Radical induced damage of *Micrococcus luteus* bacteria monitored using FT-IR spectroscopy

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Abstract. Oxidative damage induced by ascorbic acid (AA) and hydrogen peroxide (H_2O_2) was monitored by Fourier transform infrared spectroscopy (FT-IR); it appeared as a rapid and convenient means to follow the biochemical changes generated in the culture media of the yellow-pigmented *Micrococcus luteus*. Beyond a threshold of 20 mM for AA and of 40 mM for H_2O_2 (final concentration), antioxidant systems were overwhelmed and significant changes were observed in the bacterial spectra, particularly in the $1430\text{--}900\text{ cm}^{-1}$ region; this spectral window provided large information about carboxylate groups, phosphate-carrying compounds and polysaccharides implicated in the radical process. The spectroscopic results indicated that for the same final concentration, the toxicity of H_2O_2 was less important than that of AA toward *M. luteus* cells, although H_2O_2 had a more damaging effect on proteins. Thus, FT-IR spectroscopy was an appropriate physico-chemical tool suitable in biochemical and clinical research for early characterization of any type of radical aggression, and for rapid detection of the damage intensity.

Keywords: FT-IR spectroscopy, *Micrococcus luteus*, free radicals, ascorbic acid, hydrogen peroxide

1. Introduction

Oxygen free radicals and reactive oxygen species (ROS) are continuously produced during cell metabolism [1]. Aerobic organisms have developed processes for protecting against free radicals and derived toxic species. Many antioxidant defense mechanisms are known such as enzymatic (catalase, superoxide dismutase, glutathione peroxidase) or non-enzymatic (vitamins A, E, C) systems. However, when oxidative stress increases, these systems may be overwhelmed.

Ascorbic acid (AA) is considered as the most important antioxidant in cells [2]. It protects both cell membranes and intracellular constituents via its interaction with vitamin E [3]. It is a potential scavenger of superoxide anion and singlet oxygen, the forms of oxygen primarily responsible for oxygen toxicity [4]. However, increasing levels of AA do have deleterious effects; its highly reactivity with transition metals known to promote metal-dependent oxidative damage has suggested that AA may act as a pro-oxidant [5] and thus enhances oxygen radical activity. Another source of oxygen toxicity is H_2O_2 which plays a radical forming role as an intermediate in the production of more reactive ROS molecules. Once produced or added exogenously [6], it is detoxified by enzymatic systems as catalase and converted into

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water and molecular oxygen. However, catalase affinity for H_2O_2 is relatively low and a certain amount of H_2O_2 remains in cells [7]. H_2O_2 decomposition can also proceed through Fenton-reactions catalyzed by transition metal ions producing hydroxyl radicals ($\bullet\text{OH}$) capable of doing more damage to biological systems than the other ROS [8].

Since oxidative damage to biological membranes has been postulated to be one of the underlying causes of tissue injury, it was essential to make an early diagnosis of the involvement of free radicals. However, this was extremely difficult, due to the short lifetime of these species and also to the lack of sensitive technology to detect radicals directly in biological systems [9]. A means of cellular investigation is Fourier transform infrared (FT-IR) spectroscopy which has undergone enormous progress for several years. This non-destructive analytical tool provides a complete and highly specific fingerprint of cells [10]; it is a rapid method requiring minimal sample handling. Whole cells are tested in the mid IR region, between 4000 and 500 cm^{-1} , which hold characteristic bands, and their IR spectra reflect their total chemical composition. Moreover, this method is suitable for the understanding of various mechanisms occurring in cell components and/or complex biological materials, e.g. to distinguish microbial cells at different taxonomic levels [11], to identify conformational differences between normal and leukemic cells [12], to perform toxicologic studies [13].

Thus, FT-IR spectroscopy could be an appropriate and useful technique to bring to the fore free radical aggression. The purpose of this study was to investigate the potential toxicity of AA and H_2O_2 added at increasing concentrations in the culture media of *Micrococcus luteus*, a yellow-pigmented and gram-positive bacterium, which is the type species of the heterogeneous genus *Micrococcus* included into the *Micrococcaceae* family. Our objective was to analyze the cellular changes induced by radicals in *M. luteus* and to visualize results by hierarchical grouping.

2. Materials and methods

2.1. Bacterial growth and sample preparation

The yellow-pigmented *M. luteus* CIP 5345 (wild-type formerly *M. lysodeikticus*) strain was used in this study. A strict experimental protocol concerning medium, incubation time, temperature, bacterial harvest and sample preparation was first established. Then, *M. luteus* was grown under aerobic conditions [14] in a shaking water bath at 33°C in a glucose-supplemented bactotryptone-yeast extract medium (TGY). Bacterial growth was monitored by determining optical density at 600 nm (OD_{600}).

Before treatment, bacteria were grown for 24 h in order to reach the stationary growth phase (OD of 1.2 to 1.4). After this time, three independent experiments were conducted, (i) without any chemical (control cells), (ii) with H_2O_2 , (iii) with AA (1 M and 0.4 M in 1 l of distilled water, respectively). These chemicals were added to bacterial cultures at increasing final concentrations ranging from 1 to 200 mM for both AA and H_2O_2 . After an incubation time of 6 h in the shaking water bath at 33°C, the untreated and treated cells were harvested by centrifugation at 6000g for 20 min, and cell pellets were washed twice with physiological saline solution and suspended in 1 ml of distilled water. They were stored at -20°C until analysis to preserve their integrity.

2.2. FT-IR analysis

Thirty-five μl of each bacterial suspension were transferred to a ZnSe (zinc selenide) optical plate suitable for absorbance FT-IR measurements of up to 15 samples. Then, the suspensions were dried

under moderate vacuum (3–6 kPa) to form transparent films. The optical plate was protected by a KBr disk and transferred to the analysis compartment of the spectrometer purged with nitrogen.

For each bacterial sample, experiment was made in triplicate; consequently, three different spectra were obtained. The infrared spectra were recorded against a blank background within the mid-infrared region between 4000–500 cm^{-1} (wavenumbers) on an IFS 28/B FT-IR spectrometer (Bruker, Karlsruhe, Germany) equipped with a DTGS (deuterated triglycine sulfate) detector. To improve the signal-to-noise ratio for each spectrum, 64 interferograms were co-added, averaged, apodized with the Blackman–Harris 3-term function and a zero-filling factor of 4, and then Fourier transformed [11]. Spectra showing either high absorption of water vapour or peak absorbance intensities outside of the limits chosen (i.e. between 0.8 and 1.2) were excluded from the data set. Neither baseline correction nor normalization of spectra were done in our experiments. To enhance the resolution of superimposed bands (e.g. overlapping components), the second derivatives of the original spectra were calculated using the Savitsky–Golay algorithm combined with 9 smoothing points. For hierarchical grouping, Ward's algorithm was applied (using first derivative spectra) which fuses two clusters yielding the least heterogeneity, and therefore will construct the most homogeneous groups [15]. Recording of spectra, data storage and all other manipulations, such as integration of peak areas, were performed using the Opus 4.0 software (Bruker).

2.3. Statistical analysis

The IR data (i.e. band spectral areas) were expressed as mean $\pm$ SD and were paired *t*-test, using $P < 0.05$ as the limit of significance.

3. Results and discussion

3.1. Hierarchical classification

In a first set of experiments, a pool of 42 independent *M. luteus* cultures was analyzed. A first group of cultures ($n = 12$) was untreated and served as control. A second group ($n = 30$) was treated with H_2O_2 concentrations ranging from 1 to 200 mM. Hierarchical classification of the 42 sample spectra (Fig. 1A) in the whole mid-infrared region (4000–500 cm^{-1}) resulted in the formation of two main clusters (C_1 and C_2) separated by a linkage distance of 705. The first one (C_1) was subdivided into two clusters (heterogeneity of 110 between them) corresponding to untreated samples (*a*) and samples treated by low H_2O_2 concentrations ranging from 1 to 40 mM (*b*). The second one (C_2) included the other samples treated with H_2O_2 concentrations ranging from 50 to 200 mM (*c*).

In a second set of experiments, a pool of 42 independent *M. luteus* cultures was analyzed. A first group of cultures ($n = 12$) served as control (as in the first experiment). A second group ($n = 30$) was treated with AA concentrations ranging from 1 to 200 mM. Hierarchical classification of the 42 sample spectra (Fig. 1B) in the 4000–500 cm^{-1} region resulted in the formation of two main clusters (C_3 and C_4) separated by a linkage distance of 1305 (approximately two-fold higher than between C_1 and C_2). The first one (C_3) was subdivided into two clusters (heterogeneity of 95 between them) corresponding to untreated samples (*a*) and samples treated with low AA concentrations ranging from 1 to 20 mM (*d*). The second one (C_4) included the other samples treated with AA concentrations ranging from 30 to 200 mM (*e*).

Thus, we can conclude that final concentrations of up to 40 mM for H_2O_2 and 20 mM for AA, leading to similar heterogeneity between untreated and treated cells, globally induce same spectral changes in

Fig. 1. Dendrograms of the cluster analysis of *M. luteus* cells in the 4000–500 cm⁻¹ spectral region using first derivative spectra. (A) C₁: control cells (group a) and weakly H₂O₂ treated cells (group b; 1–40 mM); C₂: highly H₂O₂ treated cells (group c; 50–200 mM). (B) C₃: control cells (group a) and weakly AA treated cells (group d; 1–20 mM); C₄: highly AA treated cells (group e; 30–200 mM). Ward's algorithm was applied.

M. luteus. In an opposite side, damage was higher with AA when increasing levels of the exogenous agents were added in the culture media. However, the whole mid-infrared region gives rise to a global biochemical analysis; to obtain more details on the radical aggression, it appears necessary to study some particular spectral ranges.

3.2. IR spectral analysis

Five mean FT-IR spectra (1 to 5) were obtained from the five groups previously separated on Fig. 1A and 1B (*a* to *e*, respectively), each spectrum being the average of each spectral group. Although some complexity resulted from the spectral data which comprised features of the whole cellular biomolecules, profiles obtained after H₂O₂ and AA aggressions differed from those of controls. IR spectra of *M. luteus* were analyzed in three spectral windows.

3.2.1. Analysis of the 3000–2800 cm^{−1} region

Figure 2 depicts slight changes in this region mainly attributed to CH₃, CH₂ and CH groups in membrane fatty acids; whatever the nature of the aggressive agent, we observed an increased absorption of the CH₂ bands (at 2928 and 2854 cm^{−1}). Generally, the membrane of bacterial cells contains large amounts of polyunsaturated fatty acids which are excellent targets for free radical attack because of their multiple double bonds; however, polyunsaturated fatty acids are minor components in *M. luteus* [14]. The increased absorptions shown on Fig. 2 and those of the carbonyl (C=O) band at 1740 cm^{−1} (Fig. 3) could suggest an accumulation of lipids and/or an enhanced length of the acyl chains [16] as a consequence of lipid aggression.

3.2.2. Analysis of the 1760–1520 cm^{−1} region

This region dominated by the amide I and II bands of proteins has been used for determination of their secondary structure [17]. Figure 3 shows the result of this analysis performed on the second derivative spectra. First, the amide I band (corresponding to stretching C=O and bending C–N vibrational modes of the protein backbone) consists of a predominant α -helical segment at 1658 cm^{−1} and three component bands at 1690, 1680 and 1640 cm^{−1} due to β -sheet segments. The high and low frequency β -structure bands have components at 1694 cm^{−1} on spectrum 5, at 1630 cm^{−1} on spectra 3 and 5. Second, the amide II band (corresponding to bending N–H and stretching C–N vibrational modes of the protein backbone) consists of an α -helical segment centered at 1547 cm^{−1} and two minor bands at 1565 and

Fig. 2. Mean FT-IR spectra of *M. luteus* cells in the 3000–2800 cm^{−1} spectral region. Control cells, spectrum 1; weakly H₂O₂ treated (1–40 mM), spectrum 2; highly H₂O₂ treated cells (50–200 mM), spectrum 3; weakly AA treated cells (1–20 mM), spectrum 4; highly AA treated cells (30–200 mM), spectrum 5. Spectra are shown offset for clarity.

Fig. 3. Mean FT-IR spectra of *M. luteus* cells in the 1700–1520 cm^{-1} spectral region after second derivative procedure. Control cells, spectrum 1; weakly H_2O_2 treated (1–40 mM), spectrum 2; highly H_2O_2 treated cells (50–200 mM), spectrum 3; weakly AA treated cells (1–20 mM), spectrum 4; highly AA treated cells (30–200 mM), spectrum 5. Spectra are inverted and shown offset for clarity.

1534 cm^{-1} mainly perceptible on spectrum 3 and probably due to change in protein conformation. Indeed, it is accepted that environmental conditions (e.g. temperature, pH) brought about changes in the protein conformation [18]. The major factors responsible for conformational sensitivity of amide bands include hydrogen bonding and coupling between transition dipoles leading to the splitting of the amide I mode [19]; that can be correlated with the appearance at 1630 cm^{-1} of a shoulder in spectrum 3 and a band in spectrum 5.

3.2.3. Analysis of the 1430–900 cm^{-1} region

Spectral characteristics (Fig. 4) are firstly dominated by the symmetric C=O stretching vibrations of the COO^- groups present in free fatty acids and/or amino acid side chains [20]. The band (between 1430 and 1360 cm^{-1}) centered at 1398 cm^{-1} had a reduced intensity after radical aggression (integrated areas of 4.51 ± 0.61 , 4.18 ± 0.34 , 3.92 ± 0.28 , 4.36 ± 0.65 , 1.32 ± 0.16 for spectra 1, 2, 3, 4 and 5, respectively); the reduction was significant for spectra 3 ($P < 0.025$) and 5 ($P < 0.001$) compared with spectrum 1. Moreover, a large change in shape is visible on spectrum 5 with two shoulders at 1412 and 1378 cm^{-1} . These spectral modifications may be a consequence of $\bullet\text{OH}$ reaction on sensitive amino acid residues, occurring mainly with high AA concentrations, as observed previously on *Deinococcus radiodurans* [21]. Radicals can also interfere with the formation of the peptide cross bridges of the peptidoglycan layer inside the cell wall, and can lead to a decreased absorption of the amino acids present in the peptide part of this layer as does penicillin on *Escherichia coli* [22].

A band (between 1275 and 1210 cm^{-1}) centered at 1245 cm^{-1} and attributed to phosphate (PO_2^-) groups present in phospholipids and nucleic acids [23] is secondly observed, with increased intensity on spectra 3 and 5 compared to spectrum 1 (integrated areas of 2.40 ± 0.38 , 2.82 ± 0.32 , 3.68 ± 0.42 for spectra 1, 3 and 5, respectively) leading to significant increased areas ($P < 0.05$ and $P < 0.001$ for spectra 3 and 5, respectively compared with spectrum 1). Moreover, shifts toward low wavenumbers can be noted from 1245 cm^{-1} on spectrum 1 to 1242 cm^{-1} on spectrum 3 and to 1239 cm^{-1} on spectrum 5 suggesting hydrogen-bonding on phosphate carrying compounds.

Fig. 4. Mean FT-IR spectra of *M. luteus* cells in the 1430–900 cm^{-1} spectral region. Control cells, spectrum 1; weakly H_2O_2 treated (1–40 mM), spectrum 2; highly H_2O_2 treated cells (50–200 mM), spectrum 3; weakly AA treated cells (1–20 mM), spectrum 4; highly AA treated cells (30–200 mM), spectrum 5. Spectra are shown offset for clarity.

In the 1200–900 cm^{-1} spectral region, several macromolecules as aminosugars of the peptidoglycan, polysaccharides, phospholipids and nucleic acids absorb [24]. In particular, we observe differences in band shape between 1100 and 1000 cm^{-1} (mainly assigned to C–O stretching vibrations in osidic structures of cell membranes) with two shoulders at 1082 and 1060 cm^{-1} on spectra 1 to 4, and a band centered at 1069 cm^{-1} on spectrum 5; these differences resulted in significant increases of spectral areas ($P < 0.005$ for spectra 3 and 5 compared with spectrum 1). Among polysaccharidic components, a succinylated lipomannan has been described for *M. luteus* [25] with mannose as the major component [26]. Thus, the changes in shape observed on spectrum 5 at 1060 cm^{-1} , that we attributed to mannose, might arise from a modified composition of the cell wall during the chemical process. In addition, AA led to decreases in absorption intensity at 1155, 994 and 915 cm^{-1} and to increase at 967 cm^{-1} mainly on spectrum 5, while H_2O_2 induced slight increases at 1155 and 967 cm^{-1} mainly on spectrum 3. When the capacity of intracellular ROS scavengers and antioxidants is overcome, DNA represents the ultimate target for ROS [27]. For example, the radicals abstract hydrogens from the furanose ring and introduce hydroxyl groups at various positions on the ring structure [28]. Finally, changes in the region below 1180 cm^{-1} could be essentially ascribed to substantial modifications in the osidic (C–O–C) structures and phosphodiester (P–O–C) backbone in cell membranes.

All these results confirmed that concentrations ranging from 1 to 40 mM H_2O_2 and from 1 to 20 mM AA led globally to similar spectral changes. Beyond these thresholds, AA induced higher damage than H_2O_2 as shown on the dendrogram (Fig. 5) using two spectral ranges equally weighed (1430–1190 and 1140–945 cm^{-1}); the heterogeneity between groups *a* and *b* + *d* was 200.5, while it was 442.4 between *c* and *a*, *b* + *d*. The heterogeneity between group *e* and the others was 6709.7 demonstrating that these two spectral ranges were highly representative of the induced damage.

3.3. Protective mechanisms

When submitted to increasing AA concentrations, the main spectral alterations are attributed to $\bullet\text{OH}$ considered as important mediators of cell injury and/or death [6]. In the same way, $\bullet\text{OH}$ are the ma-

Fig. 5. Dendrogram of the cluster analysis of *M. luteus* cells using two spectral ranges equally weighed, 1430–1190 and 1140–945 cm^{-1} , and using first derivative spectra. Control cells (group *a*), weakly H_2O_2 treated cells (group *b*; 1–40 mM), highly H_2O_2 treated cells (group *c*; 50–200 mM), weakly AA treated cells (group *d*; 1–20 mM), highly AA treated cells (group *e*; 30–200 mM). Ward's algorithm was applied.

for ROS responsible for H_2O_2 killing as observed in many bacteria, owing to the ability of H_2O_2 to diffuse across the cell membrane. When ROS are prevalent in higher amounts, spectral differences resulting from the oxidative stress appear between control and treated cells. However, antioxidant defense mechanisms may contribute to the resistance of cells against oxidative damage. For example, damage to phospholipids can be prevented by the naturally occurring antioxidant vitamin E [29], considered as the major *in vivo* protector in biological membranes. Moreover, one can speculate, as described for *Deinococcus radiodurans* [30], that scavenging enzymes such as catalase and superoxide dismutase may serve at the first line of defense against oxidative stress by preventing the accumulation of ROS in *M. luteus*. Our experimental findings obviously demonstrated that *M. luteus* has developed more efficient defense mechanisms to detoxify H_2O_2 introduced exogenously in culture media than to protect against AA aggression. Indeed, catalase plays a fundamental role against H_2O_2 cytotoxicity; it decomposes H_2O_2 before it can damage cellular components. Previous data [31] clearly show that catalases for many kinds of bacteria and in particular for *M. luteus* do not become saturated with substrate and have a high specific activity. One role of catalase is to lower the risk of $\bullet\text{OH}$ formation from H_2O_2 via the Fenton-reaction catalyzed by Cu or Fe ions [32], these ions being present in culture media. Moreover, pigmentation can play a protective role; in *M. luteus*, pigments associated with membrane lipids [33] constitute a natural defense system against radical damage.

In conclusion, FT-IR spectroscopy was proved to be very sensitive for detecting and monitoring the deleterious effects of radical aggression on *M. luteus*. Thus, it allowed a clear discrimination between control and chemically treated samples. This method based on selected and discriminative spectral ranges could help to rapidly evaluate the level of oxidative damage. Consequently, FT-IR spectroscopy could be successfully used to diagnose early damage in various biological cells and to monitor the intensity of cell sensitivity toward physical or chemical agents.

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