A deeper investigation on carbohydrate cycling in *Sinorhizobium meliloti*

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Abstract Recycling of triose-phosphate and pentose-phosphate was previously reported on glucose in *Sinorhizobium meliloti*, a polysaccharide-synthesizing bacterium, but the metabolic basis of such processes remained unclear. In this work, we have used ^{13}C-labelling strategies to demonstrate that carbohydrate cycling in this organism is independent of the gluconate bypass, the alternative pathway for glucose assimilation involving its periplasmic oxidation into gluconate. Furthermore, carbohydrate cycling in *S. meliloti* is also observed on fructose, making the situation in this bacterium significantly different from that depicted for alginate-synthesizing species.

Key words: Carbohydrate cycling; β-Glucan; Gluconate bypass; Nuclear magnetic resonance spectroscopy; *Sinorhizobium meliloti*

1. Introduction

A cyclic version of the Entner–Doudoroff (ED) pathway is known to operate in several Gram-negative, polysaccharide-producing bacteria [1–5], where the glyceraldehyde-3-phosphate (GAP) molecules generated from a hexose are recycled into hexose-phosphates by gluconeogenesis (triose-phosphate cycling). This ED cycle was observed on glucose in *Azotobacter vinelandii* and other *Pseudomonas* species synthesizing alginate, a polysaccharide metabolically derived from fructose-6-phosphate (F6P). Two complementary explanations have been proposed for the operation of the ED cycle: (i) a low phosphoglucoisomerase (PGI) activity [6,7] that is further inhibited by intermediates of glucose catabolism [1] and (ii) the occurrence of the periplasmic oxidation of glucose into gluconate which feeds the catabolic pathways at the level of 6-phosphogluconate and acts as an inducer of ED enzymes [8,9] but does not enable hexose-phosphate formation (gluconate bypass) because of the irreversibility of the glucose-6-phosphate (G6P) dehydrogenase reaction. Both features generated a situation where F6P could not be obtained directly from the exogenous glucose and the anabolic demands in F6P were fulfilled by the operation of the cyclic ED pathway [5], that acted as an alternative route for the conversion of G6P into F6P. Indeed, in these organisms, the ED cycle was not observed on fructose [10], which was converted directly into F6P.

In contrast, both the ED and the pentose-phosphate pathways (PP) operate in other polysaccharide-synthesizing bacteria [3,4], leading to both triose-phosphate and pentose-phosphate cycling. The metabolic origin of the carbohydrate cycling in these species has been much less documented, but the situation differed from those utilizing only the ED cycle because: (i) anabolic demands for polysaccharide synthesis are at the level of G6P, (ii) PGI is necessarily active to complete the cycles up to G6P and (iii) cycling through the PP pathway is dissipative for carbon, which is unfavorable for polysaccharide synthesis. Nevertheless, these organisms are known to possess the gluconate bypass [11].

In previous studies, we have shown that both triose- and pentose-phosphate cycling occurred on glucose in *Sinorhizobium meliloti* [12,13], a bacterium synthesizing G6P-derived polysaccharides and possessing the gluconate bypass (Fig. 1) [14,15]. The present work was undertaken to get further details on the metabolic origin of the carbohydrate cycling in this bacterium and more specifically to answer two questions: (i) does the gluconate bypass explain the occurrence of carbohydrate cycling on glucose, and (ii) are these processes specific for glucose in this species? For this purpose, the recycling processes were assessed in a mutant lacking the glucose dehydrogenase (GDH) activity and in its parent strain on both glucose and fructose (see Fig. 1).

2. Materials and methods

2.1. Bacterial strains and growth media

S. meliloti strains used were the wild-type Rm2011 and the mutant RmH580 (= Rm2011, *gdh*::Tn5) strains, kindly provided by Prof. T.M. Finan, McMaster University, Canada. Precultures and cultures were done as previously described [16] in RC medium ($MgSO_4$, 7 H_2O: 0.2 g/l, K_2HPO_4: 1 g/l, yeast extract: 1 g/l, pH 7.2) supplemented with glucose or fructose at 6 g/l. Bacterial growth was determined spectrophotometrically at 600 nm.

2.2. Cultures for nuclear magnetic resonance (NMR) experiments

400 ml of mid-logarithmic culture (optical density at 600 nm ~0.8) was centrifuged. The bacterial pellet was washed twice with cold saline buffer, and suspended in 100 ml of RC medium containing 2.8 mM of labelled carbon source that was either [1-^{13}C], [2-^{13}C], [6-^{13}C], [1,2-$^{13}C_2$]glucose or [2-^{13}C]fructose, all 99.9% enriched from Eurisotop (Saint-Aubin, France). After 24 h of incubation with labelled hexose in shaken flasks, cell suspensions were centrifuged and the bacterial

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Abbreviations: ED, Entner–Doudoroff pathway; EMP, Embden–Meyerhof–Parnas pathway; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; GAP, glyceraldehyde-3-phosphate; GDH, glucose dehydrogenase; NMR, nuclear magnetic resonance; PFK, phosphofructokinase; PGI, phosphoglucoisomerase; PP, pentose-phosphate pathway

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pellet washed twice. The supernatant (extracellular medium) was freeze-dried and dissolved in 1 ml D_2O prior to NMR analyses.

2.3. Extraction of the β-glucans

The cell pellet was extracted twice with 75% ethanol for 30 min at 70°C, then the extract was centrifuged (12 710 × *g*, 11 min, 4°C) and the supernatant (ethanolic fraction) evaporated and freeze-dried. The β-glucans were precipitated overnight at −20°C by adding isopropanol (10 volumes) and recovered by centrifugation (20 600 × *g*, 20 min, 4°C). The resulting pellet was freeze-dried and dissolved in 0.4 ml D_2O. NMR spectra were acquired both before and after hydrolysis of the β-glucans with trifluoroacetic acid treatment (4.35 M, 100°C, 3 h and 15 min).

2.4. ^{13}C-NMR analyses and isotopic enrichment measurements

^{13}C-NMR spectra were recorded as previously described [12] on a Bruker spectrometer (AVANCE 300, wide bore) in a 5 mm ^{13}C/^{1}H probe. Isotopic enrichments of the β-glucan carbon positions were measured from ^{13}C-NMR spectra as previously described [12]. Additionally, the enrichments of the C1 glucosidic unit carbon positions obtained from ^{13}C-NMR analyses were confirmed from ^{1}H-NMR spectra by integration of the central (^{1}H bound to ^{12}C) and satellite (^{1}H bound to ^{13}C) signals.

3. Results and discussion

3.1. Gluconate synthesis

The parent Rm2011 and the *gdh* mutant RmH580 strains showed similar growth rates (Table 1) when cultured in RC medium supplemented with either glucose or fructose (0.29–0.31 h^{-1}), indicating that the lack of GDH had no apparent effect on the bacterial growth.

At the end of the culture, a slight acidification of the media (pH ranged from 6.5 to 7.0) was always observed, except for the Rm2011 strain grown on glucose for which the final pH was 5.5 (Table 1). Consistently, the synthesis of gluconate could be detected only for the latter condition by ^{13}C-NMR analyses of the extracellular medium of Rm2011 incubated with [1,2-$^{13}C_2$]glucose (Fig. 2A). Doubly labelled glucose was used here to unambiguously identify gluconate from the observation of two coupled signals at the expected chemical shifts [15]. The spectrum illustrated in Fig. 2A showed two doublet peaks located at 75.5 and 180 ppm corresponding to the gluconate C2 and C1 positions, respectively. No singly labelled gluconate was detected, indicating that gluconate was synthesized from exogenous glucose without transiting through catabolic pathways. This was consistent with the periplasmic oxidation of glucose into gluconate. The gluconate peaks were absent in spectra of extracellular media obtained from the RmH580 cells incubated with [1,2-$^{13}C_2$]glucose (Fig. 2B) and from both strains with [2-^{13}C]fructose (spectra not shown).

Table 1
Effect of hexose substrate on growth and pH of *S. meliloti* wild-type strain (Rm2011) and *gdh* mutant strain (RmH580) cultures

Strains	Hexoses	μ (h^{-1})	Final pH[a]
Rm2011	glucose	0.29 ± 0.03	5.5 ± 0.0
	fructose	0.29 ± 0.04	7.0 ± 0.0
RmH580	glucose	0.30 ± 0.02	6.9 ± 0.1
	fructose	0.31 ± 0.00	6.5 ± 0.1

No lag phase was observed for any culture condition (not shown).
[a]pH was measured at three times the mid-logarithmic phase period.

Additionally, the HCO_3^- peak located at 161.1 ppm was greater in the spectrum obtained from the Rm2011 strain compared with the RmH580 strain (Fig. 2), suggesting higher C1- and/or C2-decarboxylating activities in the parent strain.

3.2. Carbohydrate cycling on glucose

In previous works [12,13], we found that ^{13}C-labelling strategies were valuable tools for the isotopic assessment of carbohydrate cycling and that β-glucans of *S. meliloti* cells were

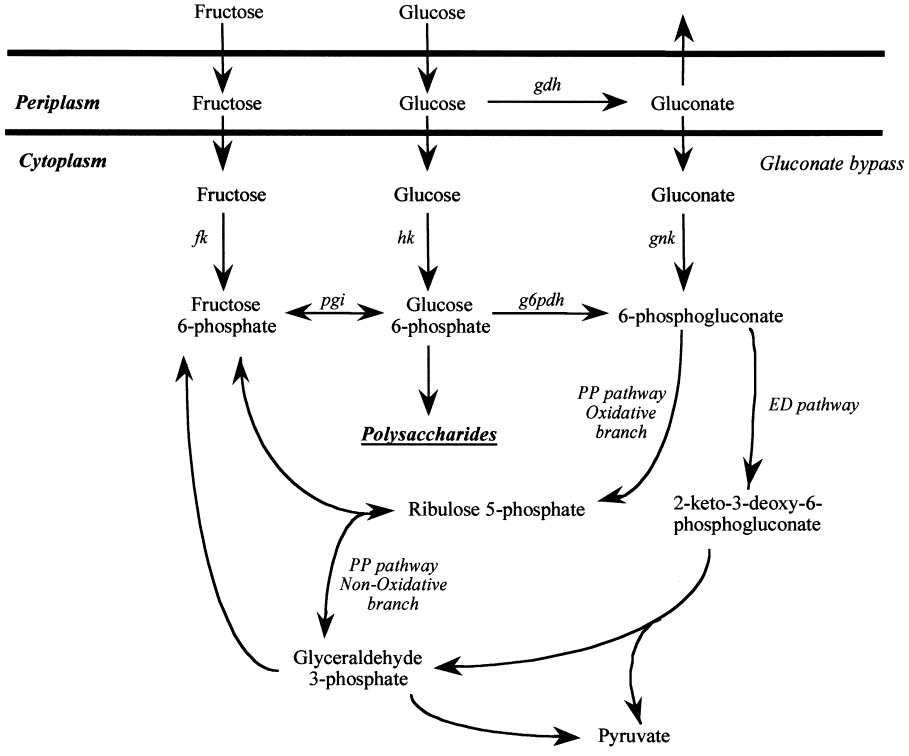

Fig. 1. Scheme of carbohydrate metabolism of *S. meliloti*.

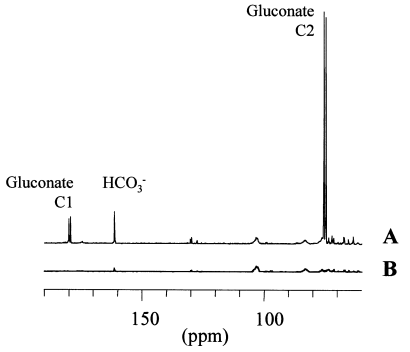

Fig. 2. ^{13}C-NMR spectra of Rm2011 (A) or RmH580 (B) extracellular culture media after incubation with [1,2-$^{13}C_2$]glucose.

suitable markers for that purpose: they are homopolymers of glucose residues [17], metabolically derived from G6P [18] (Fig. 1) and their accumulation inside the periplasm of this bacterium provides sufficient amounts of material for fine isotopic analysis. Cycling in *S. meliloti* wild-type strains Su47 and Rm1021 was accurately probed from the analysis of glucosidic units released by acidic treatment of the β-glucans [12,13]. The same experimental approach was used in this work, except that β-glucan extraction was improved by including an additional step (isopropanol precipitation) in the procedure.

3.2.1. Cycling in the parent strain Rm2011. It was first checked that the parent strain of the *gdh* mutant used in this study had the same metabolic behavior as the wild-type strains where the carbohydrate cycles were demonstrated [12,13]. The results (Table 2) were highly similar to those previously published. Briefly:

- The β-glucan glucosidic units were mainly enriched in the original position of the labelled glucose, e.g. at C1 from [1-^{13}C]glucose, indicating that a part of the exogenous glucose was directly introduced within the cells and used for polymerization.
- The labelling at C1 of glucosidic units from [6-^{13}C]glucose (Table 2) showed that [3-^{13}C]GAP molecules generated from the substrate were converted back into [1-^{13}C]- and [6-^{13}C]G6P by the action of gluconeogenetic activities (fructose-1,6-bisphosphate aldolase, fructose-1,6-bisphosphatase), resulting in triose-phosphate cycling (Fig. 3). The lack of reciprocal enrichment at C6 position from [1-^{13}C]glucose (Table 2) showed that no label from [1-^{13}C]glucose ended in [3-^{13}C]GAP, as would have resulted if phosphofructokinase (PFK) was active. Therefore, both glucose catabolism and triose-phosphate cycling occurred only via the dehydrogenating pathways (ED and PP) (Fig. 3) and not via the Embden–Meyerhof–Parnas (EMP) pathway. This is consistent with general knowledge of the carbohydrate metabolic pathways in *S. meliloti* [11] and our previous results on other wild-type strains [12,13].
- The enrichments at C1 and C3 positions from [2-^{13}C]glucose (Table 2) showed that a part of the substrate was routed through the consecutive oxidative and non-oxidative steps of the PP pathway [19], resulting in cycling of pentose-phosphates (Fig. 3).

3.2.2. Cycling in the gdh mutant RmH580. The labelling pattern of β-glucan glucosidic units obtained from the mutant was analogous to that depicted above for the parent strain (Table 2), showing that both pentose-phosphate and triose-phosphate cycling occurred via both ED and PP pathways.

As a difference, a slight label increase at C6 position from [1-^{13}C]glucose and at C5 position from [2-^{13}C]glucose could be detected (Table 2). Such labellings could be explained by the contribution of the EMP pathway to triose-phosphate cycling (Fig. 3). However, the very low level of isotopic incorporation in these two positions (2.0% from [1-^{13}C]- and 2.7% from [2-^{13}C]glucose) prevented any definitive conclusion on the operation of the EMP pathway which in any case had

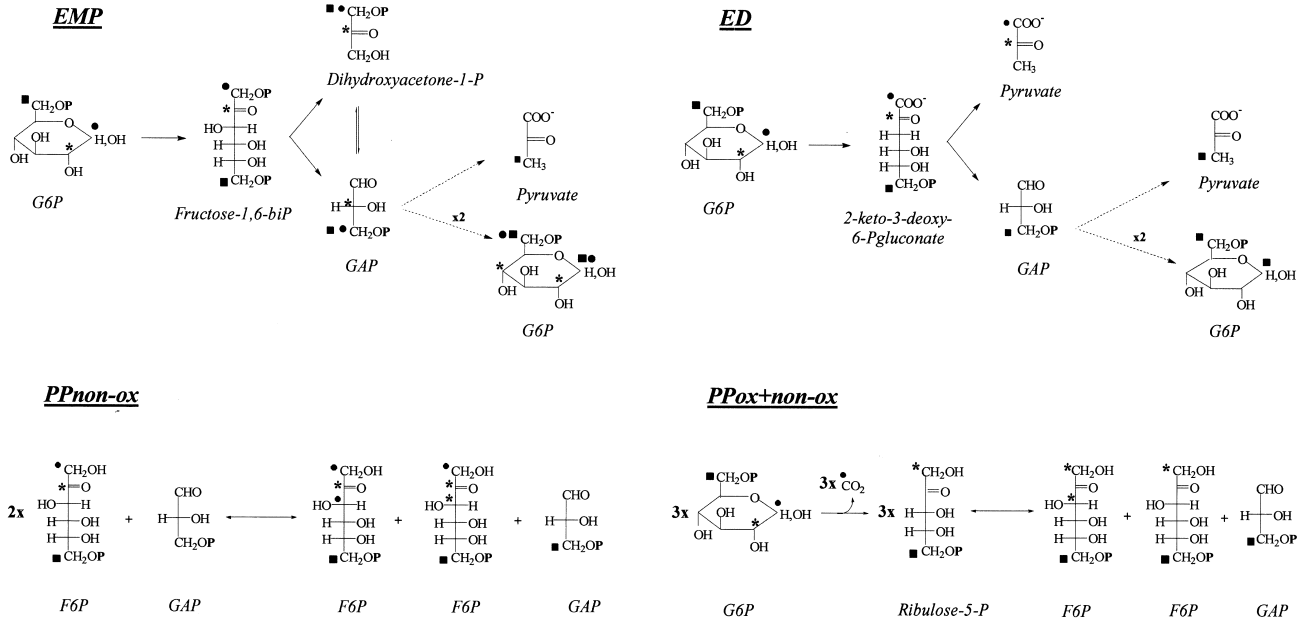

Fig. 3. Isotopic redistribution of [1-^{13}C]glucose (●), [2-^{13}C]glucose (*) and [6-^{13}C]glucose (■) through the main pathways of carbohydrate metabolism in *S. meliloti*.

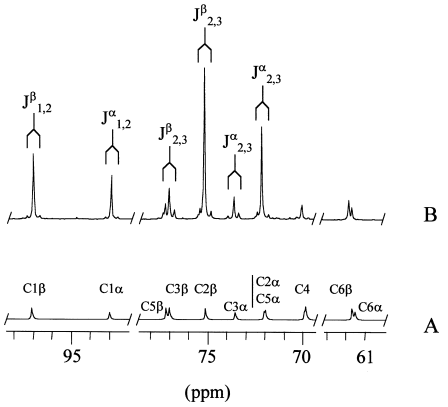

Fig. 4. ^{13}C-NMR spectra of unlabelled glucose (A) and of β-glucan glucosidic units from the Rm2011 strain incubated with [2-^{13}C]glucose (B). Only the 60–63 ppm, 70–78 ppm and 92–98 ppm regions of spectra are displayed. Coupling figures, when present, are indicated in the spectra.

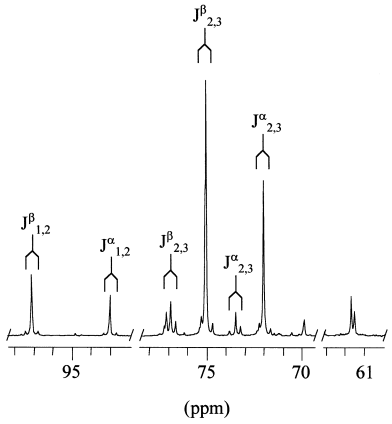

Fig. 5. ^{13}C-NMR spectra of β-glucan glucosidic units from the RmH580 strain incubated with [2-^{13}C]fructose. Only the 60–63 ppm, 70–78 ppm and 92–98 ppm regions of the spectrum are displayed. Coupling figures are indicated in the spectrum.

a very low contribution to glucose metabolism in the *gdh* mutant. Further experiments shall be performed to ascertain this observation.

For the RmH580 strain, enrichments of the redistributed carbons were in the same range as observed for the parent strain (Table 2), but more label was found in the original positions, suggesting a higher contribution of direct polymerization. Given pentose-phosphate cycling resulted in incorporation of label into C1 and C3 positions of glucosidic units from [2-^{13}C]glucose [19], the [(C1+C3)/C2] enrichment ratio from [2-^{13}C]glucose was used as an index of the contribution of pentose-phosphate cycling relative to direct polymerization. This ratio was 0.70 for the parent strain and 0.34 for the *gdh* mutant. Similarly, the (C1/C6) ratio on [6-^{13}C]glucose was representative of triose-phosphate cycling. Its value was 0.19 for the parent strain and 0.14 for the mutant. Both ratios indicated a decreased contribution of cycling in the *gdh* mutant. The decrease was lower for the triose-phosphate cycling compared with the cycling of pentose-phosphates. Such reduced operation of the PP pathway, where label at C1 is lost, was consistent with the lower C1/C2-decarboxylating activities mentioned above for the mutant strain (Fig. 2).

Taken together, these results showed that carbohydrate cycling on glucose in *S. meliloti* was not conditioned by the occurrence of the gluconate bypass, although the operation of the latter process resulted in higher cycling rates. Therefore the carbohydrate cycles in this bacterium could not be seen primarily as alternative pathways for the conversion of exogenous glucose into G6P.

3.2.3. Isotopic exchanges in the non-oxidative branch of the PP pathway. The previously reported [12] isotopic exchanges inside the pools of hexose-phosphates and triose-phosphates enabled by the reversible reactions in the non-oxidative steps of the PP pathway (Fig. 3) were observed for both strains from labelling at the C3 position from [1-^{13}C]glucose (Table 2). Further evidences of this process were obtained in the present work from the coupling figures at C2 and C3 positions of osidic units derived from [2-^{13}C]glucose (Fig. 4B, compared with control spectrum in Fig. 4A), showing the presence of two bound ^{13}C atoms in these positions. Such features were theoretically predictable but have rarely been observed so far. Their observation in *S. meliloti* emphasized the extent of the reversible activities not only of the enzymes in the non-oxidative branch of the PP pathway (epimerase, isomerase, transketolase and transaldolase) but also of PGI.

3.3. Carbohydrate cycling on fructose

The above data indicated that the metabolic basis of carbohydrate cycling on glucose in *S. meliloti* differed significantly from the situation depicted in organisms utilizing only the ED pathway. We have therefore investigated the occurrence of the recycling processes in *S. meliloti* on fructose, for which no carbohydrate cycling was observed in *A. vinelandii* and *Pseudomonas* species [1,10].

Table 2
Isotopic enrichments of β-glucan glucosidic units in Rm2011 and RmH580 strains

Strains	Exogenous [^{13}C]hexoses	Percentages of glucosidic unit isotopic enrichments[a]					
		C1	C2	C3	C4	C5	C6
Rm2011	[1-^{13}C]glucose	20.5 ± 1.4	1.1	2.0 ± 0.3	1.1	1.1	1.1
	[2-^{13}C]glucose	6.9 ± 0.7	14.0 ± 3.0	2.9 ± 0.8	1.1	1.1	1.1
	[6-^{13}C]glucose	6.4 ± 1.0	1.1	1.1	1.1	1.1	32.9 ± 3.8
	[2-^{13}C]fructose	4.9 ± 0.5	21.7 ± 3.7	3.5 ± 0.5	1.1	2.7 ± 0.2	1.1
RmH580	[1-^{13}C]glucose	26.1 ± 1.1	1.1	2.5 ± 0.6	1.1	1.1	2.0 ± 0.1
	[2-^{13}C]glucose	5.0 ± 0.5	24.6 ± 5.7	3.5 ± 0.8	1.1	2.7 ± 0.4	1.1
	[6-^{13}C]glucose	6.8 ± 0.5	1.1	1.1	1.1	1.1	47.4 ± 5.5
	[2-^{13}C]fructose	5.5 ± 0.4	21.9 ± 6.4	3.2 ± 0.9	1.1	2.5 ± 0.4	1.1

These values were obtained by comparing the peak areas in the ^{13}C-NMR analyses of the samples to that obtained for natural abundant glucose analyzed in the same conditions. The isotopic enrichment at C1 position was confirmed from ^{1}H-NMR analysis.
[a]The carbon positions for which no label was observed were assumed to have a specific enrichment equal to the natural abundance (1.1%).

We have focused particularly on the pentose-phosphate cycle by using [2-^{13}C]fructose as substrate. The label incorporation at the C1 and C3 positions of the glucosidic units (Fig. 5) showed that this cycle occurred in both strains. The specific enrichments in these positions (Table 2) were similar for both strains and were also analogous to those obtained for the RmH580 strain on [2-^{13}C]glucose. This indicated that the operation of carbohydrate cycles on fructose in *S. meliloti* was strikingly different from the situation reported for alginate-synthesizing species. The [(C1+C3)/C2] enrichment ratios on [2-^{13}C]fructose were 0.39 and 0.40 for the Rm2011 and RmH580 strains, respectively. These values were in the same range as that determined for the RmH580 strain on [2-^{13}C]glucose (0.34). Taken together, these results showed that the contribution of carbohydrate – at least pentose-phosphate – cycling was similar for all the conditions where the gluconate bypass did not operate, but was lower than observed for wild-type strains (Rm2011 or Su47 [12]) on glucose.

As for the *gdh* mutant on glucose, a very low label incorporation was found in the C5 position of β-glucan glucosidic units from [2-^{13}C]fructose for both strains (Table 2), indicating that PFK may be present but with low activity. This showed that, in our experimental conditions, the EMP pathway was not significantly involved in fructose catabolism, whereas in similar conditions 40% of fructose transited through this pathway in *Pseudomonas aeruginosa* [10].

4. Conclusion

The occurrence of carbohydrate cycling in polysaccharide synthesis is often discussed in terms of alternative pathways to compensate for insufficient direct formation of osidic precursors from the exogenous substrate. The present data indicate that such an explanation cannot apply to *S. meliloti* where the cycles are observed even if osidic precursors can be obtained directly. Together with the occurrence of carbohydrate cycling in alginate-synthesizing species on glucose (ED only) but not on fructose (both ED and EMP), and the absence of cycling in EMP-utilizing polysaccharide-synthesizing bacteria [20], our results point out that carbohydrate cycling is tightly associated with situations where the dehydrogenating pathways (i.e. ED and PP), but not EMP, were active. ED-utilizing species maintain high glycolytic rates in any growth phase [8]. It has been suggested that (exo)polysaccharide synthesis could offer an effective means of dissipating both carbon and energy under conditions where substrate is in excess to that required for growth. In that respect, the gluconeogenetic enzymes may play a key role by enabling hexose-phosphate reformation at the expense of phosphoester bonds. Further investigations on *S. meliloti* shall focus on the kinetic properties of the catabolic enzymes and on the regulation of the gluconeogenetic enzymes.

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