Exopolysaccharide and Poly- β -Hydroxybutyrate Coproduction in Two *Rhizobium meliloti* Strains

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The effects of different nitrogen and carbon sources on cell growth, pH, and exopolysaccharide (EPS) and poly- β -hydroxybutyrate (PHB) production by two strains of *Rhizobium meliloti* (M5N1 and Su47) are reported. Differences in the behavior of glucose- and fructose-grown cells were shown, in particular with the M5N1 strain. Growth in a glucose-containing medium was accompanied by acidification of the culture medium, which leads to cell death. On fructose, acidification was detected only in the medium with a mineral nitrogen supply. A lag phase in EPS production was observed with cells grown with glucose, probably related to an initial extracellular conversion of the carbohydrate into an acid. No lag phase was observed in EPS production from fructose or in PHB synthesis whatever the carbon source. A decrease in PHB content was noticed for both strains under conditions where acidification of media occurred. The extent of production, emphasized by the use of a coproduction index, indicates that the M5N1 strain is a more promising organism than is the Su47 strain for polymer production. Such a strain, put in rich medium (containing yeast extract) supplemented with fructose, accumulated PHB up to 85% of dry cell weight and excreted about 1.5 g of EPS per liter in the medium. Regulation of the coproduction of EPS and PHB by these cells is suggested.

Fast-growing rhizobia (*Rhizobium leguminosarum* subsp. *phaseoli*, *R. leguminosarum* subsp. *trifolii*, and *R. meliloti*) are able to synthesize a variety of polymers as bacteroids or free-living cells (38). These bacteria accumulate glycogen in their cytoplasm as a dynamic carbon storage product (2, 38). They also synthesize periplasmic cyclic β -(1,2)-glucans (4, 39), which can be used in food and pharmaceutical applications as inclusion agents (3). Rhizobia mainly excrete a high-molecular-weight polysaccharide (31). Exopolysaccharides (EPS) are commercially useful for producing gels and modifying the rheological properties of aqueous systems, and they have potential in replacing plant and algae EPS traditionally used in food, pharmaceutical, textile, and oil industries (25). Under specific conditions, rhizobia also synthesize poly- β -hydroxybutyrate (PHB) (12, 21, 30, 35), an intracellular carbon and energy storage polymer with potential industrial applications due to its thermoplastic and biodegradable properties (5).

The biosynthesis of these different polymers by rhizobia depends on both the genetic characteristics and culture conditions of the strain. Some studies have documented the separate production of EPS (18, 38) or PHB (10, 30, 35) by various strains, including strain Su47 of *R. meliloti*, a registered strain with well-known characteristics (2, 4, 38). However, much less attention has been paid to the relationships between the synthesis of these two major compounds by such multiproductive bacteria. It is clear that the biosynthetic pathways of these polymers are not directly linked, but they are produced at the same time. This means that the general conditions governing the operation of the biosynthetic pathways (the nature and availability of carbon and nitrogen sources, oxygenation, the energetic and redox states of cells, environmental constraints,

etc.) are the same. In particular, the extent of EPS and PHB production implies a significant energetic cost for cells.

The present work was undertaken to examine the synthesis of both EPS and PHB under different culture conditions in strains M5N1 and Su47 of *R. meliloti*. The effects of the nitrogen source were studied. Two carbon sources (glucose and fructose) which may lead to differences in EPS (7, 19) and PHB (21) production were tested. Because of different culture conditions, the coproduction of EPS and PHB was assessed in light of cell growth characteristics. The results indicate that *R. meliloti* M5N1, which coproduces large amounts of EPS and PHB, is a good candidate for further investigation of the relationships between EPS and PHB metabolism in *R. meliloti*.

MATERIALS AND METHODS

Bacterial strains. *R. meliloti* M5N1 (laboratory collection) (7, 18) and Su47 (obtained from Commonwealth Scientific and Industrial Research Organisation, Canberra, Australia) were used in this study. Both strains are able to nodulate *Medicago sativa*.

Chemicals and media. All chemicals were purchased from Prolabo (France) unless otherwise noted. Three media were used: (i) a *Rhizobium* complete (RC) medium containing 1 g of yeast extract (Difco Laboratories), 1 g of K_2HPO_4 , and 0.2 g of $MgSO_4 \cdot 7H_2O$ per liter of deionized water; (ii) R medium with mineral nitrogen, 1 g of NH_4NO_3 , 1 g of K_2HPO_4 , and 0.2 g of $MgSO_4 \cdot 7H_2O$ per liter of deionized water, supplemented with filter-sterilized biotin (10 mg liter⁻¹; Sigma Chemical Co.) and thiamine (500 mg liter⁻¹; Sigma Chemical Co.); and (iii) MM medium, a minimal medium without a nitrogen supply, containing 1 g of K_2HPO_4 and 0.2 g of $MgSO_4 \cdot 7H_2O$ per liter of deionized water. The pH of the medium was adjusted to 7.2 (H_3PO_4 [1 M]) before sterilization (120°C for 20 min). Stock solutions of glucose and fructose (Sigma Chemical Co.) were separately sterilized (105°C for 20 min) and added to a final concentration of 6 g liter⁻¹.

Growth conditions. Precultures and cultures were grown at 30°C on a rotary shaker (200 rpm). Precultures were prepared by inoculating the organism (a colony grown for 24 h in 10 ml of RC medium) into 90 ml of RC medium supplemented with a carbon source. Cells (80 ml of broth culture) were harvested at late exponential phase (24 h). For experiments using RC medium, cells were transferred directly to 720 ml of RC medium. For experiments using R or MM medium, cells were centrifuged ($12,396 \times g$ for 20 min at 4°C) and washed three times with an equal volume of the final medium. The pellets were then suspended in 800 ml of R or MM medium. The final concentration of either

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carbon source (glucose or fructose) was 6 g liter⁻¹ in all media. The cultures were grown in 2-liter Erlenmeyer flasks. Sixteen milliliters of the fermentation broth was withdrawn at 24-h intervals. One milliliter was used for cell number determination and absorbance and pH measurements, and the remaining 15 ml was used for further analyses.

Assessment of bacterial growth. Cell growth was monitored by measurements of the absorbance at 600 nm. The cell concentration was determined after dilution of the culture on RC and tryptone yeast (0.88 g of CaCl₂ · 2H₂O, 5 g of tryptone [Difco Laboratories], and 3 g of yeast extract [Difco Laboratories] per liter of deionized water) agar (Difco Laboratories) plates for *R. meliloti* M5N1 and Su47 strains, respectively. For bacterial dry weight measurement, 5 ml of culture was centrifuged; the pellet was washed twice with MM medium, suspended in 5 ml of deionized water, and transferred to a previously weighed cupel. The suspension was dried for 24 h at 100°C, cooled, and weighed. All the experiments were performed in triplicate, and the results were expressed as mean values; the standard deviations were less than 5%, except for dry biomass measurements (10%).

Assays of glucose, fructose, and PHB. Glucose and fructose were assayed with enzymatic tests (Sigma Chemical Co. and Boehringer Mannheim, respectively). The PHB content in cells was measured by gas chromatography after acidic methanolysis of cell pellets sedimented from 10 ml of fermentation broth washed twice with the same volume of MM medium as previously described (20).

EPS assay and characterization. The EPS content in the culture supernatant (0.5 ml) was determined after trifluoroacetic acid (0.5 ml of 4.25 N) hydrolysis in sealed tubes at 100°C for 3.25 h. Samples were vacuum dried and suspended in deionized water, and the glucose content was determined as described above. Under these hydrolysis conditions, glucose recovery was 80% from the EPS produced by the M5N1 strain of *R. meliloti* and purified as described below. Then the values obtained from samples were corrected accordingly and were expressed relative to the residual biomass (corresponding to the part of biomass not belonging to PHB).

The EPS produced after 144 h in 1 liter of culture medium was collected after centrifugation (40,000 × *g* for 30 min at 10°C), and the cell-free broth was concentrated by ultrafiltration (100,000 nominal-molecular-weight-cutoff membrane; Sartorius). Structural characterization was achieved by ¹H nuclear magnetic resonance analysis performed on a Bruker AM-300 spectrometer as previously described (8).

RESULTS

Growth kinetics. A good correlation between absorbance and biomass measurements was obtained for the assessment of cell growth; only the absorbance values are presented here (Fig. 1). The cell growth in RC medium was always greater than that in R or MM medium regardless of the strain or carbon source (glucose or fructose). For strain M5N1, growth on fructose was greater than that on glucose, while for strain Su47 few differences between carbon sources were observed.

A decrease in absorbance was observed for both strains growing in RC medium supplemented with glucose after 48 and 72 h of culture for strains M5N1 and Su47, respectively. This was correlated with a decrease in the number of viable cells, which occurred sooner with strain M5N1 (from 2.45 × 10⁹ to 3 × 10⁶ CFU ml⁻¹ at 48 and 144 h, respectively) than with strain Su47 (from 2.7 × 10⁹ to 5.9 × 10⁸ CFU ml⁻¹ at 120 and 144 h, respectively). Such cell death was not detected under other conditions.

As previously described (7, 19), *R. meliloti* strongly acidified RC medium supplemented with glucose (Table 1). This acidification, which was greater with strain M5N1 than with strain Su47 (4.8 and 5.5, respectively), preceded the decrease in cell number. A pH tolerance test performed on agar plates (data not shown) indicated that strains M5N1 and Su47 did not grow at a pH value lower than 5.5. This suggested that acidification was the cause of cell death. In R medium, acidification was observed with either carbon source but to a lesser extent than that observed in RC medium. In MM medium, little change in pH value was observed.

Carbon source consumption. As shown in Table 1, carbon source consumption followed cell growth. In RC medium, M5N1 consumed more fructose than did Su47, whereas the latter consumed more glucose than did M5N1. For a given strain in R medium, consumption was similar for both carbon

FIG. 1. Absorbance as a function of time for glucose and fructose as carbon sources with two *R. meliloti* strains, M5N1 and Su47. ■, RC medium; ●, R medium; ▲, MM medium.

sources, but M5N1 consumed a little more carbohydrate than did Su47. In MM medium, little carbohydrate consumption was observed.

EPS production. The M5N1 strain produced EPS with either carbon source (Fig. 2). Production on fructose was higher than that on glucose, except in MM medium, where it was slightly higher on glucose. Production was detected as soon as 24 h (time of the first sample) when media were supplemented with fructose, whereas a lag phase of about 50 h was observed in the presence of glucose. Production in MM medium supplemented with fructose began earlier and was greater than that in RC medium (23 and 5 mg liter⁻¹ h⁻¹, respectively, between 0 and 24 h of culture). Whereas in MM medium maximum production was reached after 24 h, production in RC medium continued over the entire culture period. The same phenomenon was observed on glucose.

M5N1 cells growing in RC medium supplemented with glucose produced EPS despite cell death. The high-molecular-weight fraction of the culture supernatant analyzed by ¹H nuclear magnetic resonance spectroscopy contained EPS

TABLE 1. Effects of media and carbon sources on pH, carbon source consumption, and PHB accumulation by two *R. meliloti* strains, M5N1 and Su47

Strain	Carbon source	Medium	Final pH ^a	Carbon source consumption (g/liter) ^b	PHB accumulation (%) ^c	Time (h) ^d
M5N1	Fructose	RC	6.8	4.7	84.5	122
		R	5.5	2.6	50.8	122
		MM	7.2	0.8	30	144
	Glucose	RC	4.8	2.5	40.8	72
		R	5.8	2	40	96
		MM	6.9	0.8	44.1	144
Su47	Fructose	RC	6.9	3.9	66	144
		R	6.4	1.2	32.8	96
		MM	7.2	0.7	47	144
	Glucose	RC	5.5	3.5	51	96
		R	6.3	1.5	23.6	72
		MM	7.1	0.4	70.3	144

^a pH measured at 144 h of culture.^b Consumption between 0 and 144 h of culture.^c Relative to dry cell weight.^d Culture time at which maximal accumulation of PHB was observed.

excreted (18) by living bacteria, not cell components released from lysed bacteria (data not shown).

In nitrogen-containing media, strain Su47 grown on fructose produced less EPS than did strain M5N1, but the opposite result was obtained in MM medium (Fig. 3). On glucose, strain Su47 produced EPS only in RC medium, in similar amounts and with the same lag phase as did strain M5N1.

To take into account the fraction of biomass representing EPS, EPS yields were expressed relative to the residual biomass. These results (Fig. 4) show the high productivity of cells

cultured in MM medium, except for strain Su47 fed with glucose, and confirm the low-level or nonproductive state of cells grown in R medium. For cells grown in RC medium, the transition from a nonproductive state to a productive state is correlated with the transition from the exponential to the stationary-growth phase. The data in Fig. 4 also show the lag phase in EPS production from glucose.

PHB production. PHB was produced under all conditions, and no lag phase was observed on glucose (Fig. 2 and 3). PHB production by strain M5N1 was higher in RC medium than in

FIG. 2. EPS and PHB production as a function of time for glucose and fructose as carbon sources with strain M5N1 of *R. meliloti*. ■, RC medium; ●, R medium; ▲, MM medium.

FIG. 3. EPS and PHB production as a function of time for glucose and fructose as carbon sources with strain Su47 of *R. meliloti*. ■, RC medium; ●, R medium; ▲, MM medium.

FIG. 4. EPS yield relative to the residual biomass (corresponding to the part of biomass not belonging to the PHB accumulated) as a function of time for glucose and fructose as carbon sources with two *R. meliloti* strains, M5N1 and Su47. ■, RC medium; ●, R medium; ▲, MM medium.

other media, and fructose was a better substrate than was glucose (Fig. 2). In media supplemented with fructose, PHB production by strain M5N1 was greater than that by strain Su47 (Fig. 3). For both strains grown in RC medium, PHB production was continuous on fructose. Interestingly, a decrease in PHB content was noted for both strains under conditions where acidification of the medium occurred, indicating that PHB may be mobilized in response to an environmental stress (1, 28, 38).

Because PHB is an endogenous polymer, production was examined in terms of accumulation within cells; maximal PHB accumulations for various conditions are presented in Table 1. The highest PHB accumulation was obtained with strain M5N1 growing in RC medium supplemented with fructose (85% of cell dry mass). Maximal accumulation was obtained in RC medium supplemented with fructose, whereas it was obtained in MM medium when the carbon source was glucose. In R medium, PHB accumulation for each strain was the same with either carbon source.

Coproduction index. The main purpose of the present study was to assess the coproduction of EPS and PHB in these two strains of *R. meliloti*. For this purpose, a coproduction index was defined as the logarithmic ratio of the total amount of carbon in EPS to the total amount of carbon in PHB. The values of this index as a function of time are shown in Fig. 5 for different conditions. Whatever the strain, glucose was not a good substrate for coproduction. EPS was not produced in all glucose-supplemented media, and when coproduction occurred, the index varied as a function of time, indicating a transition from a predominance of PHB synthesis to a predominance of EPS synthesis.

For both strains, coproduction indices in fructose-containing media indicated that MM medium was favorable for EPS pro-

FIG. 5. EPS and PHB coproduction index as a function of time for glucose and fructose as carbon sources with two *R. meliloti* strains, M5N1 and Su47. ■, RC medium; ●, R medium; ▲, MM medium. This index was defined as the logarithmic ratio of the total amount of carbon in EPS to the total amount of carbon in PHB.

duction. However, the coproduction index for strain Su47 in MM medium decreased over time, whereas it did not change for strain M5N1. In nitrogen-containing media, strain Su47 was more oriented toward PHB production than was strain M5N1. For both strains, the coproduction indices were comparable and had similar trends whether the nitrogen source was ammonium nitrate or yeast extract.

The best coproduction indices (near 1) were obtained for strain M5N1 in nitrogen-containing media supplemented with fructose (Fig. 5). However, much larger quantities of polymers were produced in RC medium than in R medium. In the former case, PHB within cells comprised 85% of dry cell weight and EPS was nearly 1.5 g per liter.

DISCUSSION

The natures of the carbon and nitrogen sources affected both biomass formation and biopolymer production by *R. meliloti* Su47 and M5N1. Growth in a nitrogen-depleted medium was limited and favored EPS production rather than PHB production (Fig. 5). The effect of nitrogen depletion on EPS production is consistent with previous results for *R. meliloti*

Su47, which produced EPS when nitrogen was totally consumed in a medium containing an excess of carbon (38). However, under such conditions and in the presence of glucose, PHB accumulation by Su47 cells was notable (Table 1). Although the assimilation of ammonium nitrate by *R. meliloti* has been reported previously (17), both cell growth and polymer production were low in the medium with this nitrogen source. Furthermore, the production of PHB was higher than that of EPS, which underlines the importance of nitrogen in the synthesis of PHB. As expected, the amounts of polymers produced in rich medium were greater than those in other media. PHB accumulation by strain M5N1 comprised 85% of dry cell weight even under the nonoptimized conditions of our study. This value can be compared to that (80%) observed for the high-PHB-producing microorganism *Alcaligenes eutrophus* in fed batch culture with glucose concentration control (23) and to that obtained with a recombinant strain of *Escherichia coli*, for which the accumulation was 95% of dry cell weight (11). At the same time, M5N1 cells excreted nearly 1.5 g of EPS liter⁻¹, the largest amount in our study. The increase in medium viscosity accompanying EPS production, resulting in oxygen transfer limitation, may favor PHB synthesis (10, 30, 35). It is of particular significance that the coproduction indices remained nearly constant over culture time under these conditions, suggesting that coproduction was regulated in these cells.

It has been reported that *Rhizobium* cells growing in minimal media supplemented with glucose or fructose have similar enzymatic patterns (29). However, the growth (when it occurred) of *R. meliloti* cells in glucose-containing medium was always accompanied by acidification of the medium; this was particularly marked in the complete medium in which we detected gluconate (33). Fast-growing rhizobia (particularly *R. meliloti*) are very sensitive to acidic pH (15, 16, 22, 34), which can result in either inhibition of cell growth or death of cells, depending on the strain's sensitivity (27). The extensive acidification in cultures of strain M5N1 may explain the cell death observed in the rich medium.

When cells were grown with ammonium nitrate, acidification was observed with both glucose and fructose. Furthermore, the extents of acidification were similar with both carbon sources. With fructose-grown cells, neither gluconate nor 2-ketogluconate was detected in the culture medium (33). Further investigations are necessary to better understand the origin and significance of acidification in fructose-containing media.

Strain M5N1 growing in rich medium supplemented with glucose produced EPS after cell death already had been detected. Cell growth was asynchronous, so living cells must produce EPS, although in small amounts. On the other hand, the final concentrations of the polymer were similar when acidification occurred (rich medium) and when pH variation was small (minimal medium), indicating that the pH of the culture itself does not affect EPS synthesis by *R. meliloti*. This result is consistent with studies of *R. loti* NZP 2037, which produced EPS even at pH 4.3 (28). Furthermore, it already has been shown (9) that acid-tolerant *Rhizobium* strains produce more EPS than do acid-sensitive *Rhizobium* strains.

A striking difference in the behavior of *R. meliloti* cells grown on glucose or fructose is the occurrence of a lag phase in EPS production on glucose. Such a lag phase was observed independently of strain and nitrogen supply. Its origin may be related to the extracellular conversion of glucose into gluconic (24) or 2-ketogluconic (19, 32) acids which can enter cells (26, 28, 36), wherein they are converted into 6-phosphogluconate. Because of the extent of this extracellular acid production and the irreversibility of the glucose-6-phosphate dehydrogenase reaction (14), the synthesis of glucose-6-phosphate (the EPS

precursor) is probably low. This could explain the lag phase observed for EPS production in glucose-containing media, whereas fructose (for which no lag phase was observed) enters cells directly and can yield glucose-6-phosphate. This hypothesis is supported by the fact that glucose and fructose enter *R. meliloti* cells via similar high-affinity active-transport systems (6, 13, 37), suggesting that the lag phase cannot be due to a difference at the level of entry. The gluconate bypass does not affect pyruvate synthesis and by extension acetyl coenzyme A production (the PHB precursor). This could explain the absence of a lag phase observed in PHB production. Further experiments are under way to confirm this proposed hypothesis. The elaboration of a coproduction index emphasized that the strains studied differ in their capacities to assimilate carbon and nitrogen sources for biopolymer synthesis. From a quantitative point of view, M5N1 appears to be a promising organism for the production of polymers. Because of the extent of production and the constancy of the coproduction index, this strain will be a useful model for further investigation of the regulation of biosynthetic pathways in multiproductive bacteria.

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