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Chromatographic method for diaminopimelic acid detection in calcareous rocks
Presence of a bacterial biomarker in stromatolites

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Abstract

The presence in the environment of diaminopimelic acid (DAP), a specific eubacterial marker, can be attributed to that of bacteria. We report a reliable and highly sensitive method for the quantification of DAP in calcareous rocks. It consists of acid hydrolysis of rock powder, purification of DAP by chromatography on Dowex 50W and Spherogel AA-NA^+ columns, and quantitative analysis by high-performance liquid chromatography. Addition of tritiated DAP, the internal standard, allows one to follow the relevant fractions throughout the purification procedure and to determine their yield. The analytical step consists in pre-column derivatization with *ortho*-phthaldialdehyde of purified samples, and separation through a reversed-phase C_{18} column. Chemical controls, i.e., oxidation of samples to rule out the presence of co-eluting lanthionine and cystathionine, as well as mass spectrometry, confirm the presence of DAP in analyzed samples. Our method allows the separation of *meso*- from L- and/or D-stereoisomers of DAP, and reveals their presence in the examined rocks, two stromatolites of different age and geographic origin.

Keywords: Stromatolite; Geological samples; Diaminopimelic acid

1. Introduction

Bacteria and archaebacteria are present in all biotopes on the Earth, and form no less than half of the terrestrial biomass [1]. The oldest so far uncovered microfossils, similar to extant cyanobacteria, are $3.5 \cdot 10^9$ years old [2].

The bacterial cell wall, which can represent up to 10% of the cell dry mass, contains peptidoglycan (PG), a near ubiquitous, highly specific, bacterial marker (Fig. 1A). Although the PG composition is species specific, diaminopimelic acid (DAP) occupies the third position within the muropeptide of many Gram-positive and Gram-negative bacteria, as well as cyanobacteria. It is a molecule with two asymmetric carbon atoms which allows the formation of three different stereoisomers: the L-, the D-, and the *meso*-configurations (Fig. 1B). Although the vast majority of bacteria incorporates the *meso*-dia-

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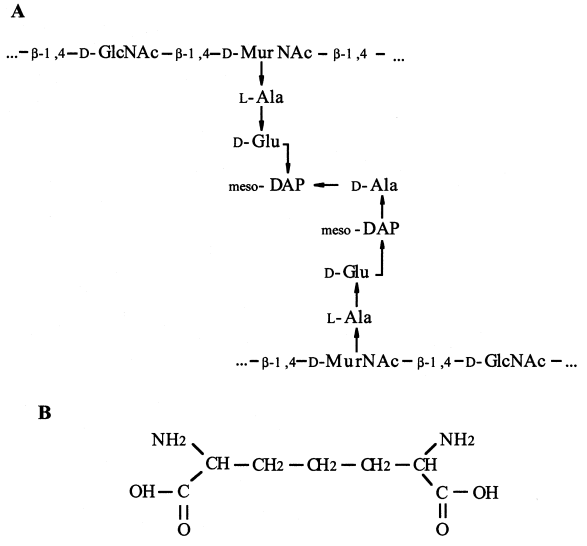

Fig. 1. Typical bacterial cell wall components. PG consists of a 50 to 150 disaccharide (*N*-acetylmuramic acid–*N*-acetylglucosamine) long chains coupled with peptide bridges (A). DAP (B), a molecule with two asymmetric carbons, capable of taking three isomeric configurations, plays a key role in transpeptidation.

stereoisomer, L-DAP has been identified in the PG of a few species.

DAP was found in peat, in marine sediments, as well as in soil [3–8]. However, although it is accepted that microorganisms, in particular bacteria, play a role in the formation of certain stromatolites [9], no method for DAP analysis in rocks is presently available [10].

We believe that a better characterization of bacterial remnants in sedimentary rocks may provide some clues to the complex and very slow processes involved in their formation. In this contribution, we describe a sensitive chromatographic method for the determination of DAP in calcareous rocks. Comparison of quantities of DAP and proportions of the *meso*-diastereoisomer in two rocks of different age and geographic origin raises a few interesting questions which will be briefly discussed.

2. Experimental

2.1. Rock samples

Two stromatolites are analyzed. A 890 Ma one from Taoudenni basin, Mauritania, and a contemporary one from Lake Thetis, Australia.

2.2. Chemicals

DAP (purum 97%), hydrochloric acid 37% (Trace Select), boric acid (puriss.), 2-mercaptoethanol (puriss.), *ortho*-phthaldialdehyde (OPA; for fluorescence applications), hexadecyltrimethylammonium bromide (HTMA; MicroSelect for molecular biology), hydrogen peroxide 30%, ethyl chloroformate, pyridine, and Dowex 50W-X8 resin were from Fluka. Sodium acetate (analytical-reagent grade), sodium citrate (analytical-reagent grade), sodium borate (analytical-reagent grade), and formic acid 98% were from Merck; methanol [high-performance liquid chromatography (HPLC) grade] was from Biosolve, and acetic acid (HPLC grade) from BDH. Milli-Q quality water (more than 18 MΩ cm) was obtained by a Millipore system. A racemic solution of tritiated DAP with a specific activity of 60 Ci/mmol (10 μCi/ml) was from American Radiolabeled Chemicals (St. Louis, MO, USA). The ion-exchange Spherogel AA-NA$^+$ 250 mm×3 mm column was from Beckman Coulter International, and the reversed-phase C_{18} 120:3 Nucleosil 150 mm×4.6 mm column from Macherey–Nagel (Switzerland).

2.3. Preparation of rock powder

A compact microcrack-free zone of the rock is first drilled to a depth of 3 mm with a 10 mm flamed twist drill. The obtained powder is discarded. At the bottom of this hole, a deeper, 7 mm wide, hole is drilled, and the generated rock powder collected and kept in sterile polypropylene tubes.

2.4. Sterility during procedure

Solutions are sterilized by filtration through 0.20-μm Nalgene filters. Disposable, sterile, polypropylene recipients were used. Hydrolysis is performed in sterile glass vials.

2.5. Hydrolysis

About 0.5 g of rock powder is dissolved in 10 ml of 6 *M* HCl, containing 10 μl of tritiated DAP (10

μCi/ml), the internal standard. Samples, hydrolyzed during 20 h at 110°C, are dried with a SpeedVac.

2.6. Purification

A 15-ml column of Dowex 50W-X8 resin, conditioned with 60 ml of 4 *M* HCl, is equilibrated with 40 ml of water to neutral pH. Samples resuspended in 1 ml of water are loaded onto the column and the resin is washed with 30 ml of water. Following elution with a 1.5 *M* NH_3 solution [11], 5-ml fractions are collected. Those containing tritiated DAP are dried with a SpeedVac.

For the next purification step, samples are resuspended in 500 μl of buffer A (see below). The separation is achieved on a 250 mm×3 mm Spherogel AA-NA^+ column, using a Dionex quaternary gradient pump GP240 and an autosampler AS3000. The mobile phase consists of 0.2 *M* sodium citrate, pH 3.28 (buffer A), and 0.2 *M* sodium borate, pH 10 (buffer B). Samples are injected into the column maintained at 50°C and eluted with a gradient from 0 to 60% of buffer B during 25 min, at a flow-rate of 0.4 ml/min. Fractions containing radioactivity are collected and dried with a SpeedVac.

2.7. Controls

At this stage, presence of DAP in samples can be confirmed by gas chromatography–mass spectrometry (GC–MS) with a system consisting of a Finnigan MAT 8430 mass spectrometer, connected to a Hewlett-Packard HP-5890 gas chromatograph equipped with a HP-7673 autosampler. Samples, derivatized with ethyl chloroformate according to the method of Husek [12], are injected into the GC–MS system in splitless mode at 280°C. They are separated on a J&W Scientific capillary column DB-5 (MS-Wil, Wil, Switzerland), 30 m×0.32 mm I.D., film thickness 0.25 mm. The temperature gradient is as follows: 60°C (1 min), 25°C/min to 150°C, 10°C/min to 300°C (2 min). The carrier gas is helium at a pressure of 10 p.s.i. (1 p.s.i.=6894.76 Pa). Mass spectra, acquired after electron impact ionization at 70 eV, are recorded from 20 to 500 amu.

An additional control consists of incubating the samples 15 min at 50°C in a solution of hydrogen peroxide–formic acid (1:9, v/v) prior to analysis [13]. This oxidation step eliminates molecules which could co-elute with DAP [14].

2.8. Quantitative analysis

Dried samples are resuspended in 250 μl of water and derivatized by mixing 50 μl of OPA reagent solution [15] with 50 μl of sample. After 2 min, derivatized samples are injected into the column. A HPLC binary gradient system with buffers having ion-pairing properties is used [16]. Buffer A: 0.1 *M* sodium acetate–methanol (60:40, v/v), 7.5 m*M* HTMA, pH 6.4. Buffer B: 7.5 m*M* HTMA, water–methanol (5:95, v/v). The HPLC system consists of a Perkin-Elmer gradient pump LC 200, a fluorescence detector serial 200, an autosampler serial 200 with a TurboChrom 4 data acquisition software, and a reversed-phase C_{18} 120:3 Nucleosil column. The sample is eluted through the column with the following concentrations of buffer B: 50% from 0 to 5 min, increasing to 80% between 5 and 25 min, remaining at 80% from 25 to 35 min. The flow-rate is 1 ml/min. The wavelength for excitation of OPA derivatives is 340 nm and the fluorescence of derivatized molecules detected at 450 nm.

3. Results and discussion

3.1. DAP determination

The procedure for the identification and the quantification of DAP in rocks, schematically represented in Fig. 2, was derived from the method devised for DAP assays in physiological fluids [14].

To avoid recent surface contamination, rocks were drilled in two steps (see Experimental). Powdered samples were resuspended in 6 *M* HCl and hydrolyzed for 20 h at 110°C, a treatment allowing one to dissolve 81.5±4.2% of the Mauritanian stromatolite and 86.1% of the Lake Thetis sample. Two purification steps consisting of separation on cationic exchangers – a Dowex 50W resin, and subsequently a Spherogel AA-NA^+ column – are performed. For each sample, the final purification yield, calculated from the radioactivity of the internal standard, was about 50%. Following OPA derivatization, purified samples were injected into a reversed-phase C_{18} 120:3 Nucleosil column, and the derivatized mole-

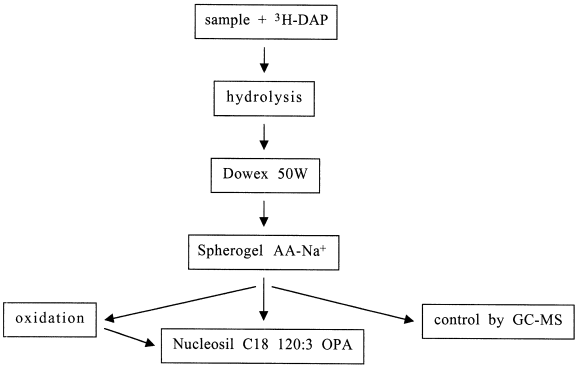

Fig. 2. Schematic representation of the method. Tritiated DAP is added to the 6 *M* HCl solution containing rock sample reduced to powder. Labeled DAP allows one to follow relevant fractions and to calculate the final yield. Following hydrolysis, samples are purified by ionic chromatography on a Dowex 50W and a Spherogel AA-NA$^+$ column. The obtained DAP-containing fraction is pure enough to be derivatized and analyzed by HPLC with a reversed-phase C_{18} 120:3 Nucleosil column. Additional controls – GC–MS and/or sample oxidation prior to HPLC analysis – are occasionally performed.

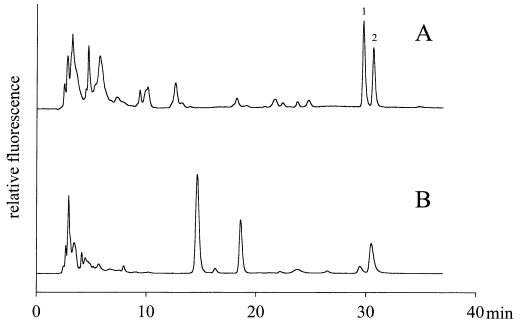

Fig. 3. (A) 50 pmol of DAP standard; peak 1 corresponds to L- and D-conformations and peak 2 to *meso*-DAP. (B) 70 mg of the Lake Thetis hydrolyzed stromatolite sample.

cules detected with a fluorescence detector. The amount of DAP in the samples was calculated from standard curves, which were obtained for each independent experiment for commercial unlabeled as well as tritiated DAP. The radioactive internal standard present in the samples was taken into account. The sensitivity of the method allows one to detect about 50 fmol of each isomer of standard DAP. A peak is considered to be detectable when its height is twice that of the baseline. However, when estimating this limit for rock samples, the solubilization and purification yields should be taken into account.

Analysis of both tritiated and commercial unlabeled DAP yielded two peaks on the chromatograms: peak 1 corresponding to L- and D-enantiomers, and peak 2 to *meso*-DAP (Fig. 3). These peaks were identified by comparison to DAP stereoisomers present in hydrolyzed *Bacillus subtilis* cell walls known to contain no less than 95% of *meso*-DAP.

The method and its repeatability were tested on the 890 Ma Mauritanian stromatolite (three samples) and the contemporary Australian stromatolite from Lake Thetis (five samples). Each sample was obtained from a different part of the rock and independently assayed (Table 1). DAP contents and corresponding standard deviations were 41.6±10.4 and 49 800±9700 pmol/g for the Mauritanian and the Australian samples, respectively (Table 1). Expressed in *Escherichia coli* equivalents, calculated according to Borruat et al. [14], the measured DAP amounts correspond to about $7{\cdot}10^6$ and $8{\cdot}10^9$ *E. coli* cells per gram, respectively (Table 1). The proportion of *meso*-DAP, about 70% for the Mauritanian sample, reached 85% for the contemporary Lake Thetis stromatolite (Table 1).

In view of the minute quantities of DAP found in

Table 1
DAP in calcareous rocks

Sample	Age	Number of samples	pmol DAP per g rock[a]	*meso*-DAP (%)	Equivalent *E. coli*·10^6 per g
Mauritania	890 Ma	3	41.6±10.4	69.5±13.7	7.2±1.8
Lake Thetis Australia	Recent	5	49 800±9700	85.6±2.8	8600±1700
Lake Thetis Australia heated at 1000°C	–	1	–	–	–
Blank[b]	–	1	–	–	–

[a] Relative to the fraction of the rock dissolved in 6 *M* HCl.
[b] The 6 *M* HCl hydrolysis solution.

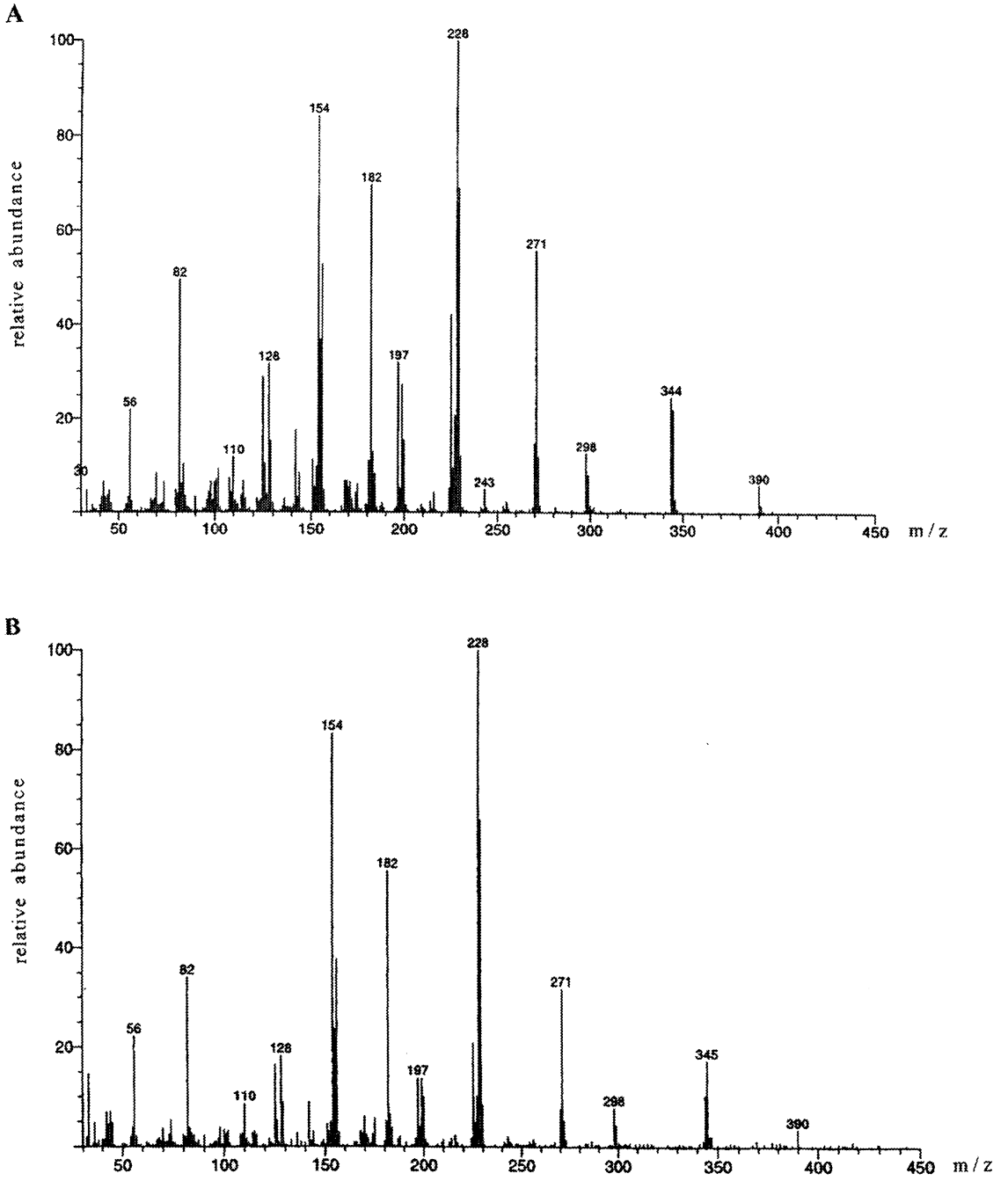

Fig. 4. Mass spectra of a DAP standard (A) and a Lake Thetis stromatolite sample (B). Spectra obtained by fragmentation of derivatized DAP (A) are characterized by a molecular ion at m/z 390 and fragments at m/z 344 ($[M-C_2H_5OH]^+$), 298 ($[M-2xC_2H_5OH]^+$), 271 ($[344-CH_3CH_2OCO]^+$), and 228 ($[M-88-73-H]^+$). The identity of the molecular ion was controlled after positive chemical ionization with ammonium as reagent gas (data not shown).

some rocks, we had to eliminate the objection of a contamination inherent to the processing. For that purpose, the Australian sample containing considerable amounts of DAP was heated during 14 h at 1000°C, a procedure intended to chemically degrade the DAP. Analysis of this material did not reveal any trace of DAP.

Oxidation of samples (Fig. 2), prior to derivatization and HPLC analysis, was performed to eliminate cystathionine and lanthionine, molecules that co-elute with the *meso*-DAP diastereoisomer [14]. Since this treatment did not affect the size of the DAP peaks on the chromatograms, it appeared that neither cystathionine nor lanthionine was present in the samples. Analysis of ethyl chloroformate derivatized samples by GC–MS clearly confirmed that both samples contained DAP (Fig. 4).

4. Conclusions

The method described here above was designed to identify DAP – a component considered to be a eubacterial taxonomic marker – in calcareous rocks. The reliability of the method was tested with a chemical control (oxidation of the samples) and by GC–MS, confirming presence of DAP in our samples.

The Australian stromatolite exhibits an extensive porosity, offering to bacteria a way to deeply infiltrate the rock and, possibly, even widen the pores. The high quantities of DAP (Table 1) strongly suggest that this rock was probably contaminated at a stage posterior to its formation by specific bacterial species containing 85% *meso*-DAP, a figure revealing a bacterial flora characteristic of the present day biosphere.

Interestingly, the proportion of *meso*-DAP in the 890 Ma compact Mauritanian sample is about 70% (Table 1). Taking into account the age of this stromatolite and assuming that it was not recently contaminated, it would appear that the DAP racemization process reached an equilibrium specific to the diagenesis conditions reigning within the rock, i.e., 7 *meso*-DAP to 3 L- or D-DAP, which was not altered subsequently.

In conclusion, analysis of rocks by our method allows us to detect and quantify DAP, a highly specific bacterial marker. Its presence in stromatolites offers a powerful evolutionary tracer throughout the 3500 Ma period during which bacteria undoubtedly colonized our planet.

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