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Detection and phylogenetic identification of labeled prokaryotic cells on mineral surfaces using Scanning X-ray Microscopy

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Abstract

The involvement of intraterrestrial microbes in geochemical cycles is now well recognized. However, owing to the small number of appropriate methods for probing these ecosystems, the exploration of their metabolic diversity, energy sources, and biogeochemical transformations remains limited. Here we demonstrate the ability of scanning X-ray microscopy using synchrotron radiation to localize and characterize the phylogenetic affiliation of individual prokaryotic cells on various mineral surfaces (e.g. carbonates, basaltic glass) when combined with a newly developed protocol based on fluorescence *in situ* hybridization coupled to ultra-small immunogold. The possibility to associate simultaneously the phylogenetic identification of microorganisms with the chemical and structural characteristics of associated mineral phases (i.e. inorganic substrate and biomineralizations), offers great interest for assessing the geochemical impact of subsurface microbial communities and unraveling microbe-mineral interactions in the deep biosphere.

Keywords: Prokaryotic cells; Mineralized environment; X-ray microscopy; Synchrotron radiation; Fluorescent *in situ* hybridization; Immunodetection

1. Introduction

It is now well recognized that the Earth subsurface is a major habitat for prokaryotes, and the number of microbes that thrive deep below the ground probably exceeds the number found in other components of the biosphere (Whitman et al., 1998). Indeed, microbial life colonized all lithospheric environments wherever carbon and energy sources, subsurface porosity, and temperature permit. As highlighted these last years in several reviews (Kieft and Phelps, 1997; Krumholz, 2000; Pedersen, 2000; Fredrickson and Onstott, 2001; Parkes and Wellsbury, 2004; Amend and Teske, 2005), the deep subsurface biosphere contributes significantly to the overall biomass and biodiversity on Earth. The study of the deep biosphere therefore has important implications for our understanding of global biogeochemical cycles and interactions between the biosphere and the geosphere. Our knowledge is however limited by the inaccessibility of subsurface

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microbial niches, the omnipresent risk of contamination and the low number of appropriate methods for the *in situ* probing of these ecosystems.

One of the key challenges in the understanding of the complexities of the subsurface and its microbial inhabitants is the exploration of the metabolic diversity of the prokaryotic populations, their energy sources, and biogeochemical transformations. Molecular methods are particularly useful for localizing and identifying the phylogenetic affiliation of microorganisms in their natural environments and have become an essential tool in microbial ecology. In particular, fluorescently labeled rRNA-targeted nucleic acid probes allow to rapidly determine specifically the abundance, location, and activity of individual microbial cells *in situ* (Amann et al., 1997). However, fluorescence *in situ* hybridization (FISH) has several limitations with regards to the study of microbes–mineral interaction and particularly the evaluation of the impact of microorganisms on the formation or dissolution of minerals. These limitations include the autofluorescence of the surrounding mineralized environment and the inability to simultaneously obtain chemical, crystallographic or spectroscopic information on associated mineral phases.

Over the past decade, advances in spectroscopic techniques have provided a large variety of opportunities to identify new microbiologically-mediated processes involving minerals, as recently reviewed in Geesey et al. (2002). In particular, owing to its high sensitivity, non-destructive character, high spatial resolution, and *in situ* multi-element analytical capability, third generation synchrotron light sources have been recognized as a powerful and promising new tool. Several methods implementing these small and brilliant X-ray beams were successfully applied to image individual cells. The most important ones include (cryo-) scanning transmission X-ray microscopy (STXM) using soft X-rays which allows to investigate single cells in 2D (Benzerara et al., 2005b) or 3D (Larabell and Le Gros, 2004), synchrotron infrared microspectrometry (Jamin et al., 1998) and single-particle X-ray diffraction (Miao et al., 2003). X-ray imaging based on X-ray fluorescence measurements has also been performed on whole cells with low energy X-rays (i.e. a few keV; Ueki et al., 2002; Foriel et al., 2004; Lemelle et al., 2004). In the hard X-ray range, single human (Bohic et al., 2001), yeast (Ortega et al., 2004), protist (Twining et al., 2003), and prokaryotic (Kemner et al., 2004) cells were mapped at the micrometric level, thus allowing the cell localization and identification by its light-element content. Notably, Kemner et al. (2004) succeeded in low-Z element localization of individual hydrated bacterial cells at a spatial resolution of 150 nm. However, the imaging approaches developed in these studies are not specific and consequently, do not permit cell phylogenetic affiliation. Moreover, the inframicrometric sample thickness required by some of the experimental set-up (e.g. STXM) does not allow to work on mineral surfaces without particular sample preparation (e.g. focused ion beam system, Benzerara et al., 2005a).

Coupling cell hybridization with specific ribosomal RNA targeted probes to spectroscopic techniques should open new venues of research in microbial physiology and ecology, particularly in the domain of geomicrobiology. It will allow the coupling of the phylogenetic identification of individual cells with the chemical and structural characterization of the mineral environment surrounding the cells. This direct mineral environment could act as a metabolic reactant and/or could have formed as a product of metabolic activity (biomineralization). We present here the first successful attempt at localizing and phylogenetically identifying prokaryotic cells in a mineralized environment by combining Scanning X-ray Microscopy (SXM) using synchrotron radiation with a newly developed protocol utilizing FISH technique coupled to ultra-small immunogold detection (Gérard et al., 2005). Sensitivity and resolution of this method were evaluated on the X-ray Microscopy beamline ID21 of the European Synchrotron Radiation Facility (ESRF, Grenoble, France) where we demonstrated the efficient specific identification of silver–gold-labeled cells on a variety of common mineral substrates such as Ca- and Fe-carbonates, quartz and basaltic glasses.

2. Materials and methods

2.1. Sample preparation

To evaluate the ability of SXM to localize and investigate the phylogenetic affiliation of prokaryotic cells on mineral surfaces, we used a simple model composed of a mixture of cultivable bacteria from different taxonomic divisions, readily distinguishable by their morphologies, as described in detail by Gérard et al. (2005). *Escherichia coli* (small rod) and *Deinococcus radiodurans* (large cocci), belonging respectively to the Gammaproteobacteria subdivision and the *Thermus/Deinococcus* group, were selected for this purpose. The cells were deposited on different types of substrates including polymer films (i.e. Ultralene® films), glass slides, basaltic glasses, calcite ($CaCO_3$), magnesian siderite ($(Fe, Mg)CO_3$), and quartz (SiO_2). Following the protocol described in Gérard et al. (2005), cells were grown in appropriate media and chemically fixed with