Rhizobia Catabolize nod Gene-Inducing Flavonoids via C-Ring Fission Mechanisms

JULURI RAGHAVENDRA RAO AND JAMES E. COOPER*

Department of Applied Plant Science, The Queen's University of Belfast, Belfast BT9 5PX, Northern Ireland, United Kingdom

Received 14 March 1994/Accepted 20 June 1994

Flavonoids are polyphenolic secondary metabolites which are synthesized by plants via the expression of two multigene-encoded enzymes: phenylalanine ammonia lyase and chalcone synthase. Subgroups of compounds such as chalcones, flavonanes, flavones, flavonols, and isoflavonoids occur in legume tissues, and they can be released (15) from roots into the rhizosphere, where some of them act as molecular signals to trigger the establishment of symbioses with bacteria in the family Rhizobiaceae (18, 19). Their principal function is to interact with the nodD gene products of rhizobia and the subsequent transcriptional activation of other nod genes (17). Other effects of flavonoids on rhizobia include promotion of chemotactic responses (1) and stimulation of growth rate by unspecified mechanisms (8).

Although rhizobia are known to utilize various aromatic compounds as carbon and/or energy sources by degrading them to catechol and protocatechuate and channeling these products after further enzymatic cleavage into the tricarboxylic acid cycle via the β-ketoadipate pathway (3, 16), their capacity to degrade flavonoids has received little attention. Only two examples have been reported: the utilization of catechin by a Rhizobium sp. isolated from Leucaena leucocephala, with attendant formation of phloroglucinol carboxylic acid and protocatechuic (6), and a novel form of C-ring cleavage in a pentahydroxy flavone, quercetin, by Rhizobium loti (20). One report (4) has described an alteration in the types and amounts of formononetin derivatives in Medicago sativa root exudate during incubation with Rhizobium mellioti, but no biotransformation mechanism was proposed. Formononetin glycosides are nod gene inducers for R. mellioti.

The purpose of the present study was to investigate the metabolic activity of rhizobia towards their nod gene-inducing flavonoids. We used gas chromatography and mass spectrometry (GC-MS) to facilitate the direct detection and identification of submilligram quantities of aromatic metabolites in supernatants of liquid media which had been supplemented with flavonoids and incubated with single-strain cultures of various Rhizobium species and biovars. We now report that rhizobia degrade nod gene-inducing flavonoids by mechanisms which originate in a cleavage of the C-ring of the molecule.

MATERIALS AND METHODS

Bacteria. The bacterial strains used and their sources were as follows: Rhizobium leguminosarum bv. trifolii P3, this laboratory; R. leguminosarum bv. viciae RBL5601 (containing the wild-type plasmid pJB511), Department of Plant Molecular Biology, Leiden University, Leiden, The Netherlands; R. leguminosarum bv. phaseoli TAL182, R. mellioti RM41, Rhizobium fredii HH1103, Pseudomonas putida DSM3226, and Agrobacterium tumefaciens C6-6, Botany Department, Marburg University, Marburg, Germany; and Rhizobium sp. strain NGR234, LBMPS, University of Geneva, Geneva, Switzerland.

Strains were grown individually in shake-flask cultures in flavonoid-free medium with mannitol replacing arabinose (20). Cultures were grown at 25°C for 72 h; this step was followed by centrifugation and washing (twice) and resuspension in sterile water.

Test media and growth conditions. For strain NGR234 the test medium was that of Broughton and Dilworth (2). For all other strains the test medium was as described by Rao et al. (20), except that mannitol was substituted for arabinose. High-pressure liquid chromatography (HPLC)-grade flavonoids were obtained from Apin Chemicals, Abingdon, United Kingdom, and rechecked for purity by GC. Flavonoids were added from stock solutions in methanol to give a final concentration of 10 μM. Media were dispensed in 1-liter
TABLE 1. Principal aromatic metabolites detected after incubation* of flavonoids with *Rhizobium, Pseudomonas, and Agrobacterium* species

<table>
<thead>
<tr>
<th>Organism</th>
<th>Flavonoid substrate</th>
<th>Ring cleavage mechanism</th>
<th>Principal degradation product(s) derived from:</th>
<th>Other partially characterized metabolites (MS data)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R. leguminosarum</em></td>
<td>Naringenin</td>
<td>C-ring</td>
<td>Phloroglucin, <em>p-Coumaric acid, p-hydroxybenzoic acid</em></td>
<td>300, 65, 91, 134, 166, 193 (peak F) <strong>[Fig. 1]</strong></td>
</tr>
<tr>
<td><em>R. leguminosarum</em></td>
<td>Quercetin</td>
<td>C-ring</td>
<td>Phloroglucin, Protocatechuic acid</td>
<td>300, 69, 77, 121, 134, 166 (peak E) <strong>[Fig. 1]</strong></td>
</tr>
<tr>
<td><em>R. leguminosarum</em></td>
<td>7,4'-Dihydroxy-flavone</td>
<td>C-ring</td>
<td>Resorcinol, <em>p-Hydroxybenzoic acid</em></td>
<td>314, 55, 134, 180, 207, 286 (peak G) <strong>[Fig. 1]</strong></td>
</tr>
<tr>
<td><em>R. leguminosarum</em></td>
<td>Luteolin</td>
<td>C-ring</td>
<td>Phloroglucin, <em>p-Coumaric acid</em></td>
<td>314, 69, 91, 134, 152, 180, 207, 271, 297 (peak H) <strong>[Fig. 1]</strong></td>
</tr>
<tr>
<td><em>R. meliloti</em></td>
<td>Genistein</td>
<td>C-ring</td>
<td>Caffeic acid, protocatechuic acid, phenylacetic acid</td>
<td>298, 69, 132, 135, 171, 269 (corresponds to apigenin)</td>
</tr>
<tr>
<td><em>Rhizobium</em> sp. strain NGR234</td>
<td>Daidzein</td>
<td>C-ring</td>
<td><em>p-Hydroxybenzoic acid</em></td>
<td>174, 51, 77, 87, 102, 115, 131, 159 (umbelliferyl derivative)</td>
</tr>
<tr>
<td><em>Rhizobium</em> sp. strain NGR234</td>
<td>Apigenin</td>
<td>C-ring</td>
<td>Resorcinol, <em>p-Hydroxybenzoic acid</em></td>
<td>174, 51, 77, 87, 102, 115, 131, 159 (umbelliferyl derivative)</td>
</tr>
<tr>
<td><em>P. putida</em></td>
<td>Quercetin</td>
<td>A-ring</td>
<td><em>p-Hydroxybenzoic acid</em></td>
<td>174, 51, 77, 87, 102, 115, 131, 159 (umbelliferyl derivative)</td>
</tr>
<tr>
<td><em>A. tumefaciens</em></td>
<td>Naringenin</td>
<td>Non-specific</td>
<td>Protocatechuic acid</td>
<td>174, 51, 77, 87, 102, 115, 131, 159 (umbelliferyl derivative)</td>
</tr>
</tbody>
</table>

* The incubation period was 24 h for all metabolites depicted except the partially characterized products of naringenin degradation by *R. leguminosarum* bv. viciae, which were detected within 2 h.

* Each flavonoid is an effective nod gene inducer for its corresponding *Rhizobium* sp.

amounts in 3-liter Erlenmeyer flasks and inoculated with single cultures of bacteria to give an initial concentration of 1.5 × 10⁸ cells per ml. Unless otherwise specified, incubation was at 25°C on a rotary shaker (200 rpm) for a maximum period of 24 h. Control treatments required for confirmation of bacterial flavonoid transformations were also included as follows: basal medium plus flavonoid with no inoculum and basal medium minus flavonoid with inoculum. Flasks were wrapped in aluminum foil to prevent photosensitive reactions from occurring.

**Analysis of culture extracts.** After incubation, 1-liter aliquots of media were centrifuged (8,000 × g for 10 min) and the supernatants were extracted by hand (six times) with ethyl acetate after saturation with sodium chloride. This was followed by drying with Na₂SO₄ and concentration on a rotary evaporator. Residues were taken up in 0.5 ml of methanol and treated with an excess of freshly prepared ethereal diazomethane using a Diazald kit (Aldrich Chemicals) at 0°C. After 12 h, the solvents, along with excess diazomethane, were evaporated, and the remaining residues were dissolved in 0.5 ml of diethyl ether prior to GC-MS analysis (20).

**RESULTS**

**Principal degradation products.** The main aromatic products detected in the supernatants of flavonoid-supplemented media are presented in Table 1. Rhizobia consistently degraded flavonoids via C-ring cleavage, whereas *P. putida*, as expected, degraded quercetin by an initial fission in the A-ring of the molecule. *A. tumefaciens* displayed a nonspecific mode of degradation towards naringenin, which yielded no conserved A- or B-ring products. Analysis of mass spectral data from derivatized samples obtained from control flasks failed to detect any of the monocyclic, hydroxylated aromatics which were found in the inoculated, flavonoid-supplemented treatments.

In the presence of *Rhizobium* spp., flavonoids with OH substitutions at the 5 and 7 positions yielded phloroglucinol as the conserved A-ring product. 7,4'-Dihydroxyflavone, which has a single OH substitution on its A-ring, yielded resorcinol as the conserved product from this part of the molecule. A wider range of metabolites was found among the B-ring derivatives. For example, *R. leguminosarum* bv. viciae yielded p-coumaric acid and its by-product, p-hydroxybenzoic acid. The same metabolites would be predicted from a C-ring fission of 7,4'-dihydroxyflavone by *R. leguminosarum* bv. trifolii, but only p-hydroxybenzoic acid was detected. *R. leguminosarum* bv. phaseoli generated protocatechuic acid, as expected from the OH substitution pattern of its nod gene inducer, quercetin. *R. meliloti* generated three compounds originating in the B-ring of luteolin: phenylacetic acid, caffeic acid, and protocatechuic acid. The isoflavonoids genistein and daidzein were metabolized by *R. fredii*, while the broad-host-range *Rhizobium* sp. strain NGR234 also degraded daidzein and apigenin.

The total ion gas chromatogram of the extracted and derivatized supernatant from naringenin-supplemented medium after incubation for 2 h with *R. leguminosarum* bv. viciae is shown in Fig. 1. The retention times and mass spectra of peaks A, B, and C were identical to those of similarly derivatized authentic phloroglucinol, p-hydroxybenzoic acid, and p-coumaric acid, respectively. Peak D represents residual, unmetabolized naringenin, peaks E and F represent new flavanone (M⁺ 300) structures, and peaks G and H represent new chalcone (M⁺ 314) structures. The relative quantities of derivatives in the supernatant extracts may not truly reflect their concentrations in the culture medium. This is most likely...
with RHIZOBIA CATABOLIZE nod GENE-INDUCING FLAVONOIDS

FIG. 1. Total ion gas chromatogram of methylated ethyl acetate extracts of naringenin-supplemented medium after incubation for 2 h with R. leguminosarum bv. viciae. Peaks: A, phloroglucinol; B, p-hydroxybenzoic acid; C, p-coumaric acid; D, naringenin; E and F, new flavanone structures; G and H, new chalcone structures. Compounds represented by peaks E, F, G, and H do not persist at detectable levels upon further incubation.

Our results clearly show that rhizobia degrade a wide range of flavonoids by means of primary fissions located exclusively in the C-rings of the compounds. This type of flavonoid catabolism has previously been reported for members of two other bacterial genera: some intestinal Clostridium strains and an Eubacterium species (14, 25). While A. tumefaciens was able to catabolize naringenin, the fission mechanisms differed from those observed in R. leguminosarum bv. viciae, and no conserved A- or B-ring products were detected. The general catabolic mechanism displayed by rhizobia can be regarded as a reversal of plant flavonoid biosynthesis, which is characterized by the condensation of 3 U of malonyl coenzyme A (the phloroglucinol A-ring unit) and 1 U of p-coumaroyl coenzyme A (the B- and C-ring component) to yield the basic three-ring structure. More aromatic products were detected in the GC-MS analyses than are shown in Table 1 and Fig. 2. However, we have deliberately concentrated on those products associated directly with the ring cleavage mechanisms operating in these experiments and on other monomeric aromatics which can be regarded as satellite molecules derived from the conserved A- or B-ring metabolites.

Two particularly interesting features of the rhizobial degradation pathways are the formation of chalcones as the first intermediates of C-ring fission in most cases and the appearance of new chalcone, flavanone, and flavone structures among the metabolites when naringenin and luteolin were incubated with R. leguminosarum bv. viciae and R. meliloti, respectively. Compounds with these structures are themselves potential nod gene inducers and could be involved in interactions with the nodD gene product. Chalcones especially have been reported to serve as more potent nod gene inducers than other flavonoid molecules (9), and this feature has largely been attributed to their open C-ring system (7), which offers increased spatial flexibility during interactions with NodD proteins (5). Chalcones emerging from an initial C-ring fission in the inducer flavonoid may provide additional conformational flexibility during binding to NodD proteins, thereby increasing transcriptional activation of other nod genes.

Our data suggest that flavonoid C-ring cleavage is conserved in all Rhizobium species tested and might be a form of natural catabolic response to the basic flavonoid ring system. This implies that such biotransformations could also be anticipated for other, non-gene-inducing flavonoids, which, because of their incompatible hydroxylation patterns or the steric arrangement of their ring systems, do not interact with NodD proteins (9). The preference of NodD proteins for certain flavonoids further suggests that the receptor protein is in direct contact with the inducer (7), perhaps in the cytoplasmic membrane wherein the NodD protein is located (24), and the inducer has also been shown to accumulate in R. leguminosarum bv. viciae (22). Further studies of inducer-NodD protein binding mechanisms would be required to ascertain the significance of flavonoid C-ring cleavage at the subcellular level during nod gene induction. Since all products were detected in culture supernatants, it is not possible to say whether they represent compounds that are released from cells into the medium or whether degradation is an intracellular process.

It is interesting to speculate on the significance of flavonoid degradation pathways for the general biology of rhizobia. As with other aromatic compounds (16), degradation could allow rhizobia to utilize flavonoids as carbon or energy sources.
Degradation of this type could also provide an effective means of detoxifying certain phytoalexin-like molecules.

New flavonones and chalcones have also been observed in the root exudates of *Vicia sativa* subsp. *nigra* after inoculation with *R. leguminosarum* bv. *viciae* (21), and their origin was attributed to de novo biosynthesis in root tissue followed by rapid release into the rhizosphere (23). Our results suggest that the new compounds could arise from the degradation of naringenin in root exudate by free-living rhizobia. Although the new chalcones and flavonones formed by *R. leguminosarum* bv. *viciae* from naringenin in the present study could not be fully characterized, it was established that their GC retention times and the mass spectra of their derivatives were different from those of authentic naringenin and naringenin chalcone. In inoculated, naringenin-supplemented medium, a mixture of naringenin and naringenin chalcone was detected within 1 h of incubation. The addition of naringenin chalcone alone to a sterile basal medium resulted in cyclization of the C-ring and complete isomerization to naringenin after 2 h, thus confirming that no spontaneous isomerization in the reverse direction occurred under the test conditions and that the initial C-ring cleavage in naringenin was bacterially mediated.

When incubated with *R. meliloti*, luteolin yielded several other closed C-ring metabolites (e.g., a tetrahydroxy flavanone and apigenin), despite the concurrent existence of C-ring fission (Fig. 2). Speculation on additional roles for phenolic compounds as signal molecules in legume- *Rhizobium* interactions has increased since the discovery that some flavonoids function as natural auxin transport regulators in plants by competing with naphthylphthalamic acid for binding to the naphthylphthalamic acid receptor in plant cells (13) and acting as auxin transport inhibitors. An extra dimension has been added to this concept following reports that auxin transport inhibitors induce nodule-like structures on legume roots (10, 12) and that *R. meliloti*, in both wild-type and pSym-cured forms, produces a luteolin-induced metabolite which competes with naphthylphthalamic acid for its binding site on plant membranes (11). The fact that this compound is produced by rhizobia which are devoid of *nod* genes suggests that it is unlikely to possess a structure of the type found in Nod factors and that it could be either a novel microbial metabolite or a product derived from luteolin degradation.

ACKNOWLEDGMENTS

We thank the Leverhulme Trust, the Agricultural and Food Research Council, and the British Council for financial support.

We thank the following researchers for donations of strains: W. Streit, Marburg, Germany; W. J. Broughton, Geneva, Switzerland; and R. J. H. Okker, Leiden, The Netherlands. We also acknowledge the valuable assistance of D. R. Boyd and N. D. Sharma, Department of Chemistry, The Queen's University of Belfast, with interpretation of MS data, and we thank J. T. Hamilton, Department of Agriculture for Northern Ireland, for MS analyses.
REFERENCES
cens sp. nov. requiring H₂ or formate to degrade gallate, pyro-