and radical activities ($\cdot\text{OH}$ and NO_3^-) have been quantitatively evaluated. Clearly, measured biological and chemical reaction rates are in the same range of magnitude, and their relative contribution varies according to the scenarios we have tested, including the temperature of the clouds (5 or 17 °C), the origin of the clouds (urban and remote) and the diurnal cycle (day and nighttime). The general conclusions are the following: i) bacteria activity is always more favored at higher temperatures, therefore it will be limited to warm clouds; ii) during daytime, $\cdot\text{OH}$ are obviously the preponderant actors.

However biotransformation processes could be the main sink for C1 compounds during nighttime where the radical chemistry is less efficient than enzymatic reactions. This is explained by the kinetic constant of NO₃ with these organic compounds that are lower than those with ·OH and by the lower NO₃ concentration during the night compared to those of ·OH during the day. The same conclusions were reached in our previous study where we had considered organic acids such as formate, succinate and acetate (Väitilingom et al., 2010); iii) the origin of air masses modulates the extent of the previous conclusion. Indeed NO₃ reactivity which is negligible at night compared to bacteria in remote clouds becomes of some importance in urban clouds, this is due to the higher concentration of NO₃ in polluted clouds compared to non polluted ones. However, we are aware that our experimental approach leads to some simplifications where some stressful factors for microorganisms are not considered (very acidic medium, presence of highly toxic chemical species that can characterized polluted clouds).

In conclusion, our results reinforce the hypothesis of the existence of a microbial activity in cloud droplets. It is therefore timely to consider biodegradation in parallel to free radicals oxidation among the processes catalyzing atmospheric transformation of organic matter in clouds and thus to complete cloud chemistry models integrating both chemical and biological components.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.atmosenv.2011.06.035.

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Electronic supplementary material

Biotransformation of methanol and formaldehyde by bacteria isolated from clouds. Comparison with radical chemistry

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Table S1: Summary of the various techniques used during the incubations with the 4 bacterial strains.

	¹ H NMR	¹³ C NMR	<i>In vivo</i> ¹³ C NMR	Aerolaser
Strains	(S) CH ₃ OH (C) CHO ₂ H (C) CH ₃ OH	(S) ¹³ CH ₂ O (C) ¹³ CH ₃ OH (C) ¹³ CHO ₂ H (C) ¹³ CH ₂ O	(S) ¹³ CH ₂ O (C) ¹³ CH ₃ OH (C) ¹³ CHO ₂ H (C) ¹³ Cglycerol (C) ¹³ C propanediols (C) ¹³ CH ₂ O	(S) CH ₃ OH (C) CH ₂ O
<i>Bacillus</i> sp 3B6	X	X	X	
<i>Pseudomonas</i> <i>graminis</i>	X	X		X
<i>Pseudomonas</i> <i>syringae</i>	X	X		X
<i>Frigobacterium</i> sp	X	X		X

(S): Initial substrates; (C): Compound analysed by the technique.

Figure legends

Figure S1: Methane metabolism. Formaldehyde is transformed in L serine.

(Adapted from <http://www.genome.jp/kegg/pathway.html>).

Figure S2: Serine metabolism. Serine is transformed in phosphatidyl-L-serine.

(Adapted from <http://www.genome.jp/kegg/pathway.html>).

Figure S3: Glycerophospholipid metabolism. Connection between serine metabolism and glycolipid metabolism *via* glycerophospholipid metabolism.

(Adapted from <http://www.genome.jp/kegg/pathway.html>).

Figure S4: Glycerolipid metabolism. Production of glycerol, propane-1,2-diol and propane-1,3-diol.

(Adapted from <http://www.genome.jp/kegg/pathway.html>).

Serine metabolism

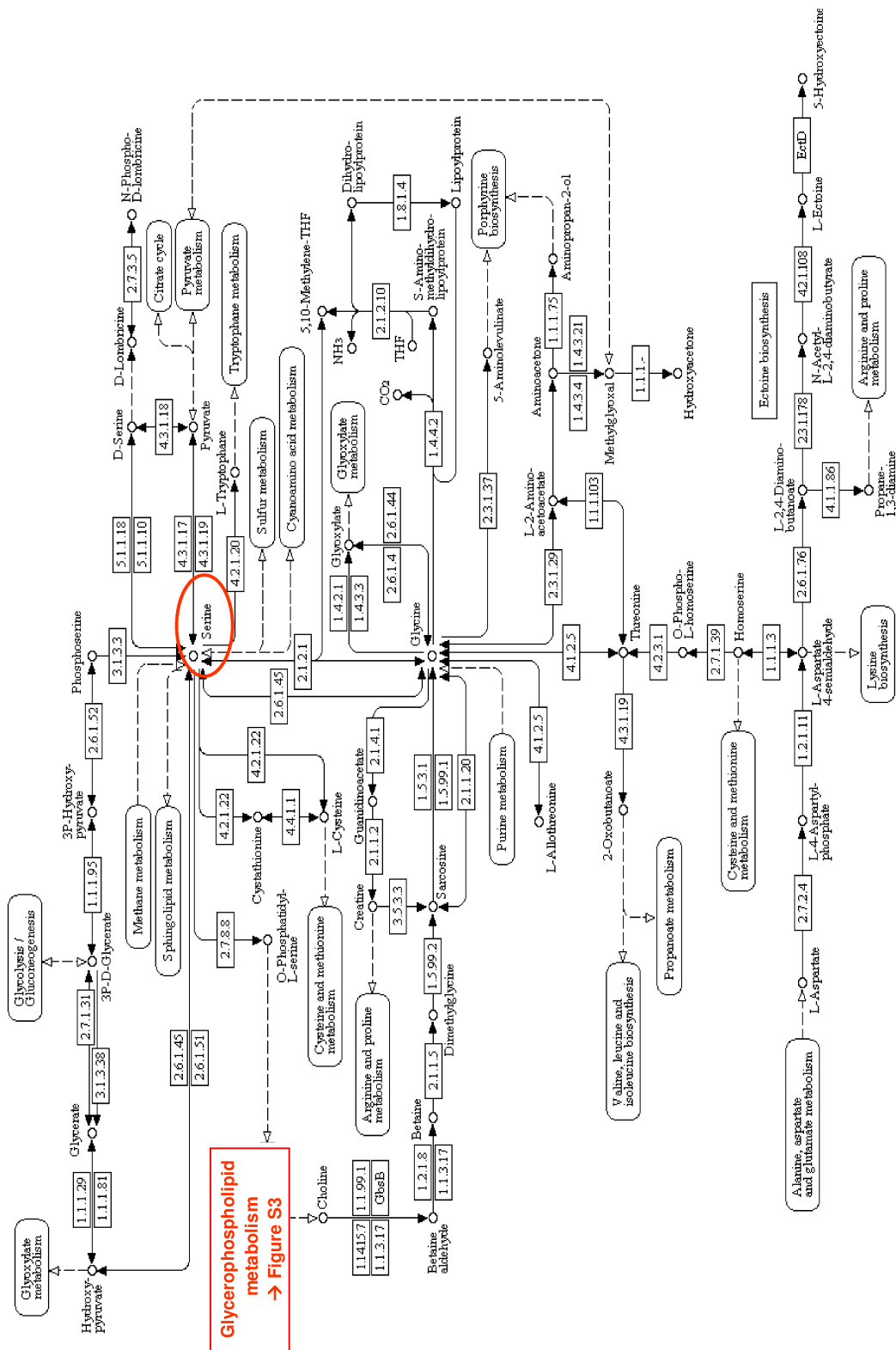
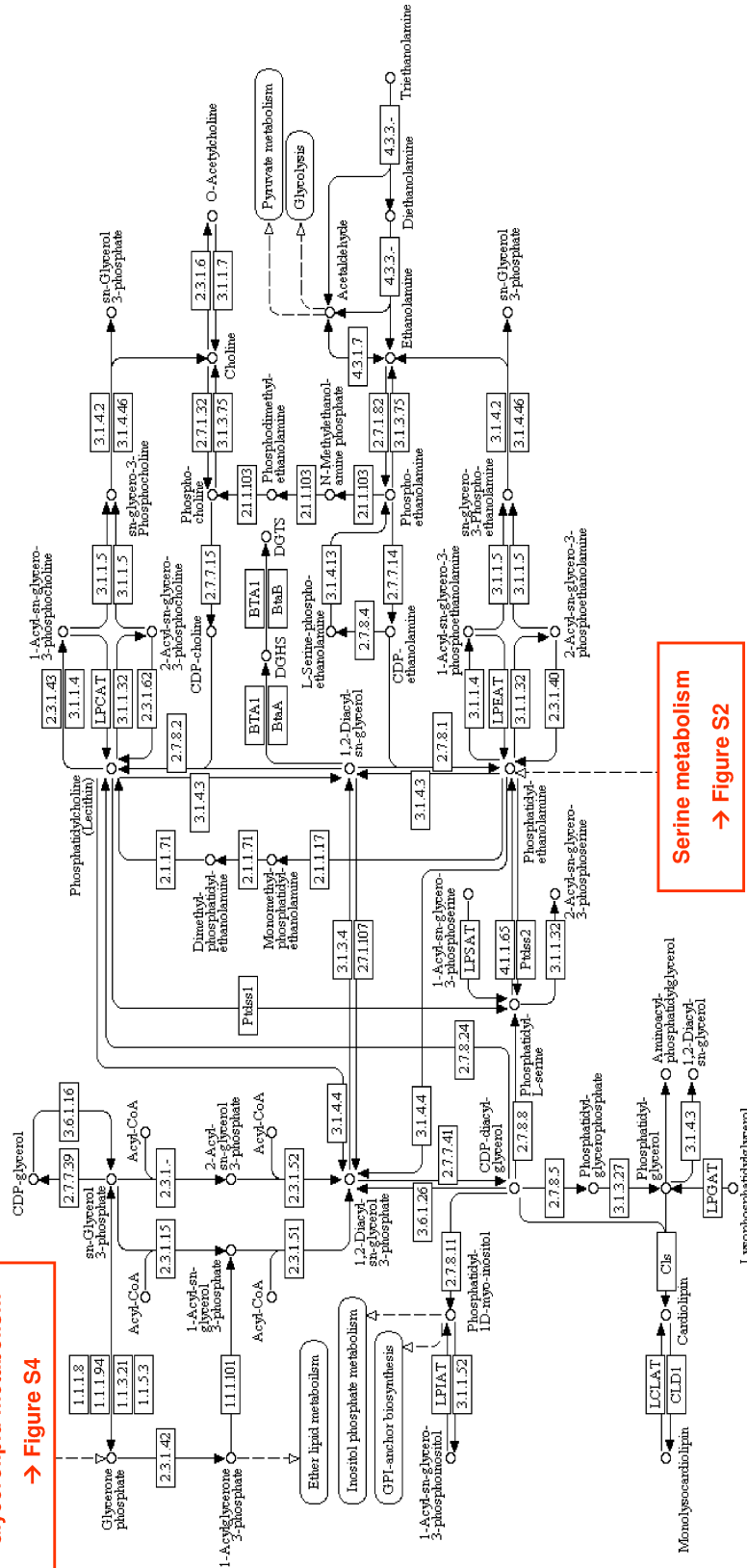


Figure S2

Glycerophospholipid metabolism

Glycerolipid metabolism
→ Figure S4



Serine metabolism
→ Figure S2

Figure S3

4.2.3 Conclusion on C1 Compounds Metabolism

The main conclusions of this paper are the following:

Formaldehyde and methanol metabolic routes

All the bacteria investigated were able to transform formaldehyde and methanol, even at low temperature (5 °C, average temperature in warm clouds).

The metabolic pathways of photo- and biodegradation of methanol and formaldehyde presented some similarities. As an example, photochemistry, through the production of radicals, is involved in the progressive oxidation of organic compounds to CO₂ in cloud water. Methanol can be oxidised into formaldehyde by the four strains tested or by reactivity with free radicals. The main difference between radical chemistry and biological activity is the ability of some microbes to catalyse the reduction of formaldehyde into methanol even under very oxidative conditions.

In addition, we showed the ability of *Bacillus* sp. 3B6 to produce complex compounds such as propane-1,2-diol, propane-1,3-diol and glycerol *via* the serine pathway. This last point is of importance concerning atmospheric chemistry as it suggests that not only successive oxidations that lead to shorten molecules can occur in cloud water but also reactions that can result in compounds of larger molecular size. In our study, C3 molecules have been built from a C1 compound. It is of major importance to be aware that such functionalisation processes in the atmosphere, identified as determining for atmospheric sciences, can be catalysed by microorganisms.

Relative contribution of microbial activity versus radical chemistry

The relative contributions of microbial and radical activities ($\cdot\text{OH}$ and

NO₃·) have been quantitatively evaluated. Clearly, measured biological and chemical reaction rates are in the same range of magnitude, and their relative contribution varies according to the scenarios we have tested, including the temperature of the clouds (5 or 17 °C), the origin of the clouds (urban and remote) and the diurnal cycle (day- and night-time). More precisely it was shown that:

i) During daytime, ·OH are obviously the preponderant actors. However, biotransformation processes could be the main sink for C1 compounds during night-time where the radical chemistry is less efficient than enzymatic reactions. This is explained by the kinetic constant of NO₃· with these organic compounds that are lower than those with ·OH and by the lower NO₃· concentration during the night compared to those of ·OH during the day. The same conclusions were reached in our previous study where we had considered organic acids such as formate, succinate, and acetate (Vaïtilingom *et al.*, 2010).

ii) The origin of air masses modulates the extent of the previous conclusion. Indeed NO₃· reactivity, which is negligible at night compared to bacteria in remote clouds, becomes of some importance in urban clouds, this is due to the higher concentration of NO₃· in polluted clouds compared to non polluted ones. However, we are aware that our experimental approach leads to some simplifications where some stressful factors for microorganisms are not considered (very acidic medium, presence of highly toxic chemical species that can characterised polluted clouds).

In conclusion, our results, together with those already obtained with carboxylic acids, suggest that microbial activity could be an alternative route to photochemistry processes in cloud waters. Of course, these experiments were performed in bulk phase while atmospheric chemistry is a multiphase process. So, to go further, cloud chemistry models should integrate both chemical and biological components.

5

GENERAL CONCLUSION

AND PERSPECTIVES

The discovery of the presence of living and active microorganisms in clouds is rather recent. Their survival ability under such hostile environment and their potential role in atmospheric processes are open questions in the scientific community. The objective of this thesis was to bring some answers to these topics.

What have we learned??

1. For living in the atmosphere

We have shown that *Bacillus* sp. 3B6 has a very active metabolism towards the compounds tested, many of them are present in the atmosphere (formaldehyde, methanol, mannitol, sorbitol, arabitol, glucose, sucrose, trehalose, and cellulose) providing efficient sources of energy for this bacterium and thus allowing this strain to live in this environment.

The second important feature is the synthesis of ExoPolymericSubstances (EPSs), polymers containing saccharides (FructoOligoSaccharides (FOSs) together of levan and inulin type; 1,6-linked α -galactan) or not PolyHydroxyAlkanoates (PHAs). All these compounds have been shown to

protect microorganisms to various environmental stresses, mainly by forming biofilms. In the particular context of the atmospheric environment these EPSs should contribute to help this strain to survive facing desiccation events, UV exposure, radical reactivity, acidity, freezing–defreezing *etc.* EPS synthesis could be a strategy complementary to the unique ability of *Bacillus* strains to form endospores for survival under extreme condition.

2. For atmospheric chemistry

Until very recently, only radical chemistry, and particularly photochemistry, was considered in atmospheric chemistry processes.

It has been already shown that the metabolism of microorganisms present in cloud waters can be a possible alternative route of dicarboxylic acid transformations to those of abiotic processes. Here we bring some evidence that microorganisms could also transform methanol and formaldehyde with rates close to those of due to radical reactivity. These biocatalytic reactions could be of major importance during the night.

More, we have also shown that *Bacillus* sp. 3B6 is able to degrade other types of compounds present in the atmosphere (see above), thus it can also contribute to the transformation of a wide range of organic matter in cloud water.

Finally, the use of NMR as an “objective tool” allowed showing that *Bacillus* sp. 3B6 was able to synthesise molecules of larger molecular weight than the initial substrate. This was the case of the synthesis of EPS, large polymers from smaller saccharide units and also for C3 compounds synthesised from C1 compounds. These compounds could be released into the cloud water and could be considered as Secondary Organic Aerosols (SOAs). This concept is rather new and of great interest at the moment; even concerning photochemistry processes in cloud water.

3. For atmospheric microphysics

The production of various metabolites that will be excreted into the medium can have some importance on microphysics. For instance, EPSs which are highly functionalised molecules could have an impact on CCN formation by interacting with water, or due to their ability to change the “wettability” of the cell surface. In this latter case they will act by changing the CCN properties of bio-aerosols.

What do we need in the future??

Our results have brought some new insights in the potential role of microorganisms in atmospheric processes and the ability of microorganisms to survive in clouds. However, they represent only a first step and a lot of experiments are needed to get final answers to these open scientific questions.

1. For atmospheric microphysics and chemistry

Our results have shown that *Bacillus* sp. 3B6 was able to metabolise various substrates present in the atmosphere; however, the experimental conditions used were very far from real cloud environment. Therefore new experiments should be conducted using “artificial cloud water” containing most of the metabolites of interest and also carboxylic acids, inorganic ions, *etc.* with more acidic pH and lower temperatures in order to mimic real cloud waters.

Under these conditions, biodegradation rates should be measured and compared to real photodegradation rates measured under the same conditions. Photo-bioreactors are now available in our laboratories. This will give some indications of the relative contribution of biodegradation processes versus photodegradation ones.

Also the synthesis of EPSs should be tested at lower temperatures close to those in clouds in order to confirm their potential role both in the

chemistry (“SOAs hypothesis”) and the microphysics (“CCN hypothesis”) of clouds.

The further step should be to work with real cloud water containing its endogenous microflora and the complex mixture of organic and inorganic compounds. Now it is possible to collect large amounts of cloud water at the summit of the Puy de Dôme thanks to the rehabilitation of the observatory equipped with chemistry and microbiology laboratories.

However, if one is interested in monitoring the degradation of compounds such as methanol, mannitol, sorbitol, arabitol, glucose, sucrose, and trehalose or other carbohydrates, it is necessary first to develop new tools to show their occurrence and to quantify their concentrations in cloud waters. Our laboratories are fully equipped with sensitive NMR probes and NMR spectrometers as well as Mass Spectrometry instruments, these tools should be used to better characterise the water phase content.

2. For living in the atmosphere

We have shown that *Bacillus* sp. 3B6 was able to synthesise numerous EPSs that could potentially protect it against stresses related to atmospheric environment. This hypothesis could be tested under laboratory conditions by exposing the bacterium to UV light, by proceeding to freezing–defreezing cycles, by acting on the composition of the medium to induce acidic, or osmotic stresses. The survival abilities could be evaluated by counting the remaining living cells or by assessing their metabolic changes. This last point could be studied using a metabolomic approach, which is currently under development in our laboratories.

6

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