Luteolin uptake by *Rhizobium meliloti*: evidence for several steps including an active extrusion process

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Evidence for several steps in luteolin uptake by *Rhizobium meliloti* is presented. The first step is highly reversible and strongly pH dependent absorption, greatly enhanced at acidic pH. Studies with liposomes suggest that this step consists of a passive dissolution of the flavonoid in its non-ionized state in the lipidic matrix of the membrane. This first step is enhanced in bacteria killed by high temperature. The second step, a slower and more stable accumulation, is not pH dependent. The nature of the sites involved in this more stable binding remains to be determined. Low temperature and treatment with the metabolic inhibitor potassium cyanide resulted in an increase of luteolin uptake and a decrease of extrusion of flavonoids, suggesting an active extrusion process. Studies with spheroplasts or isolated membranes showed that under physiological conditions most of the luteolin was entrapped in the outer membrane while only a small amount was kept in the cytoplasmic membrane. Arguments are presented supporting the view that *R. meliloti* has developed a mechanism to keep luteolin in its membranes which helps avoid the toxic effect of the flavonoid on the respiratory chain by trapping most of it in the outer membrane and to maintain the *nod* operon in a transcriptionally active form in conditions where this strong inducer is no longer exuded by the host plant.

Keywords: *Rhizobium meliloti*, luteolin uptake, flavonoid

INTRODUCTION

One of the first steps in the establishment of symbiotic interaction between *Rhizobium meliloti* and *Medicago sativa* is the reception of plant signals by the bacterial partner. Plant roots exude flavonoids, several of which can act as *nod* gene inducers while others can inhibit the expression of these genes (Phillips *et al.*, 1991). Among these flavonoids, luteolin (3',4',5,7-tetrahydroxyflavone), mainly exuded by germinating seeds of *M. sativa*, is a potent *nod* gene inducer in *R. meliloti* (Peters *et al.*, 1986). Even if the physiological role of this flavonoid in the induction of nodulation in natural conditions is not fully elucidated, it remains an excellent model to study the interactions between bacteria and plant signals. Luteolin, like other flavonoid inducers, induces *nod* gene expression through an interaction with a protein constitutively expressed in *R. meliloti*, the NodD protein, which is bound to the promoter regions of the *nod* gene operons (Kondorosi, 1991). When flavonoids are bound to NodD, a change in conformation of the protein takes place

allowing the RNA polymerase to initiate transcription of the *nod* genes lying downstream. The presence of large hydrophobic domains in the NodD protein, as well as several other observations, suggest that the interaction between the NodD protein and the flavonoid could take place in or on the cytoplasmic membrane of the bacterium (Schlaman *et al.*, 1989).

The first step in the interaction is the uptake of flavonoids by the bacteria. This process, however, remains poorly documented. Naringenin exuded by roots of *Vicia faba* is a potent *nod* gene inducer in *Rhizobium leguminosarum*. Absorption of naringenin by *R. leguminosarum* is a rapid, passive and highly reversible process (Recourt *et al.*, 1989) and it was suggested that the flavonoid was simply dissolved in the lipidic matrix. A more complex picture was presented by our laboratory (Hubac *et al.*, 1993) concerning luteolin absorption by *R. meliloti*. We have demonstrated that the first reversible step is followed by a slower stable accumulation of luteolin, mainly in the outer membrane. Luteolin was shown to inhibit NADH oxidase

activity in the cytoplasmic membrane and it was proposed that the accumulation of the flavonoid in the outer membrane protects the respiratory chain against the toxic effect of an excess of the flavonoid. On the other hand, the uptake of luteolin was shown to exhibit a certain degree of host-specificity. For example, other Gram-negative bacteria did not absorb luteolin at the same rate as *R. meliloti*. Luteolin uptake was also altered in several mutants affected in the symbiotic plasmid and the level of *nodD* gene expression influenced luteolin accumulation.

In this work, a more extensive study on the characteristics of luteolin uptake by the wild-type *R. meliloti* strain Rm41 was performed. The effect of pH was investigated at different steps of the luteolin uptake in whole bacteria, spheroplasts, isolated membranes and liposomes. Luteolin uptake and its extrusion were also studied under several conditions, including the effects of temperature and metabolic inhibitors.

METHODS

Bacterial strain. The wild-type *R. meliloti* strain Rm41 (first isolated by K. Szende and F. Ordögh) was used.

Media and culture conditions. Liquid cultures were grown at 30 °C on TA medium as described previously (Hubac *et al.*, 1992). Growth of bacteria was followed spectrophotometrically by measuring OD₅₄₀. Samples were collected during the exponential phase of growth (OD₅₄₀ = 0.9).

Fractionation of membranes. Fractionation on isopycnic sucrose gradients was as described by Hubac *et al.* (1992). The KDO (2-ketodeoxyoctonate) content served as marker for the outer membrane, which contained lipopolysaccharides. The exopolysaccharides which were located outside the outer membrane were largely lost during the fractionation process. NADH oxidase activity was the marker for the inner membrane (de Maagd & Lugtenberg, 1986).

Whole-membrane preparations (including both the outer and the inner membranes) were recovered after disruption of the cell walls by passage through a French press and were in the first pellet of the subsequent centrifugation at 15000 *g* for 2 h.

Spheroplast isolation. The technique was adapted from the procedure of Witholt *et al.* (1976) described for *Escherichia coli* and from Schmidt *et al.* (1984) for *R. meliloti*. After centrifugation of the bacterial cultures at 3000 *g* for 20 min at 20 °C the pellet was washed twice in two buffers containing, respectively, 100 mM Tris/HCl, 2% (w/v) sucrose (pH 8.0) (TES suspension) and 100 mM Tris/HCl, 25% sucrose, 2 mM EDTA (pH 8.0). Lysozyme was added to the TES suspension at a final concentration of 250 µg ml⁻¹. This suspension was incubated for 30 min at 37 °C. Spheroplasts were obtained after centrifugation at 22000 *g* for 20 min at 20 °C. Spheroplasts were found to be devoid of KDO; they exhibited significant NADH oxidase activity but had a very low level of oxygen consumption. This result is generally observed with Gram-negative bacteria and can be explained by the loss of most of the cytochrome *c* during the separation of the two membranes.

Measurement of NADH oxidase activity. This was done spectrophotometrically as described in previous work (Hubac *et al.*, 1993).

Determination of luteolin content. Samples (40 ml) of *R. meliloti* growing in the presence of luteolin (10 µM) were

centrifuged at 5000 *g* for 30 min at 4 °C. The bacterial pellet was suspended in 4 ml ethanol. After a second centrifugation at 5000 *g* for 30 min, the supernatant was collected, evaporated under a nitrogen stream and resuspended in 1 ml ethanol. This extract was then analysed for luteolin content by spectrophotometry at 350 nm or by HPLC (Hubac *et al.*, 1992). For fractionation of the membranes, the analysis was done with 500 ml of bacterial culture and luteolin was added before or after fractionation.

Luteolin uptake by whole bacteria was expressed in ng (ml bacterial culture)⁻¹. During incubation with flavonoids for 1–12 h, the mass of bacteria increased markedly; therefore it could not serve as reference. Under standardized starting conditions, for an OD₅₄₀ of 0.9, the bacterial fresh weight was around 10 mg (ml culture)⁻¹.

Luteolin uptake by liposomes. Large multilamellar vesicles were prepared either from a mixture of phosphatidylcholine and phosphatidylethanolamine from soybean (*Glycine max*; Sigma), or from a total lipid extract of *R. meliloti*, by mild shaking of the dry lipids in Tris/HCl buffer (pH 7.5). Vesicles of more than 0.2 µm were discarded by filtration. To measure uptake of luteolin by vesicles, the following technique was developed. C18 cartridges (Waters) were used; these do not retain liposomes (the fatty acid tails of lipid are fully turned inside the vesicle) while free luteolin in aqueous solution is retained in the solid phase. When luteolin was mixed with liposomes, some of it was incorporated in the lipidic phase. Luteolin uptake by liposomes was determined by two methods. (1) The amount of luteolin eluted in a sodium-phosphate buffer in the absence or presence of liposomes was measured. In the absence of liposomes, no luteolin was eluted in the aqueous buffer at pH values lower than 9, while in the presence of liposomes, luteolin was detected in the phosphate buffer (pH 5, 6, 7.5 or 8) which represented the luteolin taken up by liposomes. (2) The luteolin that remained on the C18 cartridge after elution with the phosphate buffer, in the absence or presence of liposomes, was measured. The pool of luteolin bound to the cartridge was eluted with ethanol, which detached more than 90% of the flavonoid content. All these experiments were performed at room temperature.

Respiratory activity. Oxygen uptake was measured with a Clark-type oxygen electrode built in the laboratory.

RESULTS

Evidence for a two-step process of luteolin uptake by *R. meliloti*

After overnight incubation of *R. meliloti* strain Rm41 in TA medium with 10 µM luteolin at three different pH values, the luteolin content was determined in the first bacterial pellet recovered by centrifugation at 5000 *g* for 30 min, as described in Methods. This first pellet was then resuspended, washed with fresh TA medium 1–4 times and the luteolin content was determined in the successive washed bacterial pellets. Most of the luteolin was lost during the first washing, while the luteolin content only slightly decreased during subsequent washings (Fig. 1).

Bacteria were incubated with luteolin for 30 s to 12 h then pelleted and re-incubated for 1 h in fresh TA medium. Luteolin content was determined in bacteria after the first incubation (with luteolin) and after the second one (without luteolin). The amount of more stably fixed luteolin increased progressively with the time of in-

Fig. 1. Effect of pH on the two steps of luteolin uptake by intact *R. meliloti* cells. Bacteria were incubated overnight in TA medium with luteolin (10 μ M) at pH 5 (—), 6 (---) or 7 (—). At the end of the incubation, bacteria were pelleted, resuspended and washed up to four times in fresh medium without luteolin. Then, the luteolin content [ng (ml bacterial culture)⁻¹] was determined in each pellet. Values are means of five experiments $\pm$ SE.

Fig. 2. Effect of incubation of *R. meliloti* with luteolin on the stability of its binding to the bacteria. Intact bacteria were incubated for different times (from 30 s to 12 h) with luteolin (10 μ M) at pH 7.4 and then pelleted and re-incubated for 1 h in fresh medium without flavonoid. The stability of binding of luteolin was expressed as a percentage of luteolin remaining in the bacteria after 1 h in the fresh medium.

cubation in the presence of the flavonoid (Fig. 2). These characteristics of luteolin binding by *R. meliloti* are clearly indicative of a two-step process: a highly reversible first step followed by a slower more stable binding. As seen in Fig. 1, the first, reversible, step was greatly enhanced at acidic pH.

Effect of pH

The reasons for the strong pH effect were studied in more detail. We observed that the growth rate of bacteria was affected by the pH. Growth was very slow at pH 5; it

Fig. 3. Effect of pH on luteolin uptake by intact *R. meliloti* cells. Bacteria were incubated for 4 h with luteolin (10 μ M). Values for luteolin uptake were expressed in ng (ml bacterial culture)⁻¹.

reached a maximum at pH near 7.0, then decreased slowly at basic pH values (data not shown).

As seen in Figs 1 and 3, the reversible luteolin uptake was higher at acidic pH and decreased when the pH of the culture medium became basic. The amount of stably bound flavonoid was only weakly affected by the pH, suggesting that the first step of binding is a passive and reversible process while the second step corresponds to a more stable binding of luteolin which is independent of pH.

As previously suggested by Recourt *et al.* (1989) for the first step of naringenin uptake by *R. leguminosarum*, flavonoids can be simply solubilized in a lipidic matrix depending on their ionization state. This hypothesis was tested with liposomes which were prepared either from a mixture of phospholipids from soybean (containing mainly phosphatidylcholine and phosphatidylethanolamine rich in linoleic acid) or from a total lipid extract of *R. meliloti* which contained, as previously reported, a mixture of phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine and also phosphatidylcholine in lower amount, all rich in vaccenic acid (Hubac *et al.*, 1992). These liposomes were mixed with luteolin and luteolin uptake was measured as described in Methods. This uptake was found to be strictly pH-dependent with the two types of liposomes (Fig. 4). The wavelength of the maximum of absorption of luteolin alone in the phosphate buffer at different pH values was also determined in order to calculate the equilibrium between ionized and non-ionized states of the flavonoid. At pH 5, all flavonoid was in its non-ionized state and the uptake was maximal, while at pH 8 the flavonoid was in an ionized state and uptake was near zero. This result is in good agreement with the hypothesis that the lipidic matrix plays a major role in the first passive and reversible step in luteolin uptake.

The effect of pH was also tested on isolated membranes. When a whole-membrane preparation (including both outer and inner membranes), was incubated for 2 h at different pH values with luteolin, then pelleted and the

Fig. 4. Luteolin uptake by liposomes. Liposomes (at a concentration of 1 mg ml^{-1}) were mixed with luteolin ($10 \text{ }\mu\text{M}$) in phosphate buffer at different pH values and the luteolin uptake was determined as described in Methods. $\square$, Luteolin in non-ionized state (% of luteolin); $\blacksquare$, luteolin uptake by liposomes from soybean phospholipids [$\mu\text{g (mg liposomes)}^{-1}$]; ▨ , luteolin uptake by liposomes from total extract of *R. meliloti* [$\mu\text{g mg liposomes}^{-1}$].

Table 1. Effect of pH on luteolin uptake by isolated membranes

Membrane preparation	pH				
	5.5	6.5	7.5	8.0	9.5
Whole membrane*	1.02	0.84	0.56	0.18	0
Isolated outer membrane†	0.18		8.1		
Isolated inner membrane†	3.33		0.94		
Whole membrane fractionated into‡:					
Outer membranes	0.33		0.45		
Inner membrane	1.06		0.08		

* Whole membrane preparation: 15.5 mg protein were incubated for 2 h with luteolin ($10 \text{ }\mu\text{M}$) in TA medium and the pH was adjusted to different values. At the end of incubation, membranes were pelleted by centrifugation at $150\,000 \text{ g}$ for 2 h and the luteolin content was expressed as $\mu\text{g (mg protein)}^{-1}$.

† Isolated outer and inner membranes: 3.5 and 12 mg protein, respectively, were incubated at pH 5.5 and 7.5 for 2 h in the presence of luteolin ($10 \text{ }\mu\text{M}$), then repelleted by centrifugation at $150\,000 \text{ g}$ for 3 h and analysed for their luteolin content which was expressed as $\mu\text{g (mg protein)}^{-1}$.

‡ Whole membrane preparation: 15 mg protein was first incubated at pH 5.5 with luteolin ($10 \text{ }\mu\text{M}$), then outer and inner membranes were separated on a sucrose gradient and analysed for their luteolin content which was expressed as $\mu\text{g (mg protein)}^{-1}$.

luteolin content determined, the same general effect of pH as with the intact bacteria was observed (Table 1).

When isolated outer and inner membranes were incubated

Table 2. Effect of pH on luteolin incorporation in spheroplasts

Methods: 1, intact bacteria were incubated in TA medium for 3 h with luteolin ($10 \text{ }\mu\text{M}$) at different pH values, then spheroplasts were isolated as described in Methods and the luteolin content determined and expressed as $\mu\text{g (mg protein)}^{-1}$; 2, isolated spheroplasts were incubated in TA medium for 3 h at different pH values, then pelleted and analysed for their luteolin content, which was expressed as $\mu\text{g (mg protein)}^{-1}$.

Method	pH:			
	5.0	6.0	7.4	8.0
1	0.62	0.75	0.62	0.02
2	3.5	2.1	1.0	0.4

with luteolin at pH 5.7 and 7.4, luteolin uptake in the inner membrane was greatest at acidic pH while, conversely, uptake was highest at neutral pH in the outer membrane (Table 1). This result suggests that a specific site having high affinity for luteolin is involved in its stabilization at neutral pH and not at acidic pH; in this membrane, this could be then related to the ionized state of the flavonoid.

To repeat in our system the experiment previously reported by Recourt *et al.* (1989) with *R. leguminosarum* and naringenin, a whole-membrane preparation was first incubated for 2 h with luteolin ($10 \text{ }\mu\text{M}$) and then the outer and inner membranes were separated and the luteolin content was determined. Under these conditions at pH 5.7, luteolin was found in significant amounts only in the inner membrane (Table 1), confirming that the site of luteolin stabilization has low affinity for the flavonoid in its non-ionized state, while at neutral pH more flavonoid was found in the outer membrane.

The effect of pH was also tested, in two ways, with spheroplasts from *R. meliloti*. In the first experiment, intact bacteria were incubated with luteolin at different pH values and then spheroplasts were isolated as described in Methods. The luteolin content in the spheroplasts was similar for pH values from 5 to 7.4; it became very low when bacteria were incubated at pH 8 (Table 2). In the second experiment, luteolin content was measured with isolated spheroplasts incubated for 3 h with luteolin at different pH values. Strong pH dependence was observed and the amount of luteolin found in the spheroplasts incubated in this manner was higher, especially at acidic pH, than when spheroplasts were isolated after incubation of living bacteria with the flavonoid. These results, in perfect agreement with our previous results (Hubac *et al.*, 1993), indicate that, in intact bacteria, the outer membrane trapped most of the luteolin, thus avoiding an accumulation of a large amount of the flavonoid in the inner membrane. It is clear that isolated spheroplasts were able to incorporate luteolin in its non-ionized state, as were isolated inner membranes.

Table 3. Luteolin uptake and extrusion by intact bacteria exposed to different conditions or treatment

Luteolin extrusion was expressed as the percentage of luteolin taken up which was lost during the incubation in the fresh medium without luteolin. Values are means of five experiments $\pm$ SE.

Conditions*	Luteolin uptake [ng (ml culture) ⁻¹]		Luteolin extrusion (%)
	After 4 h incubation with luteolin	After 1 h more without luteolin	
1. Control (30 °C)	95 $\pm$ 6	18 $\pm$ 1	81
2. Killed by heating at 100 °C	190 $\pm$ 12	170 $\pm$ 20	0.1
3.0 + Luteolin for 4 h at 30 °C, then -luteolin for 1 h at 4 °C	95 $\pm$ 6	49 $\pm$ 4	48
4. + Luteolin for 4 h at 4 °C, then -luteolin for 1 h at 4 °C	164 $\pm$ 10	48 $\pm$ 4	71
5. + Luteolin and KCN for 4 h, then -luteolin + KCN for 1 h	95 $\pm$ 6	63 $\pm$ 5	34
6. + Luteolin for 4 h then -luteolin + KCN for 1 h	166 $\pm$ 11	63 $\pm$ 5	62

*1. Bacteria were incubated at 30 °C with luteolin (10 μ M) for 4 h, then, for the analyses of luteolin extrusion, bacteria were pelleted and resuspended in fresh TA medium in the bacterial pellet. 2. Bacteria were killed by heating at 100 °C for 30 min and then incubated at 30 °C with luteolin (10 μ M) for 4 h. Luteolin extrusion was studied at 30 °C. 3. Bacteria were incubated at 30 °C, and the luteolin extrusion was analysed at 4 °C. 4. Bacteria were incubated at 4 °C with luteolin (10 μ M) for 4 h; luteolin extrusion was analysed at 4 °C. 5. Bacteria were incubated with KCN (10 mM) and luteolin (10 μ M) for 4 h; luteolin extrusion was analysed with KCN (10 mM). 6. Bacteria were incubated at 30 °C and luteolin extrusion was analysed in the presence of KCN (10 mM).

Evidence for an active extrusion of luteolin

When bacteria killed by boiling were incubated with luteolin, the uptake was enhanced (Table 3). That could be explained by protein denaturation; only the lipidic phase became freely accessible to luteolin. When killed bacteria were put in a fresh medium without flavonoid for 1 h, the luteolin content was almost the same as in the first bacterial pellet after 4 h of incubation with luteolin.

Luteolin uptake was markedly increased when bacteria were incubated at 4 °C, but in this case the loss of luteolin decreased when bacteria were put in a fresh medium without luteolin, indicating strong inhibition of luteolin extrusion. Moreover, when bacteria were treated with

Fig. 5. Effect of luteolin on the activity of NADH oxidase in spheroplasts from *R. meliloti*. Spheroplasts were incubated at 30 °C in Tris (pH 8), 0.2 mM DTT and 0.12 mM NADH with or without luteolin. Oxidation of NADH was followed at 340 nm for 6 min. (A), (C), (D), Spheroplasts were isolated from bacteria incubated without luteolin then added directly into the cuvette at concentrations of 0 μ M (A), 10 μ M (C) or 50 μ M (D) luteolin. (B), Spheroplasts isolated from bacteria incubated overnight with luteolin (10 μ M).

KCN, conditions in which growth and respiratory activity were totally but reversibly inhibited (data not shown), the same effects were observed as under low temperature: an increase of luteolin uptake and a decrease of its extrusion when bacteria were put in fresh medium without luteolin. These results can be interpreted as showing an active flavonoid extrusion process by *R. meliloti*.

Evidence for a protective role of the outer membrane against luteolin excess inhibiting NADH oxidase

In a previous paper (Hubac *et al.*, 1993), we suggested that the outer membrane can protect the respiratory chain located in the inner membrane against an excess of flavonoid. We tested this hypothesis with spheroplasts prepared from bacteria incubated with or without luteolin (Fig. 5). The NADH oxidase activity was not affected in spheroplasts isolated from bacteria first incubated with the flavonoid, while in spheroplasts directly treated with luteolin (10 or 50 μ M) in the cuvette, strong inhibition of NADH oxidase was observed.

DISCUSSION

This work confirms results reported in previous papers (Recourt *et al.*, 1989; Hubac *et al.*, 1993) but also brings new insights into the process of luteolin uptake by *R. meliloti*. A large fraction of the flavonoid is absorbed rapidly and highly reversibly by the living bacteria. It is tempting to think that this first pool is simply dissolved into the lipidic matrix of the membrane (probably mostly of the outer one). Experiments with liposomes are in agreement with this hypothesis. This process probably constitutes the first step of luteolin uptake by the bacteria after the flavonoid has crossed the exopolysaccharide barrier. This step is strongly pH-dependent; it can be assumed that luteolin dissolves in the lipidic matrix when it is in a non-ionized state. This step seems to be necessary for binding of luteolin to bacteria, since no significant

uptake was observed at basic pH, under which condition all the flavonoids are in an ionized state. After this first step, luteolin undergoes more stable interactions with other sites in the membrane. The second step is slower and its stabilization can require several hours. It occurs over a wide range of pH values in whole bacteria. This result is in agreement with our previous work determining that luteolin mostly accumulates in the outer membrane after incubation of bacteria at neutral pH. We have also found that a low, but significant amount of luteolin was always stabilized in the inner membrane when intact bacteria were incubated in a wide range from acidic to neutral pH. The results obtained with spheroplasts also confirm the presence of luteolin in a relatively low amount in the cytoplasmic membrane (even if the presence of flavonoids in the cytoplasm of bacteria cannot be ruled out in our experiments) when the spheroplasts were isolated after incubation of intact bacteria with luteolin.

Here we provide evidence for an active luteolin extrusion process by *R. meliloti* as shown by the results with killed bacteria and the effects of low temperature or of metabolic inhibitors such as KCN. A similar increase in naringenin uptake by *R. leguminosarum* at low temperature or in the presence of metabolic inhibitor has been reported (Recourt *et al.*, 1989) and allows us to conclude that such a mechanism is not restricted to *R. meliloti* but can be generally found in Gram-negative bacteria. Indeed, Nikaido (1993) developed the hypothesis that diffusion of non-ionized small molecules across the outer membrane of Gram-negative bacteria may occur through lipid bilayer domains. The outer membrane bilayer is then a very effective barrier against amphiphilic compounds containing charged groups. The lipophilic uncharged agents traverse the cytoplasmic membrane and the only defence that Gram-negative bacteria can use against these compounds is their inactivation or active extrusion.

Finally, our work suggests that, in order to protect the respiratory chain against the toxic effect of flavonoids, *R. meliloti* has evolved a system allowing it to keep some luteolin in its membranes, mainly in the outer one. Luteolin, one of the most powerful *nod* gene inducers, is exuded by the host plant at an early stage of germination, well before the plants become competent for establishing symbiosis with the bacteria (Phillips *et al.*, 1991). It could be hypothesized that this mechanism of flavonoid storage allows maintenance of the *nod* operon in the transcriptionally active state until the roots of the plant become able to form functional nodules.

ACKNOWLEDGEMENTS

We thank Dr G. Dubertret for excellent assistance and C. Deforet for typing the manuscript.

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Received 13 April 1994; accepted 13 June 1994.