Osmoprotection by Pipelic Acid in Sinorhizobium meliloti: Specific Effects of D and L Isomers

KAMILA GOUFFI,* THÉOPHILE BERNARD, AND CARLOS BLANCO

Equipe Osmoadaptation chez les Bactéries, UMR CNRS 6026, Université de Rennes 1,
Campus de Beaulieu, F-35042, Rennes, France

Received 15 December 1999/Accepted 3 April 2000

Dl-Pipelic acid (dl-PIP) promotes growth restoration of Sinorhizobium meliloti cells facing inhibitory hyperosmolality. Surprisingly, D and L isomers of this imino acid supplied separately were not effective. The uptake of L-PIP was significantly favored in the presence of the D isomer and by a hyperosmotic stress. Chromatographic analysis of the intracellular solutes showed that stressed cells did not accumulate radiolabeled L-PIP. Rather, it participates in the synthesis of the main endogenous osmolytes (glutamate and the dipeptide N-acetylglutaminylglutamine amide) during the lag phase, thus providing a means for the stressed cells to recover the osmotic balance. 13C nuclear magnetic resonance analysis was used to determine the fate of D-PIP taken into the cells. In the absence of L-PIP, the imported D isomer was readily degraded. Supplied together with its L isomer, D-PIP was accumulated temporarily and thus might contribute together with the endogenous osmolytes to enhance the internal osmotic strength. Furthermore, it started to disappear from the cytosol when the L isomer was no longer available (during the late exponential phase of growth). Together, these results show an uncommon mechanism of protection of osmotically stressed cells of S. meliloti. It was proved, for the first time, that the presence of the two isomers of the same molecule is necessary for it to manifest an osmoprotective activity. Indeed, D-PIP seems to play a major role in cellular osmoregulation through both its own accumulation and improvement of the utilization of the L isomer as an immediate precursor of endogenous osmolytes.

All microorganisms have to cope with fluctuations in the osmolality of their environment. The mechanisms of osmoregulation are very similar in all living organisms (plants, animals, and bacteria). In response to the elevated osmolality of their environment, they accumulate, at relatively high intracellular concentrations, inorganic ions and neosynthesized low-molecular-mass organic molecules called compatible solutes. Amino or imino acids (glutamate, proline, pipelic acid [PIP], and ectoine), carbohydrates (trehalose, sucrose, and glycerol) and methylated onium compounds (glycine betaine [GB] and dimethylsulfiniopropionate [DMSP]) are the most frequently accumulated compatible solutes (6, 8, 10, 19, 37, 42). After a sudden decrease in osmolality, accumulated compatible compounds can be liberated into the surrounding environment and subsequently used as osmoprotectants by the same or other organisms if they are under a hyperosmotic stress (25, 27). Such compounds that are able to improve growth of cells under inhibitory osmoralties are thus called osmoprotectants.

Hence, in natural environments, the concept of an osmoprotectant supposes an ecological cycle in which the compatible solutes are shuttled from producers to consumers exposed to hyperosmotic constraint. Several osmoprotectants produced by plants are therefore of prime environmental interest for soil bacteria. Osmoprotection of the soil bacterium Sinorhizobium meliloti has been reported to be effective with a variety of compounds: GB; GB derivatives or analogues, such as proline betaine, γ-butyrobetaine, trigonelline, dimethylglycine, and DMSP (5, 11, 22, 34); ectoine (39); sucrose (16); and some disaccharides derived from plant polymers (15).

PIP is a nonprotein imino acid the occurrence or accumula-
Anisms, we have investigated the osmoprotective effect and the fate of PIP in osmotically stressed cells. The differential effect of d- and l-enantiomers of the inosine acid was analyzed.

**MATERIALS AND METHODS**

**Bacterial strain and growth conditions.** *S. meliloti* 102F34, a standard laboratory strain, was grown aerobically at 30°C overnight in mannitol-salts-yeast extract (MSY) medium until the late exponential phase of growth was reached (16). Bacteria were inoculated at a final concentration of 1% (vol/vol) into minimal LAS medium containing 10 mM sodium L-α-aspartate, 10 mM sodium L-α-lactate, and the same mineral salts as in MSY medium. The osmolarity of the medium was increased by the addition of 0.5 M NaCl or an isotonic concentration of the nonolactate mannitol (0.8 M). d-, l-, and dl-PIP, dL-lactate, and -α-aspartate stock solutions (Sigma Chimie, Saint-Quentin Fallavier, France) were sterilized by filtration. Bacterial growth was monitored by optical density at 570 nm (OD570 nm). The protein contents of the cultures were determined by the method of Lowry et al. (24).

**Extraction of cellular solutes.** The pellet of freshly harvested and washed cells was extracted twice with 80% (vol/vol) ethanol under vigorous magnetic stirring at room temperature for 30 min. After centrifugation, the supernatant (ethanol-insoluble fraction or EIF) contained the intracellular macromolecular components and cell envelopes.

**Production of L-[14C]PIP.** L-[14C]PIP was synthesized by *B. ammonium* ATCC 6872 from L-[14C]jasmine monochloride (11.1 GBq/mmol; Amersham, Les Ulis, France). The labeled lysine and 1 M NaCl were added to a cell suspension growing in M63 medium. The mixture was incubated for 2 h, and L-[14C]PIP was purified as described previously (13). The specific activity (1.83 GBq/mmol) was determined after quantification by high-performance liquid chromatography.

**Radiolabeling assays.** Cells of *S. meliloti* were grown in Erlenmeyer’s flasks containing 10 ml of LAS medium with 0.5 M NaCl and 1 mM L-[14C]PIP or a mixture of unlabeled d-PIP and L-[14C]PIP (0.5 mM each isomer). Respired CO2 was trapped on a strip of filter paper (3 by 1 cm) moistened with 30 μl of 5 M KOH. Samples were removed periodically, washed twice in 1 ml of osmotic LAS medium without PIP, and submitted to an ethanolic extraction as described above. The radioactivity of the ESF and EIF was measured by liquid scintillation counting. An aliquot of the soluble fraction was analyzed by paper chromatography as described below.

**Chromatographic analysis and purification of glutamate and NAGGN.** Identical numbers of cells were extracted at each stage of growth in LAS medium—0.5 M NaCl in the presence of 1 mM L-[14C]PIP or d-PIP as the carbon source. The amino acids and PIP were detected by amino acid analysis as described previously (16). The cytoplasmic levels of glutamate, NAGGN, and trehalose were determined by paper chromatography as described below.

**Transport assays.** Cells were cultivated in defined LMS medium with NaCl or mannitol added. PIP was supplied as either 1 mM dl-PIP, 1 mM D- or L-PIP, or 1 mM D- and L-PIP (0.5 mM each isomer). GB (1 mM), the most potent osmoprotectant known for *S. meliloti* so far (5), was used in this experiment as a positive control (Table 1). Addition of 1 mM d-, l-, or dl-PIP to the growth medium had no significant effect on unstressed cells. In sharp contrast, dl-PIP enhanced both the growth yields and the growth rates of NaCl- and mannitol-stressed cells about twofold and was as effective as GB (Table 1). Similar results were obtained when a mixture of d- and l-isomers of PIP was supplied to osmotically stressed cells. However, provided separately, neither d- nor l-PIP was able to improve the growth of cells under hyperosmotic conditions (Table 1).

To determine whether the response of bacterial cells is dependent upon their ability to assimilate the different forms of the inosine acid, D-, L-, and DL-PIP were supplied to the unstressed and stressed cultures as potential carbon sources. Thus, in this experiment, the lactate of LAS medium was used within the substitution of d-, l-, or dl-PIP (10 mM each). No growth was observed without added PIP, showing that aspartate could not be used as a carbon source. Figure 1 shows that in the absence of osmotic stress, all of these compounds were able to support growth of *S. meliloti* to different degrees. Indeed, the growth rates were about 0.06 and 0.04 generation h−1 in the presence of d- or d-PIP as the carbon sources, respectively; maximal OD570 were about 1 and 0.7 U, respectively. Hence, each of these two isomers can be imported by the L or D isomer provided separately.

**RESULTS**

Osmoprotection of *S. meliloti* is effective by dl-PIP but not by the l or d isomer provided separately. Cells were cultivated in defined LAS medium with NaCl or mannitol added. PIP was supplied as either 1 mM dl-PIP, 1 mM d- or l-PIP, or 1 mM D- and L-PIP (0.5 mM each isomer). GB (1 mM), the most potent osmoprotectant known for *S. meliloti* so far (5), was used in this experiment as a positive control (Table 1). Addition of 1 mM l-, d-, or dl-PIP to the growth medium had no significant effect on unstressed cells. In sharp contrast, dl-PIP enhanced both the growth yields and the growth rates of NaCl- and mannitol-stressed cells about twofold and was as effective as GB (Table 1). Similar results were obtained when a mixture of d- and l-isomers of PIP was supplied to osmotically stressed cells. However, provided separately, neither d- nor l-PIP was able to improve the growth of cells under hyperosmotic conditions (Table 1).

### Table 1. Comparative effects of dl-, d-, and l-PIP on the growth of *S. meliloti* 102F34 subjected to an hyperosmotic stress

<table>
<thead>
<tr>
<th>Putative osmoprotectant added to LAS medium</th>
<th>None</th>
<th>Result with osmotic stress</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μ (h−1)</td>
<td>OD&lt;sub&gt;570&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dl-PIP</td>
<td>0.17</td>
<td>1.9</td>
</tr>
<tr>
<td>dl-PIP</td>
<td>0.18</td>
<td>1.8</td>
</tr>
<tr>
<td>d-PIP</td>
<td>0.15</td>
<td>2.0</td>
</tr>
<tr>
<td>d- + l-PIP</td>
<td>0.17</td>
<td>2.0</td>
</tr>
<tr>
<td>GB</td>
<td>0.18</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* Cultures were grown in LAS medium with or without osmotic (0.5 M NaCl or 0.8 M mannitol), in the presence or the absence of 1 mM the indicated compound assayed.

* Growth parameters are expressed as the growth rate (μ [generation per hour]) and the maximal OD<sub>570</sub> at the stationary phase.

* LAS medium with 0.8 M mannitol develops the same osmotic pressure as LAS medium containing 0.5 M NaCl (2.4 MPa).

* FIG. 1. Growth of *S. meliloti* 102F34 in the presence of d-, l-, and dl-PIP as carbon sources. Cells were cultivated in aspartate-S medium containing 10 mM dl-PIP (●), l-PIP (○), or d-PIP (▲).
and catabolized by *S. meliloti*. The main point of interest was that the simultaneous addition of D- and L-PIP as carbon sources (5 mM each) had a synergistic effect on growth parameters of *S. meliloti* cells. The growth rate and maximal OD<sub>570</sub> were about 0.12 generation h<sup>-1</sup> and 2.7 U, respectively. A similar phenomenon was observed when these carbon sources (D-, L-, or DL-PIP) were supplied to *S. meliloti* cells grown under hyperosmotic conditions. Total bacterial growth was greater when stressed cells were grown in the presence of the L and D isomers of PIP, LAS medium containing 0.5 M NaCl and 1 mM DL-PIP showed.

**TABLE 2. Effects of D-PIP and 0.5 M NaCl on the uptake of L-[14C]PIP by *S. meliloti* 102F34**

<table>
<thead>
<tr>
<th>PIP in transport medium&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Uptake rate (nmol min&lt;sup&gt;-1&lt;/sup&gt; mg of protein&lt;sup&gt;-1&lt;/sup&gt;) in medium&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 M NaCl</td>
</tr>
<tr>
<td></td>
<td>No PIP</td>
</tr>
<tr>
<td>L-[14C]PIP</td>
<td>8</td>
</tr>
<tr>
<td>DL-[14C]PIP</td>
<td>18</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cells were grown to mid-exponential phase in LAS medium without or with 0.5 M NaCl. D-, L-, or DL-PIP (1 mM) was added or not during 4 h to these cultures, and uptake experiments were performed as described in the text.

<sup>b</sup> Initial rates of PIP uptake were measured in fresh isotonic growth medium containing 0.5 mM L-[14C]- or DL-[14C]PIP (0.25 mM each isomer [1.83 GBq mmol<sup>-1</sup>]).

Fate of PIP in stressed growing cells of *S. meliloti.* To investigate the intracellular fate of DL-PIP (osmoprotective form of PIP) in stressed cells of *S. meliloti,* an analysis of cell extracts was undertaken. Spectra of ethanolic extracts (Fig. 2) from cells cultivated in LAS medium containing 0.5 M NaCl and 1 mM DL-PIP showed both the characteristic peaks of PIP and those of the three major endogenous osmolytes: glutamate, NAGGN, and trehalose. These three compounds were also observed in the absence of exogenous osmoprotectant (data not shown). The signals of PIP appeared together with those of glutamate and NAGGN in the early and mid-exponential phases of growth (Fig. 2A and B) and with those of glutamate, NAGGN, and trehalose in the late exponential phase (Fig. 2C). The resonances of PIP were absent thereafter in spectra from cells in the stationary phase (Fig. 2D). Extracts obtained from stationary-phase cells exhibited a spectrum similar to that of cells grown under osmotic stress without osmoprotectant and harvested at stationary phase.

Nevertheless, <sup>13</sup>C NMR analysis did not allow determination of which isomer (D- and/or L-PIP) was accumulated by stressed cells of *S. meliloti.* To answer this question, the intracellular fate of L-[14C]PIP was observed over the growth cycle of stressed cells grown in LAS medium–0.5 M NaCl plus 1 mM DL-PIP (i.e., the conditions under which osmoprotection by...
PIP was effective). Under such conditions, about 8% of the supplied radioactivity was taken up from the medium after 5 h of culture (lag phase of growth) (Fig. 3A). During this period, the percentages of the radioactivity incorporated into the EIF, \( ^{14}\text{CO}_2 \), and the ESF were about 21, 15, and 63% of the intracellular radioactivity, respectively. After 10 h of culture, the uptake rate of \( \text{L-}^{14}\text{C-PIP} \) increased to reach a constant value as soon as the growth started (Fig. 3A). Taking into account the specific radioactivity of the supplied \( \text{L-}^{14}\text{C-PIP} \), this value was estimated to be 40 nmol min\(^{-1}\) mg of protein\(^{-1}\). The radioactivity in the ESF decreased, and the levels of radiocarbon were quite similar in the three fractions (ESF, EIF, and \( ^{14}\text{CO}_2 \)), respectively. After 10 h of culture, the uptake rate of \( \text{L-}^{14}\text{C-PIP} \) increased to reach a constant value as soon as the growth started (Fig. 3A). Taking into account the specific radioactivity of the supplied \( \text{L-}^{14}\text{C-PIP} \), this value was estimated to be 40 nmol min\(^{-1}\) mg of protein\(^{-1}\). The radioactivity in the ESF decreased, and the levels of radiocarbon were quite similar in the three fractions (ESF, EIF, and \( ^{14}\text{CO}_2 \)). Indeed 30, 33, and 37% of the intracellular radioactivity was recovered in the EIF, \( ^{14}\text{CO}_2 \), and the ESF, respectively. At this time, bacteria which were inoculated at an initial OD\(_{570}\) of 0.1 had reached an OD\(_{570}\) of only 0.2 U, which corresponds to the early exponential phase of growth (Fig. 3A). After 15 h of growth, 50% of the supplied radioactivity was incorporated into the cells. The radioactivity of the ESF still continued to decrease, and the radiolabeled carbon was recovered mainly in \( ^{14}\text{CO}_2 \) (51%) rather than in the EIF (30%) as the cells entered the mid-exponential growth phase. Labeled \( ^{14}\text{CO}_2 \) reached its maximal level (62% of supplied radiocarbon) in the late exponential growth phase, when only 10% of the supplied \( \text{L-}^{14}\text{C-PIP} \) remained in the growth medium. The labeling decreased in parallel in both the ESF and in the EIF, which retained 17 and 21% of the intracellular radiocarbon, respectively (Fig. 3A).

Chromatographic analysis of the ESF of cells harvested at different periods of culture was undertaken to identify the radiolabeled compounds accumulated in these cytosolic fractions. PIP gave the predominant ninhydrin-colored spot detected in the ESF of cells collected at the early, mid-, and late exponential growth phases. This spot was absent from the ESF of cells harvested in stationary phase. Another spot corresponding to glutamate was also revealed after ninhydrin spraying of the chromatogram. Surprisingly, autoradiographic analysis of the chromatograms clearly demonstrated that the radioactivity was not localized in spots corresponding to PIP. The labeling was found in the spot of glutamate and in a spot not revealed with ninhydrin, but comigrating with the dipeptide NAGGN. Spots attributed to PIP, glutamate, and NAGGN were eluted from the chromatograms, and their identity was confirmed by \( ^{13}\text{C} \) NMR. The radiocarbon from

![FIG. 3. Fate of \( \text{L-}^{14}\text{C-PIP} \) in the presence of \( \text{D-PIP} \) in stressed cells of \( \text{S. meliloti} \). Cells were cultivated in LAS medium–0.5 M NaCl containing \( \text{L-}^{14}\text{C-PIP} \) (1 mM, 5 GBq/mmol) with (A and B) or without (C and D) \( \text{D isomer} \). \( \text{OD}_{570} \), growth expressed as OD\(_{570}\). Other symbols express radioactivity in the medium (●), \( ^{14}\text{CO}_2 \) (▲), EIF (□), and ESF (■). Histograms B and D represent the percentages of ESF radiolabeling: ▲, glutamate; and ■, NAGGN.](http://aem.asm.org/)

![FIG. 3. ...](http://aem.asm.org/)

![FIG. 3. ...](http://aem.asm.org/)
Corresponding to PIP, we consequently infer that the accumulation of the D isomer and (ii) no radioactivity was detected in the spot corresponding to the D-PIP (data not shown).

These results suggest that L-PIP acts as a precursor of the main endogenous compatible solutes (glutamate and NAGGN) in stressed cells of *S. meliloti*. Thus, since (i) we have provided a mix of nonradiolabeled D-PIP plus L-[14C]PIP in the growth medium and (ii) no radioactivity was detected in the spot corresponding to PIP, we consequently infer that the accumulated PIP observed by 13C NMR and in chromatograms does correspond to the D isomer of PIP.

To determine the behavior of PIP in unstressed cultures, *S. meliloti* cells were grown on LAS medium containing 1 mM DL-PIP plus L-[14C]PIP. Chromatographic analysis of cell extracts showed that the radiocarbon was distributed over several primary metabolites and not preferentially in glutamate and NAGGN. Nevertheless, chromatographic analysis of the ESF from cells harvested after 2 h of growth revealed an unlabeled spot corresponding to the D-PIP (data not shown).

In summary, all of these data indicate that stressed cells of *S. meliloti* cultivated in the presence of 1 mM DL-PIP were able to accumulate at least transiently the D isomer, whereas they catabolized immediately the L isomer of PIP.

**Stressed cells of *S. meliloti* immediately catabolize both the L and D isomers of PIP supplied separately.** The fate of L-PIP was observed over the entire bacterial growth cycle in LAS medium–0.5 M NaCl containing 1 mM L-[14C]PIP. In contrast to the situation observed with DL-PIP (Fig. 3A), bacterial cells incorporated labeled PIP at a lower velocity (Fig. 3C), corresponding to about 19 nmol min⁻¹ mg of protein⁻¹. Consequently, 48% of the supplied L-[14C]PIP still remained in the growth medium after 30 h of culture. During the lag phase of growth, the intracellular radiocarbon was recovered in the ESF, CO₂, and EIF at 63, 16, and 20% of the incorporated radioactivity, respectively. As observed with DL-PIP, the radioactivity increased in CO₂ as soon as the cells started to grow (i.e., after 10 h of incubation). The labeling of the EIF increased until 30 h of culture and then remained constant over the entire growth cycle (about 25 to 30% of the incorporated radioactivity). That of 14CO₂ reached a maximal value of 67% when cells entered the late exponential phase of growth (Fig. 3C).

Chromatographic analysis of the ESF of stressed cells cultured in the presence of 1 mM radiolabeled L-PIP and harvested at different stages of growth also revealed that the radioactivity was recovered only in the spots corresponding to glutamate and NAGGN. Figure 3D shows that 100% of the radioactivity of the ESF was recovered only in glutamate when cells were harvested in the lag and early-exponential phases of growth. In parallel, maximal labeling of NAGGN (80% of the total ESF labeling) was attained as the cells entered the stationary phase. Thus, L-[14C]PIP acts as a precursor of glutamate, which itself gives rise to NAGGN.

Furthermore, chromatographic and NMR analyses of the ESF of stressed cells cultivated in the presence of 1 mM D-PIP (unlabeled) did not show any accumulation of PIP (data not shown). That suggests that the D isomer supplied alone was catabolized under conditions of high osmolality.

In summary, both the nonosmoprotective forms of PIP (D- and L-PIP provided separately) were catabolized and thus not accumulated by stressed cells of *S. meliloti*.

**DL-PIP, the only osmoprotective form of PIP, increases endogenous osmolyte concentrations in stressed cells of *S. meliloti*.** The amounts of intracellular glutamate, NAGGN, and trehalose were determined during the growth cycle of *S. meliloti* cultivated in LAS medium containing 0.5 M NaCl without and with 1 mM D-PIP, L-PIP, or DL-PIP (1/1 D-/L-PIP ratio).

In the absence of exogenously supplied PIP, the intracellular level of glutamate reached a maximal value of 650 nmol mg of protein⁻¹ in the first hours of the growth and decreased during the growth process (Table 3). The level of NAGGN increased from 150 nmol mg of protein⁻¹ in the beginning of growth to 510 nmol mg of protein⁻¹ when cells entered the late exponential phase of growth. Trehalose remained at a low level during the exponential growth phase and increased up to 150 nmol mg of protein⁻¹ during the stationary phase. Similar results were obtained when 1 mM D- or L-PIP was supplied to the growth medium. In other words, when each isomer of PIP was provided separately, no significant effect on the level of the major endogenous osmolytes was observed.

In contrast, when cells were cultivated in medium containing 0.5 M NaCl and 1 mM DL-PIP (1/1 D-/L-PIP ratio), a strong increase in the intracellular glutamate level, from 690 nmol mg of protein⁻¹ at the beginning of growth to more than 1,200 nmol mg of protein⁻¹ in the late exponential growth phase, was observed (Table 3). The NAGGN level also increased significantly during the exponential phase and reached about 700 nmol mg of protein⁻¹ at the end of growth. The trehalose content reached a steady-state level of about 150 nmol mg of

**TABLE 3. Effects of D-, L-, and DL-PIP on amounts of endogenous osmolytes in salt-stressed cultures of *S. meliloti* 102F34**

<table>
<thead>
<tr>
<th>Form of PIP added</th>
<th>Early exponential phase</th>
<th>Late exponential phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glutamate</td>
<td>NAGGN</td>
</tr>
<tr>
<td>None</td>
<td>650</td>
<td>150</td>
</tr>
<tr>
<td>D-PIP</td>
<td>700</td>
<td>190</td>
</tr>
<tr>
<td>L-PIP</td>
<td>710</td>
<td>180</td>
</tr>
<tr>
<td>DL-PIP</td>
<td>690</td>
<td>190</td>
</tr>
</tbody>
</table>

* a Cultures were grown in LAS medium with 0.5 M NaCl plus the indicated isomeric form(s) of PIP (1 mM).

b Cytosolic osmolytes in cells at early and late exponential phases of growth were quantified enzymically after 5 to 8 and 24 to 30 h of culture, respectively. Data are means of triplicate assays with standard deviations of less than 10%.

c Similar levels of the indicated osmolyte were also found in cells at the stationary phase.
protein when the cultures entered the stationary phase in both the presence and the absence of PIP.

Together these results indicate that DL-PIP, but neither D- nor L-PIP provided separately, induced an enhancement of intracellular glutamate and NAGGN levels in stressed cells of S. meliloti.

**DISCUSSION**

In this study, we have demonstrated for the first time the atypical and the differential behavior of D and L enantiomers of PIP in bacterial osmoprotection. Osmoprotection of S. meliloti cells was effective only when the two isomers of PIP were present together in the stressing medium of culture. Such a response differs from that of E. coli cells, where exogenously supplied L-PIP, but not D-PIP, exerts a protective effect under an inhibitory osmolarity (14). To understand this uncommon behavior, we have focused our attention on the uptake activity and the fate of L-PIP in hyperosmotically stressed cells of S. meliloti grown in the presence and the absence of D-PIP. The most important observations were that (i) NaCl was able to stimulate L-PIP influx within cells of S. meliloti as described previously for osmoprotective compounds such as betaines (5, 11) and DMSP (34), in both S. meliloti and other bacteria; and (ii) the uptake of L-PIP was stimulated by the D isomer regardless of the osmolarity of the growth medium.

Salt-stressed cells of S. meliloti were able to catabolize L-[1-13C]PIP, in both the presence and the absence of D-PIP, into glutamate and the dipeptide NAGGN, the two main endogenous osmolytes neosynthesized by this bacterium. Because the radioactivity of glutamate decreased during the exponential growth phase and that of NAGGN increased in parallel, a close metabolic relationship might exist between these two cytosolic solutes.

Analysis of cellular soluble extracts by 13C NMR and chromatography revealed that (i) in stressed cells grown in the presence of DL-PIP, D-PIP accumulated until the late exponential phase of growth and then disappeared as L-PIP was completely consumed; and (ii) both D- and L-PIP were immediately catabolized when they were provided separately to osmotically stressed cultures of S. meliloti. Thus, in cells grown under hyperosmotic conditions in the presence of DL-PIP, the catabolism of L-PIP might inhibit that of D-PIP, leading to the accumulation of the D isomer. The complete consumption of L-PIP from the medium triggers the catabolism of D-PIP. Consequently, the isomer is the main (if not the sole) precursor of the endogenous osmolytes neosynthesized by stressed cells of S. meliloti. Because no intermediate between PIP and glutamate was detected in our chromatographic analyses, we can only speculate on the nature of the catabolic pathway of PIP. As already shown for Pseudomonas putida P2, which catabolizes PIP to glutamate (33), 1-Δ1-piperideine-6-carboxylate and L-α-aminoacidic acid could be the products of oxidation of the imino acid. α-Hydroxyglutarate could be an intermediate leading to α-ketoglutarate and glutamate. Since PIP oxidase, the first enzyme involved in the catabolism of PIP, was shown to be highly specific for the L isomer (3, 29), one can expect that catabolism of the D isomer should proceed through a racemization as a first step. PIP racemase might therefore be considered as a key enzyme in the osmoprotection of S. meliloti cells in the presence of DL-PIP. Its activity would be negatively regulated by the L isomer under hyperosmotic conditions.

Several studies have reported the preferential utilization of one enantiomer of a molecule over the other as a carbon source by many microorganisms. This was observed, for example, in P. putida, which degrades only the L-carnitine when it grows in the presence of DL-carnitine (20, 28). In Pseudomonas sp. strain AK 1, grown on DL-carnitine, the L isomer is degraded first (30). Acinetobacter calcoaceticus is able to metabolize L-carnitine as a carbon source, but no growth was observed with D-carnitine (20); nevertheless, A. calcoaceticus is able to catabolize D-carnitine in the presence of L-carnitine (20).

Surprisingly, the transient intracellular accumulation of the D isomer when L-PIP is present in the growth medium does not inhibit the synthesis of endogenous osmolytes (glutamate and NAGGN), because it was usually observed in the presence of the other accumulated osmoprotectants GB and DMSP (5, 34, 40). Similarly to ectoine, sucrose, and a few other disaccharides (15, 16, 39), the imported D-PIP leads to a significant increase in intracellular levels of glutamate and NAGGN in stressed cells of S. meliloti. The actual concentration of these osmolytes can be calculated according to the cell volumes determined elsewhere (39) for the same bacterium under identical conditions of culture. That leads to maximal concentrations of 320 mM for glutamate and 180 mM for NAGGN in the presence of 0.5 M NaCl and 1 mM DL-PIP. Since glutamate occurs mainly as potassium glutamate in osmotically stressed cells (8) and the dipeptide behaves as a neutral solute, the osmotic pressure developed by the cytosolic solution of these two osmolytes might account for almost 80% of that of the external stressing medium. Moreover, it should be mentioned that D-PIP itself, as long as it remains accumulated, might contribute with the endogenous osmolytes to the recovery of cell turgor.

In all, our data bring a novel insight into the versatile osmo-adaptation processes in S. meliloti. That two isomers of a given molecule can exert together a beneficial effect under stressing conditions may confer to the bacterium a significant advantage over other organisms requiring osmoprotectants in the same habitat. The mechanism of action of the two isomers remains still unclear. Further work will pay attention to the regulation of both D-PIP catabolism and the level of endogenous osmolytes.

**ACKNOWLEDGMENTS**

This research was supported by grants from the Direction de la Recherche et des Etudes Doctorales and by the Centre National de la Recherche Scientifique.

We acknowledge J. Hamelin for 13C NMR analysis and M. Uguet and C. Monnier for technical assistance.

**REFERENCES**