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Biotransformation of the Pentahydroxy Flavone Quercetin by *Rhizobium loti* and *Bradyrhizobium* Strains (*Lotus*)

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***Lotus* rhizobia catabolized quercetin in an arabinose-based medium via a novel form of C-ring cleavage, yielding phloroglucinol and protocatechuic acid. Conservation of the A and B rings of the flavone suggests that a chalcone could be formed as a transient intermediate.**

Flavonoids are commonly synthesized phenolic secondary metabolites in the tissues of higher plants which, together with other aromatic compounds, can be exuded into the rhizosphere (17). With regard to the legume-*Rhizobium* symbiosis, intense interest in the subgroup of flavonoids containing the flavone ring structure has been generated by the finding that they are specifically responsible for induction of nodulation genes in several *Rhizobium* species (for example, see reference 10).

Suggestions have also been made (3, 5, 11), prompted in part by the knowledge that such compounds induce *Rhizobium fredii* genes not involved in nodulation (14), that flavonoids might be catabolized by rhizobia. Evidence for this function is, however, scarce.

One study (1) demonstrated that a *Rhizobium* strain from *Leucaena leucocephala* could degrade the flavan-3-ol catechin, a component of condensed tannin in tannery effluent, but there are no reports of rhizobia cleaving the flavone-type ring structure.

Quercetin (3,3′,4′,5,7-pentahydroxy flavone) is one of the major flavonoid constituents in roots and nodules of *Lotus* species (12). In this study, quercetin was used as a supplement in a defined, arabinose-based culture medium, and we now report the detection of its degradation products by gas chromatographic-mass spectrometric (GC-MS) analysis of samples extracted after incubation with *Lotus* rhizobia.

Growth medium was based on that described by Munns and Keyser (8), without galactose and KH_2PO_4 and with the following additions: Na_2HPO_4 (25 mM), biotin and thiamine hydrochloride (each 0.5 mg liter^{-1}), and calcium pantothenate (10 mg liter^{-1}). The L-arabinose concentration was 0.25%, and quercetin (Sigma Chemical Co., Poole, Dorset, England) was added as required from a stock solution in methanol to give a final concentration of 30 mg liter^{-1}. The pH was adjusted to 7.6.

The *Rhizobium loti* strains (NZP2042 and LC22) and the *Bradyrhizobium* sp. (*Lotus*) strains (CC814s and CC829) were from the following sources: Department of Scientific and Industrial Research, Palmerston North, New Zealand (*R. loti* NZP2042); Division of Plant Industry, Commonwealth Scientific and Industrial Research Organisation, Canberra City, Australia (*Bradyrhizobium* sp. strains CC814s and CC829); Department of Agriculture for Northern Ireland, Belfast, Northern Ireland (*R. loti* LC22).

Strains were grown as single cultures in quercetin-free medium, harvested by centrifugation, washed twice, and resuspended in sterile water. The strains were used to inoculate media in 300-ml amounts in 500-ml dark-glass bottles covered with aluminum foil as a precaution against any photosensitized reactions. The initial concentration of cells was 1.5×10^3 ml^{-1}, and incubation was at 25°C on a rotary shaker for 72 h. Treatments involved medium only (control), medium plus quercetin (control), medium plus *R. loti* or *Bradyrhizobium* sp. strain (controls), and medium plus quercetin plus *R. loti* or *Bradyrhizobium* sp. strain.

Subsamples were withdrawn aseptically at various intervals during all treatments to check for the presence of free quercetin and any evidence of degradation or polymerization products by thin-layer chromatography (TLC) on silica gel (thickness, 0.2 mm). After centrifugation (8,000 × *g* for 10 min), the supernatant was decanted, and its pH was adjusted to 6.0 with dilute hydrochloric acid, followed by extraction (three times) with ethyl acetate and concentration by evaporation. Chromatograms were developed in toluene-ethyl formate-formic acid (5:4:1) and examined for color reactions under UV light at 365 nm in the absence of spray reagents (7).

After 72 h, an additional aseptically drawn sample was taken for viable cell counts on yeast extract-mannitol agar in the case of rhizobial treatments. At this time the remaining volumes of all treatments were centrifuged as above, and the supernatants were hand extracted with ethyl acetate (5 × 60 ml) after saturation with sodium chloride, followed by drying with Na_2SO_4 and concentration on a rotary evaporator. The residues were taken up in 0.5 ml of methanol and treated with an excess of ethereal diazomethane solution at 0°C. After 12 h, the solvents, along with the excess diazomethane, were evaporated and the residues were dissolved in 0.5 ml of diethyl ether prior to GC-MS analysis on a Hewlett-Packard HP5890 gas chromatograph directly linked to a Hewlett-Packard HP5970 mass selective detector. The gas chromatograph was equipped with an Ultra 2 fused silica wall-coated open tubular column (12 m by 0.2 mm) with 5% diphenyl polysiloxane–95% dimethyl polysiloxane (0.33 μm) as the bonded phase. Helium was the carrier gas at a flow rate of 1 ml min^{-1}, and a split ratio of 20:1 was employed. After injection, the oven temperature was held at 80°C for 2 min and then programmed to rise by 10°C min^{-1} up to 150°C, followed by 25°C min^{-1} up to 300°C, which was held for 5 min. The mass selective detector was operated in the full

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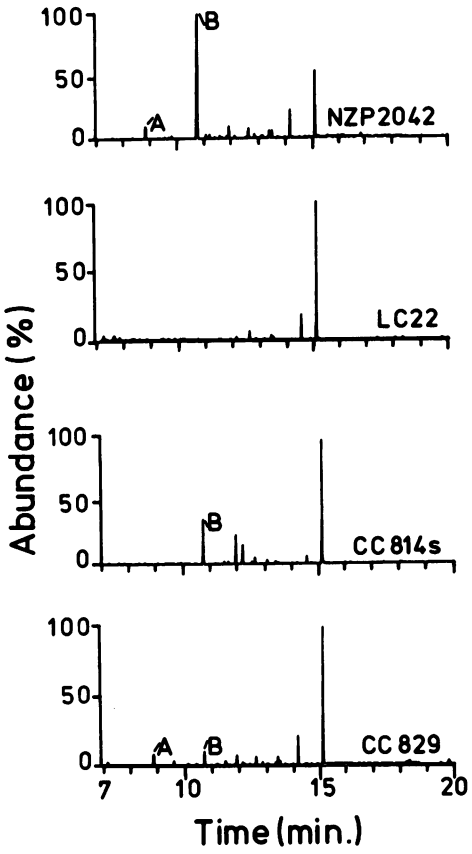

FIG. 1. Total ion chromatograms of methylated ethyl acetate extracts of quercetin-supplemented media, inoculated with *R. loti* NZP2042 and LC22 and *Bradyrhizobium* sp. strains CC814s and CC829 and incubated for 72 h. Component peaks are labeled for phloroglucinol (A) and protocatechuic acid (B).

scanning electron impact mode over the range of 50 to 550 atomic mass units at 0.86 scans s^{-1}.

All rhizobial treatments developed viable populations of $>10^7$ cells ml^{-1} after 72 h. Total ion chromatograms of extracts from quercetin-supplemented medium inoculated with four rhizobial strains are shown in Fig. 1. Component peaks for protocatechuic acid (Fig. 1, peak B) were present for three strains, and accompanying phloroglucinol peaks (Fig. 1, peak A) were present for two of these. The mass spectra of both components (Fig. 2) were identical to those of authentic compounds. Mass spectral analyses of peaks in the total ion chromatograms from the control treatments failed to detect these quercetin degradation products or any other hydroxy-aromatic compounds. Structural characteristics suggest that protocatechuic acid originated from the B-ring of quercetin, while phloroglucinol was derived from the A-ring. The formation of these cometabolites by strains NZP2042 and CC829 indicates a form of quercetin C-ring cleavage which has not been previously reported in bacteria (Fig. 3). Some intestinal clostridia and a *Eubacterium* species can also degrade quercetin via C-ring cleavage (16, 6), but the end products are not the same, suggesting that both the pattern of ring fission and the ensuing metabolic pathways are different from those in rhizobia.

Flavone degradation has also been reported in *Pseudomonas* species (15) but with fission initiating in the A-ring via

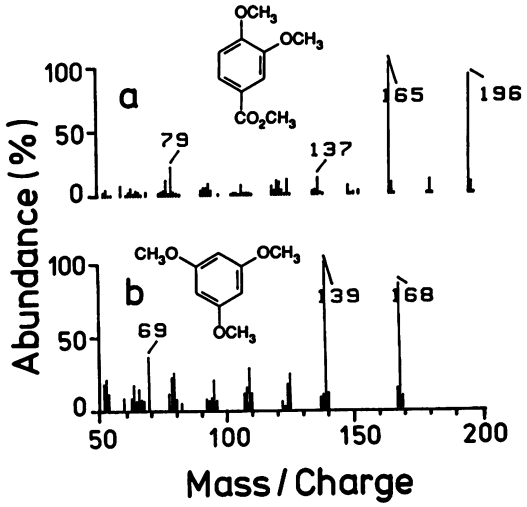

FIG. 2. Mass spectra of degradation products. (a) Spectra of the component in peak B corresponding to methylated authentic protocatechuic acid; (b) spectra of component in peak A corresponding to methyl ether of authentic phloroglucinol.

hydroxylation at C-8. Other bacterial transformations of quercetin include sulfation (4) and glucosidation (13). Although no chalcones were detected among the metabolites in this study, the absence of quercetin A- or B-ring cleavage suggests that rhizobia could generate such compounds as transient intermediates. Chalcones are capable of inducing *Rhizobium nod* genes at lower concentrations than flavones (11), and the possibility therefore arises that intermediates from flavonoid catabolism could be involved in *nod* gene regulation.

TLC analyses throughout the incubation period showed that, in treatments involving inoculation with strains NZP2042, CC814s, and CC829, free quercetin was present in the early stages but was fully metabolized at 72 h. In the uninoculated control and the treatment inoculated with strain LC22, free quercetin was also present in the early stages of incubation, but at 72 h the TLC analyses indicated that extensive polymerization of quercetin had occurred, most probably as a consequence of its autooxidation (2). In both cases, no free quercetin was available for methylation prior to GC-MS analysis, and thus quercetin itself did not register in the total ion chromatograms of any treatment. The detection of protocatechuic acid and phloroglucinol by TLC analysis concurred with identification by GC-MS for all strains.

Recovery of phloroglucinol in the ethyl acetate extracts may have been adversely affected by the high solubility of

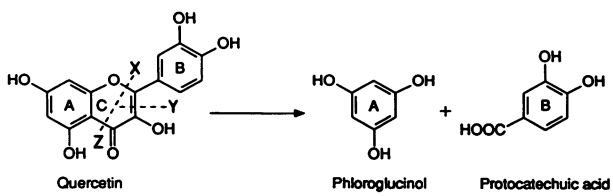

FIG. 3. Proposed pattern of quercetin C-ring cleavage by *Lotus* rhizobia. Cleavage of the C-O bond at X could yield a chalcone intermediate. Further cleavages at Y and Z release the A- and B-ring units as phloroglucinol and protocatechuic acid, respectively.

this compound in water. Detection of metabolites might be improved by growing larger volumes of cultures, extracting with three volumes of methylene chloride or chloroform, and using high-performance liquid chromatography for product separation.

These results demonstrate the ability of *Lotus* rhizobia to catabolize quercetin, yielding products including protocatechuic acid, which has previously been shown to serve as a sole carbon and energy source for some rhizobia after further conversion to succinyl coenzyme A and acetyl coenzyme A via 3-oxoadipate (9).

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