Mechanism of gluconate synthesis in *Rhizobium meliloti* by using in vivo NMR

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Received 6 May 1997; revised version received 21 June 1997

Abstract The dehydrogenation of [1-^{13}C]- and [2-^{13}C]glucose into gluconate was monitored by NMR spectroscopy in living cell suspensions of two *Rhizobium meliloti* strains. The synthesis of gluconate was accompanied, in the cellular environment, by the formation of two gluconolactones, a γ-lactone being detected in addition to the expected δ-lactone. These lactones – as well as the gluconate – could be further metabolized by the cells. The δ-lactone was utilized faster than the γ-lactone. The presence – in significant amounts – and the relative stability of the lactones raise the question of their possible physiological significance.

Key words: Gluconate; Gluconolactone; Glucose dehydrogenase; NMR spectroscopy; *Rhizobium meliloti*

1. Introduction

The dehydrogenation of glucose into gluconate is a common feature of glucose metabolism in microorganisms. The reaction can be catalyzed by either a NAD(P)$^+$-dependent glucose dehydrogenase (EC 1.1.1.47) or a pyrroloquinoline-quinone (PQQ)-dependent glucose dehydrogenase (EC 1.1.99.17). Both enzymes mechanism is believed to result in the formation of a glucono-1,5-lactone (or δ-gluconolactone), which can be further hydrolyzed either chemically or enzymatically by a gluconolactonase (EC 3.1.1.17). The resulting gluconate will be used in either the Entner-Doudoroff [1,2], the pentose-phosphate [3] or alternative [4,5] pathways.

By using ^{13}C-NMR techniques, Jarori and Maitra [6] have shown that the reaction catalyzed by the glucose 6-phosphate dehydrogenase (EC 1.1.1.49) – a reaction similar to that catalyzed by the glucose dehydrogenases – resulted in the formation of two lactones, a phosphoglucono-1,4-lactone (or γ-phosphogluconolactone) being generated in addition to the expected δ-phosphogluconolactone. This result led the authors to reconsider the reaction mechanism of this enzyme.

Concerning the glucose dehydrogenase reaction, the occurrence of the two lactones has not been examined. We recently undertook in vivo ^{13}C-NMR experiments to investigate the synthesis of various polymers in *Rhizobium meliloti* (unpublished work). During incubations with [1-^{13}C]glucose, a NMR signal could clearly be assigned to gluconate, since it is known that *Rhizobium meliloti* produce this compound [7]. Two unidentified peaks near the gluconate peak were also detected during these experiments. Here, we present data that demonstrate that these peaks belong to the δ- and γ-gluconolactones. For this, two strains (M5N1 and Su47) of *Rhizobium meliloti* were fed with [1-^{13}C]- or [2-^{13}C]glucose, allowing unambiguous assignment of resonances to gluconolactones (see [6]).

2. Experimental

2.1. Micro-organisms and preparation of cell suspension for NMR studies

Rhizobium meliloti M5N1 and Su47 strains (laboratory collection) able to nodulate *Medicago sativa* were cultured as previously described [8] using yeast extract containing medium. For NMR experiments, mid-logarithmic (OD_{600}=0.7) phase harvested cells were centrifuged, washed, suspended at a final density of 5×10^{10} cells/mL into 7 mL of fresh medium – containing 15% D_2O – deprived of carbon source. These solutions were introduced into an airlifted NMR tube [9]. The aeration was set at 4.2×10^{-7} m^3/s (3.6 vvm). The air-lifted NMR tube was then placed into the magnet, and a first spectrum acquired to check for the natural abundant signals. The labelled carbon source (99% [1-^{13}C]glucose or [2-^{13}C]glucose, both purchased from Eurisotop-CEA, France) was then added at a final concentration of 30 mM. Spectra were accumulated in consecutive blocks of 6000 scans each (70 min).

2.2. Carbon-13 NMR measurements

Carbon-13 NMR spectra at 30°C were obtained in the Fourier-transform mode at 75.47 MHz on a Bruker spectrometer (AM 300, wide bore) equipped with a dual ^{13}C and ^{31}P 20-mm probe, using a spectral width of 15 kHz (16 K memory size), a 30° pulse angle, an interpulse delay of 0.7 s and bilevel proton decoupling. The FIDs were exponentially multiplied (6 Hz) prior to Fourier transformation. Chemical shifts were expressed as ppm relative to the resonance of benzene (129.2 ppm) added in a sealed capillary. The chemical shifts of the resonances of commercial gluconate, 2-ketogluconate and δ-gluconolactone (both from SIGMA) and of purified γ-gluconolactone (generous gift of Prof. Beaupère, Amiens, France) were obtained from solutions prepared with fresh culture medium, unless otherwise noted.

3. Results

3.1. Metabolism of [1-^{13}C]- and [2-^{13}C]glucose in Rhizobium meliloti

The same strategy as Jarori and Maitra [6] was used with aerated cell suspensions of *Rhizobium meliloti*, incubated with 30 mM of either [1-^{13}C]- or [2-^{13}C]glucose. In our experimental conditions, glucose was totally consumed after approximately 9 h. The dehydrogenation of [1-^{13}C]glucose into [1-^{13}C]gluconate resulted in the presence, in the ^{13}C-NMR spectra, of the expected resonance at 179.6 ppm (Fig. 1). Two additional resonances were observed at 177.9 and 174.6

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Abbreviations: PQQ, pyrroloquinoline quinone

PII S0014-5793(97)00832-6

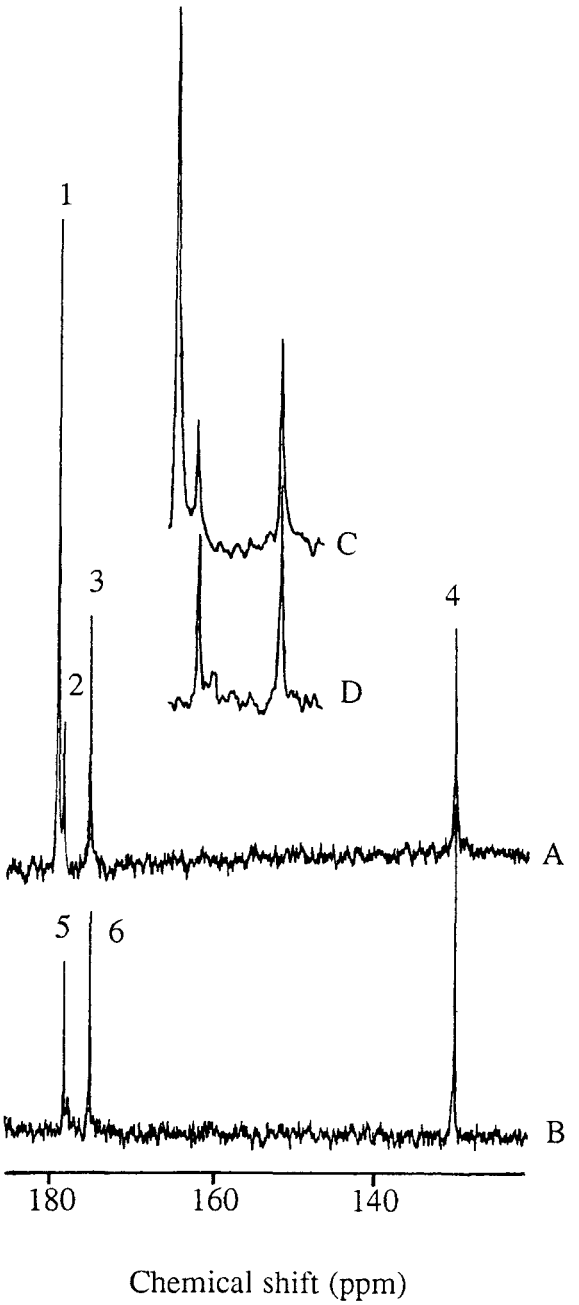

Fig. 1. Characterization of products of the [1-^{13}C]glucose metabolism in *Rhizobium meliloti* M5N1. (A, B) 120–185 and (C, D) 173–180 ppm regions of proton-decoupled ^{13}C-NMR spectra, from (A, C) a suspension of mid-exponential phase harvested *R. meliloti* cells incubated during 8 h with [1-^{13}C]glucose and (B, D) a mixture of purified γ- and δ-gluconolactone dissolved in fresh culture medium at pH 4.5. Chemical shifts are in ppm according to the benzene signal (129.2 ppm). Assignments: 1, gluconate C1; 2, 3, initially unassigned peaks; 4, benzene; 5, γ-gluconolactone C1; 6, δ-gluconolactone C1.

ppm. These chemical shifts were very similar to that of, respectively, the γ- and δ-lactones of gluconate-6-phosphate obtained by Jarori and Maitra [6] (note that, in their work, the frequency scale was referenced approximately 2.1 ppm downfield as compared to the present work). Such comparison is possible because the phosphoryl group at the C6 poorly influences the chemical shifts of C1 carbons (and slightly that of C2). The chemical shifts of the additional peaks fully agree with that of purified γ- and δ-gluconolactones analyzed in culture medium (Fig. 1). In *Rhizobium* species, the gluconate can undergo further dehydrogenation, yielding 2-ketogluconate [10]. However, the chemical shift of the C1 carbon of this compound (175.9 ppm) did not agree with those observed. The peak of gluconate as well as the two unidentified peaks were not detected in the spectra of living *Rhizobium meliloti* cells incubated with [1-^{13}C]fructose (data not shown), a sugar that does not undergo the glucose dehydrogenase reaction but which is believed to have an intracellular metabolism similar to that of glucose in these cells [11].

In the ^{13}C-NMR spectra obtained from *Rhizobium meliloti* cells incubated with [2-^{13}C]glucose (Fig. 2), the decrease of the glucose C2 peaks was accompanied by the presence of three peaks located at 72.0, 73.7 and 75.2 ppm. These chemical shifts were similar to that obtained by Jarori and Maitra [6] for the C2 of, respectively, the δ-phosphogluconolactone, the γ-phosphogluconolactone and the phosphogluconate. They fully agreed with those obtained for the C2 of purified δ-, γ-gluconolactone and gluconate, but not for the 2-ketogluco-

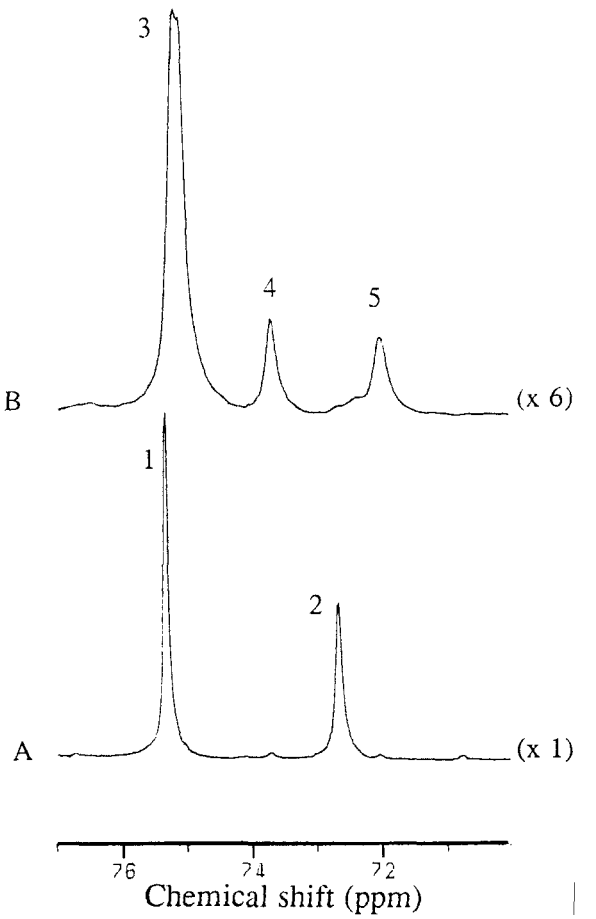

Fig. 2. Characterization of the products of [2-^{13}-C]glucose metabolism in *Rhizobium meliloti* M5N1. 70–77 ppm region of the proton-decoupled ^{13}C-NMR spectra from a cell suspension of mid-exponential phase harvested *R. meliloti* incubated for 0.5 (A) or 8 h (B) with [2-^{13}C]glucose. Chemical shifts are in ppm according to the benzene signal (129.2 ppm). Assignments: 1, glucose C2 (β anomer); 2, glucose C2 (α anomer); 3, gluconate C2; 4, 5, initially unassigned peaks.

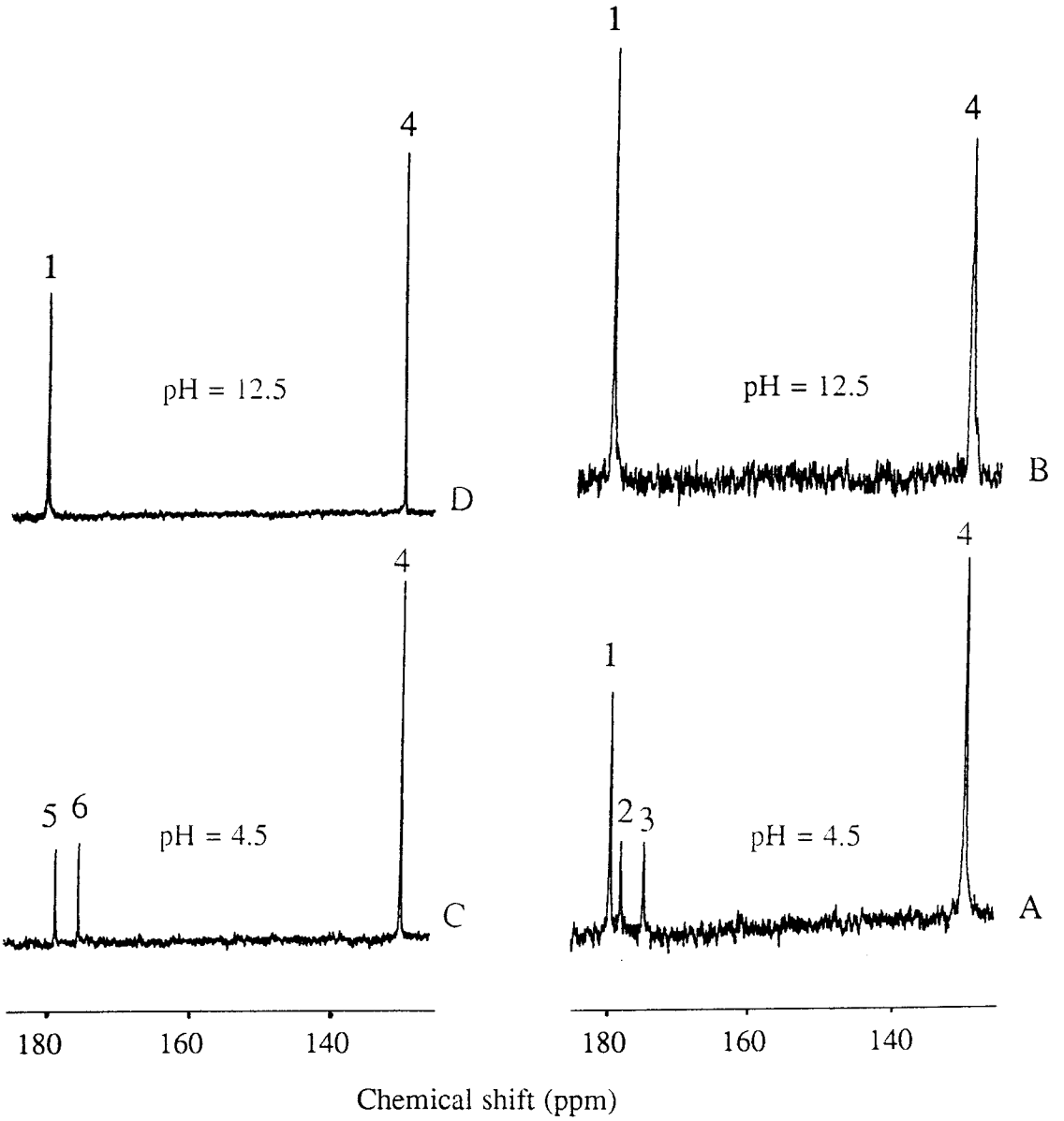

Fig. 3. Effect of alkalinization on the metabolic products of [1-^{13}C]glucose in *Rhizobium meliloti* Su47. 125–185 ppm regions of proton-decoupled ^{13}C-NMR spectra from the medium removed from a suspension of mid-exponential phase harvested *R. meliloti* incubated for 8 h with [1-^{13}C]glucose (A, B) and from a mixture of purified γ- and δ-gluconolactone dissolved in fresh culture medium (C, D). The solutions were analyzed at pH 4.5 (A, C) and at pH 12.8 (B, D) after addition of KOH (10 M). Chemical shifts are in ppm according to the benzene signal (129.2 ppm). Assignments: 1, gluconate C1; 2, 3, initially unassigned peaks; 4, benzene; 5, γ-gluconolactone C1; 6, δ-gluconolactone C1.

nate (98.3 ppm). They were not detected in spectra of cells incubated with [2-^{13}C]fructose.

These experiments were performed initially with the strain M5N1. Similar results were obtained with the strain Su47 (data not shown), i.e. occurrence of the two lactones.

3.2. *Effect of alkalinization*

The PQQ-dependent glucose dehydrogenase is located at the periplasmic side of the inner membrane of gram negative bacteria [12], a location allowing the release of the products out to the medium. Different series of experiments were then performed where the incubation of the cells with [1-^{13}C]glucose was stopped after 8 h and the medium removed by centrifugation. The pH of this medium was 4.5–5, indicating that the gluconate synthesis was accompanied by significant acidification. One part of the removed medium was NMR analyzed, which evidenced the presence of the three peaks (Fig. 3, spectrum A). The other part was alkalinized to pH 12.5 with KOH (10 M) and also NMR analyzed (Fig. 3, spectrum B). Under alkaline conditions, the lactones are known to be hydrolyzed into gluconate, as controlled with a mixture of the two purified gluconolactones (Fig. 3, spectra C and D). As shown in Fig. 3 (spectra A and B), alkalinization of the medium resulted in the disappearance of the two unassigned peaks and in the increase in the intensity of the gluconate peak, indicating that the hydrolysis product of the chemical species giving these peaks was gluconate. All the data presented above demonstrate that the glucose dehydro-

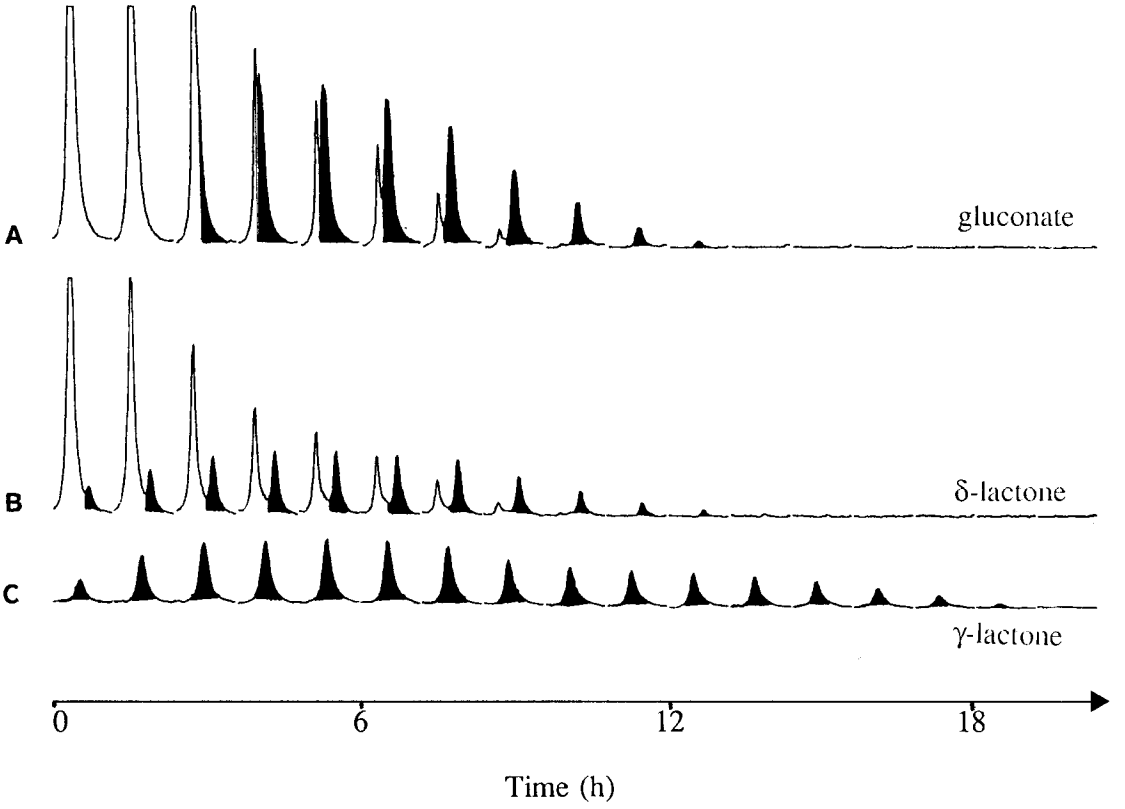

Fig. 4. Time-lapse evolution of the signals from gluconolactones and gluconate during incubation of an aerated cell suspension of *Rhizobium meliloti* with [2-^{13}C]glucose. The peaks from gluconate (line A), δ-gluconolactone (line B) and γ-gluconolactone (line C) are filled in black.

genase reaction of cells of *Rhizobium meliloti* involves the formation of two gluconolactones.

Additionally, the following control experiment was performed. Purified gluconate was incubated at 30°C and at a pH of 4.5 in either fresh medium or in a medium removed from a culture with glucose as carbon source. Whatever the medium, no lactone was observed in the NMR spectra, even after 15 h of incubation. These observations indicate that the lactones observed in the presence of cell suspensions were not simply the result of a chemical conversion. All of these experiments show that the glucose dehydrogenase reaction with cells of *Rhizobium meliloti* is associated with the NMR detection of two gluconolactones.

3.3. Kinetics of lactone evolution

The time-lapse evolution of the ^{13}C-NMR resonances of the two lactones and of the gluconate are shown in the Fig. 4 for incubations with [2-^{13}C]glucose, in the case of the strain Su47. The three resonances increased with time until approx. 6 h of incubation and then decreased, indicating their utilization by the cells. The decrease of these signals was observed at a time for which the [2-^{13}C]glucose was not totally consumed but had still a concentration around 4 mM. The rates of synthesis of the two lactones appeared very similar, but their disappearance did not follow the same kinetics. The decrease of the δ-lactone signal was similar to that of the gluconate, the two chemical species were not detected after 14 h of incubation. The rate of disappearance of the γ-gluconolactone was significantly lower than that of the two other chemical species. A slight signal was still observed at 19 h of incubation.

4. Discussion

The occurrence of δ-lactones as intermediates of either the glucose or the glucose 6-phosphate dehydrogenation reactions is the object for the discussion of the reaction mechanism. It is thought that this reaction proceeds via the direct dehydrogenation of the pyranosyl form of either glucose or glucose 6-phosphate [13]. Based on the NMR detection of two lactones produced during the reaction catalyzed by purified glucose 6-phosphate dehydrogenase, Jarori and Maitra [6] revisited this mechanism. These authors proposed a mechanism involving the opening of the pyranose ring of glucose 6-phosphate before the actual dehydrogenation reaction. The lactones are then formed by intramolecular esterification occurring at the catalytic site, and released. Our results show that gluconate synthesis in *Rhizobium meliloti* cells is accompanied also by the formation of two lactones, indicating that the occurrence of two lactones may be a common feature of glucose or glucose 6-phosphate dehydrogenation. However, the glucose dehydrogenase of *Rhizobium meliloti* is a member of the membrane associated PQQ-dependent family [7], while the type VII glucose 6-phosphate dehydrogenase studied by Jarori and Maitra [6] is NAD(P)$^+$-dependent. These two coenzymes have different catalytic mechanisms, but both reactions are leading to the formation of the two lactones. This suggests that the formation of the two lactones may not be linked to the dehydrogenation step but rather to the substrate structure.

The gluconolactones were observed in living cell suspensions. They were stable enough to persist for hours, a stability favoured by the acidification of the medium (from pH 7.3 to

4.5–5) accompanying the gluconate synthesis. The presence – in significant amount – and the relative stability of the lactones raise the following question: is this just a (bio)chemical curiosity or can it be of physiological significance? If the occurrence of γ-gluconolactone is a new feature, the δ-gluconolactone is a known inhibitor of a wide variety of enzymes involved in polysaccharide metabolism [14–17]. Can such an inhibition by the lactone(s) explain the lag phase observed in the exopolysaccharide production when *Rhizobium meliloti* cells were grown on glucose (but not present when grown on fructose) [8], which was also the period during gluconate was produced? The δ-gluconolactone was also shown to induce, in *Saccharomyces cerevisiae*, the oxidative branch of the pentose-phosphate pathway, i.e. the pathway through which gluconate can be metabolized in these cells [3], while in *E. coli*, the Entner-Doudoroff pathway was shown to be induced via PQQ-dependent glucose dehydrogenase [2]. These two pathways are present in *Rhizobium* species, the Entner-Doudoroff being considered as the key route for glucose metabolism in these cells [18,19]. All the above data indicate that the physiological significance of the mechanism of glucose dehydrogenation (including both lactones) should be examined.

The two gluconolactones were utilized by the *Rhizobium meliloti* cells once the medium was near depletion in glucose, but at different kinetics, suggesting that their utilization may not proceed through the same way. As the δ-gluconolactone is utilized faster than the γ-gluconolactone, it could be suggested that only the former lactone undergoes a catalytic way, but the higher chemical stability of the latter precluded any conclusion. In addition, the 6-phosphogluconolactonase (EC 3.1.1.31), involved in the hydrolysis of the δ-phosphogluconolactone that is formed in the pentose-phosphate pathway, was shown to hydrolyze also the γ-phosphogluconolactone [20], suggesting that a gluconolactonase may utilize both gluconolactones. *Rhizobium* species are able to utilize gluconate [11,19], suggesting the presence of a gluconate permease. The occurrence of a gluconolactonase has not been investigated. The mechanisms by which the two gluconolactones are utilized in *Rhizobium meliloti* remain to clarify.

Acknowledgements: P. Tavernier is supported by a fellowship from the Biopôle Végétal de Picardie, France. The careful reading of the manuscript by Christine Moresoli was gratefully appreciated.

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