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Hemoproteins of *Bradyrhizobium japonicum* Cultured Cells and Bacteroids

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The hemoprotein content of 17 strains of *Bradyrhizobium japonicum* bacteroids from field-grown plants and the corresponding strains of cultured cells was determined spectrally. The major terminal oxidases, cytochromes (cyt) aa_3 and *o*, were present in all strains of cultured cells. cyt aa_3 was present in significant amounts in bacteroids only in strains of DNA homology group II. cyt *o* appeared to be present in bacteroids of all strains, and the average level was the same as in cultured cells. cyt *b* and *c* in the membrane fractions were higher in bacteroids of all strains compared with cultured cells. cyt P-450 was present in both the membrane and soluble fractions of bacteroids of most strains. The total P-450 content varied sixfold among strains. A CO-reactive hemoprotein, P-422, was present in the soluble fraction of all strains of cultured cells. P-422 may be a hemoglobinlike protein, and it was present in significant amounts in bacteroids only in DNA homology group I strains.

The major hemoprotein oxidases of aerobically grown cells of various species of rhizobia are cytochromes (cyt) aa_3 and *o* (2, 12, 16, 18, 24, 25). Some of these reports also indicated that cyt aa_3 and *o* were not found in bacteroids of the corresponding species. Instead, new CO-binding hemoproteins including cyt c_{552}, P-450, and P-420 were found in bacteroids which were not found in cultured cells (1, 18). Earlier we reported that bacteroids of *Bradyrhizobium japonicum* 61A76 contained cyt aa_3 and possibly *o* (15). To determine the pattern of hemoproteins among strains of *B. japonicum* and which ones warrant further characterization of their role in symbiosis, we determined the major hemoproteins of 17 strains grown aerobically from three DNA homology groups and of the corresponding bacteroids isolated from the root nodules of field-grown soybean plants.

MATERIALS AND METHODS

B. japonicum USDA 38, 62, 83, 86, 110, and 117 were from the USDA-ARS National *Rhizobium* Culture Collection, Beltsville, Md.; strains 5633, D193, 8°, 10324, USDA 29, USDA 58, USDA 130, and USDA 140 were obtained from G. Elkan, North Carolina State University; 61A50 and 61A76 were from The Nitragin Co. The strains were grown in a yeast extract–gluconate–inorganic salts medium (13) containing 25 mM MOPS (3-[*N*-morpholino]propane sulfonic acid) buffer unless otherwise noted. For preparation of extracts of aerobically grown cells, succinate or malate was substituted for gluconate for the growth of a few strains. This was done to reduce the extracellular polysaccharide production which made harvesting the cells difficult. How much the carbon source influenced the hemoprotein levels is not known. Cultured cells and bacteroids were suspended in 0.1 M potassium phosphate (pH 6.8–0.1 mM $MgCl_2$–1 μg of DNase per ml and then ruptured by passage through a French pressure cell. Cell debris was removed by centrifugation at 18,000 × *g* for 15 min. The membrane fraction sedimenting at 183,000 × *g* (90 min) was isolated, washed, and used for analysis.

Taproot nodules were harvested about 45 days after emergence from field-grown soybeans (*Glycine max* cv. Williams) as previously described (15). The soil contained low numbers of indigenous *B. japonicum* cells, and uninoculated plants had less than 1 taproot nodule per plant, whereas inoculated plants had an average of about 30. Bacteroids were isolated from taproot nodules and purified by sucrose density centrifugation as previously described (15; also see Table 2). The hemoprotein content was determined by spectroscopy by using difference spectra, dithionite reduced minus ferricyanide oxidized. For the CO-reactive hemoproteins, the difference spectrum between (dithionite plus CO) minus dithionite was used. The extinction coefficients were those used by Appleby (1, 2) except for cyt *o*, which was derived from Daniel (10). Protein was determined by the Lowry method after heating for 10 min in 0.5 N NaOH to solubilize the membrane proteins (15).

RESULTS

Fractionation of bacteroids. Ching et al. (9) reported that bacteroids from soybean nodules could be separated into three fractions by discontinuous sucrose density gradient centrifugation. They suggested that the heaviest fraction corresponded to bacteria, the intermediate fraction corresponded to transforming bacteria, and the lightest fraction corresponded to mature bacteroids. The mature bacteroid fraction was devoid of cyt aa_3, had a higher P-450 content, and had higher β-hydroxybutyrate dehydrogenase and nitrogenase activities compared with the other fractions, characteristics of bacteroids compared with cultured rhizobial cells. In a previous study (15), we used a similar gradient with *B. japonicum* 61A76 bacteroids and found that about 90% of the bacteria were in the lightest fraction. From the present study, it is apparent that the density of mature bacteroids is a characteristic of the specific rhizobial strain (see columns 2 to 4, Table 2). With most of the strains, the major fraction of bacteroids was found in the lighter fraction

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TABLE 1. Hemoprotein content of the soluble and particulate fractions of cultured cells

DNA homology group	Strain	Hemoprotein content (nmol of heme/g of protein)								
		Particulate					Soluble			
					CO reactive			CO reactive		
		aa_3	*b*	*c*	*c*	*o*	*c*[a]	*c*	P-450	P-422
I	10324[b]	180	415	275	47	165	85	19	4	++++[e]
	USDA 58[c]	195	335	230	57	120	145	31	–	+++
	D193[b]	185	345	255	42	230	105	19	–	+++
	USDA 38[c]	225	470	355	68	165	20	11	4	+++
	5633[d]	170	500	330	48	135	100	23	–	+++
	USDA 115[b]	170	440	345	60	135	170	28	5	+++
Ia	USDA 110[c]	110	310	225	69	100	170	35	–	+++
	USDA 62[c]	85	295	205	65	160	140	30	–	+++
	61A50[b]	190	360	270	59	100	140	53	–	++
	USDA 140[c]	125	340	255	48	110	75	33	–	+++
	8°[c]	305	510	370	71	130	100	26	–	+++
II	61A76[d]	140	335	270	65	150	200	66	–	+++
	USDA 117[b]	190	395	285	78	85	125	37	–	+++
	USDA 86[b]	125	285	195	47	90	185	57	–	++
	USDA 29[d]	200	340	280	73	80	175	53	–	++
	USDA 130[d]	140	355	265	77	80	140	39	–	+++
?	505[d]	70	180	130	39	65	160	50	–	++
	Avg	159	365	267	55	124	131	36		

[a] Includes all cyt *c*.
[b] Carbon source for growth was succinate.
[c] Carbon source for growth was malate.
[d] Carbon source for growth was gluconate.
[e] Extinction coefficients for P-422 are unknown. +, Relative amounts. –, Not detected.

TABLE 2. Hemoprotein content of the soluble and particulate fractions of bacteroids

DNA homology group	Distribution on analytical[a] sucrose gradient (% in band):			Strain	Hemoprotein content (nmol of heme/g of protein)[b]									
					Particulate						Soluble			
								CO reactive				CO reactive		
	2	3	4		aa_3	*b*	*c*	*c*	*o*	P-450	*c*	*c*	P-450	P-422
I	18	59	21	10324	Tr	650	750	125	65	35	100	22	15	++
	29	22	46	USDA 58	Tr	820	1,020	210	80	43	140	38	24	+++
	70	19	08	D193	56	505	480	95	77	14	110	23	31	+
	86	08	02	USDA 38	40	530	620	57	94	24	170	13	28	++
	44	48	05	5633	Tr	600	490	68	210		270	96	42	+
	87	06	03	USDA 115	Tr	550	690	74	70	47	160	15	44	++
Ia	15	33	49	USDA 110	Tr	650	740	75	150	87	220	58	70	–
	86	07	02	USDA 62		520	590	53	140	32	250	75	34	–
	06	10	79	61A50	Tr	620	650	82	170	58	280	83	99	–
	93	03	01	USDA 140		475	560	54	140	40	250	73	29	–
	31	64	03	8°	Tr	520	460	44	185	4	195	22	98	–
II	97	01	01	61A76	91	500	470	40	130	Tr	180	41	44	–
	89	05	02	USDA 117	80	470	420	47	110	6	200	72	31	–
	96	02	01	USDA 86	60	580	450	60	175	4	225	73	35	–
	86	10	02	USDA 83	126	410	380	48	80		185	63	25	–
	40	52	05	USDA 29	100	585	540	73	170	12	120	50	45	–
	93	04	01	USDA 130	88	360	315	65	160	9	230	52	18	–
?	86	09	02	505	Tr	486	530	68	71	9	375	99	62	–
				Avg		546	564	74	126		203	54	43	

[a] The analytical sucrose density gradient was composed of 6 ml of sample, 8 ml of 45% sucrose (wt/wt), 10 ml of 50% sucrose, 8 ml of 52% sucrose, and 5 ml of 57% sucrose, all prepared in 50 mM potassium phosphate, pH 7.5. The tubes were centrifuged for 4.25 h at 90,000 × *g* in a Beckman SW27 rotor (15). Band 1 did not enter the 45% sucrose and consisted mostly of mitochondria. The preparative gradient did not contain the 52% sucrose and thus collapsed bands 3 and 4 together. The band containing the major fraction, band 2 or bands 3 plus 4, was analyzed. % in band is the percentage of recovered bacteroids in each band.
[b] Average of two preparations. Variation typically was less than 20%.

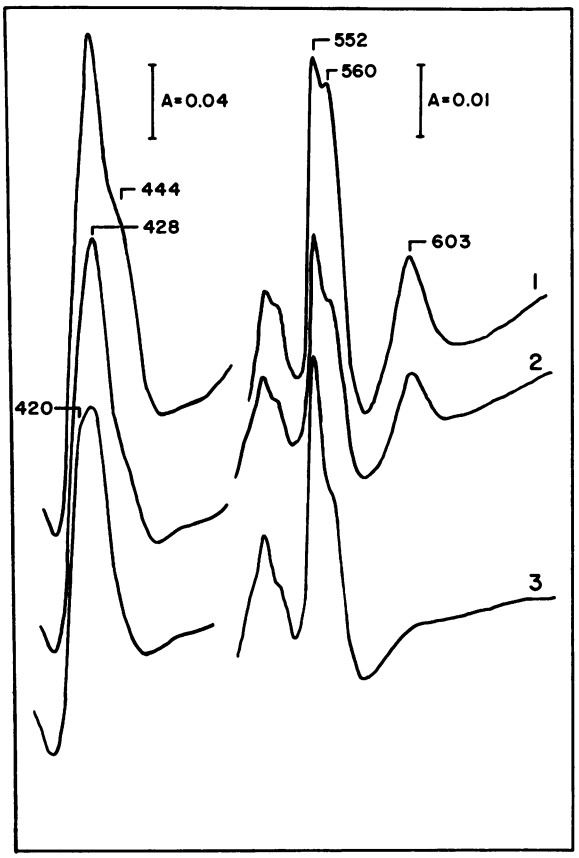

FIG. 1. Reduced minus oxidized difference spectra of particulate fractions. Samples of the particulate fractions were reduced with dithonite or oxidized with air. 1, Cultured cells of USDA 130; 2, bacteroids of USDA 130; 3, bacteroids of USDA 140.

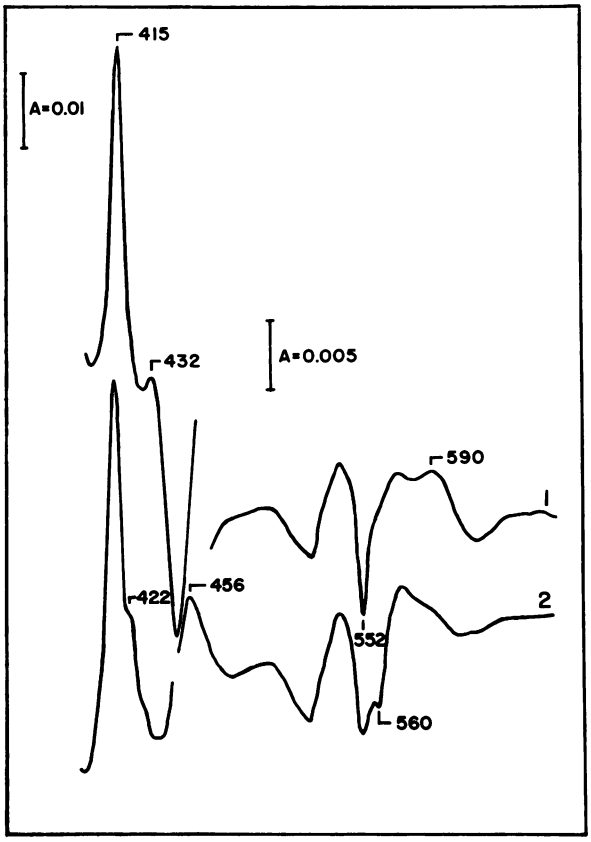

FIG. 2. (Reduced plus CO) minus reduced difference spectra of particulate fractions of USDA 130. Samples of the particulate fractions were reduced with dithionite and equilibrated in a syringe with CO or with argon for 30 min. 1, Cultured cells; 2, bacteroids.

banding at the 45 to 50% sucrose interface. With a few strains such as USDA 110 and 61A50, the major fraction was found in the heavier fraction banding at the 52 to 57% sucrose interface; and with other strains (8° and USDA 29), the major fraction banded at the 50 to 52% sucrose interface. Cultured cells also banded at various densities depending on the strain. The major bacteroid fraction corresponded to mature bacteroids in all strains, based on reduced cyt aa_3 and P-422 content and increased amounts of P-450 and membrane-bound cyt *b* and *c*. For preparative work, a modified gradient was used which did not contain 52% sucrose and therefore fractionated bands 3 and 4 together (15). As can be determined from the distribution shown in Table 2, this preparative gradient would not influence interpretation of the results. In a few cases in which about equal distribution was found in two major bands, e.g., strains 5633 and USDA 29, we found that the hemoprotein composition was essentially the same in bacteroids from both fractions.

Hemoprotein content of cultured cells. The hemoprotein content of cultured cells of *B. japonicum* is illustrated in Table 1. The hemoprotein concentrations were determined from spectra similar to those shown in Fig. 1 to 3. The strains are grouped according to DNA homology (14). Qualitatively, the major hemoproteins in all strains appeared similar. Quantitatively, the amounts of the various hemoproteins varied severalfold among the different strains. No particular significance is attributed to the quantitative differences as it is well known that the hemoprotein content of cells is variable and somewhat dependent on the growth conditions. Thus, no hemoprotein phenotype appears to be associated with the DNA homology group with cultured cells, although there is a hemoprotein phenotype associated with bacteroids (see below).

One important characteristic of all strains is that they appeared to contain a hemoprotein among the soluble proteins in the cell-free preparations which we labeled P-422. The (reduced plus CO) minus reduced difference spectrum of the supernatant fraction of strain USDA 130 is shown in Fig. 3. The minimum at 552 nm and the peak at 414 nm are due primarily to the CO-reactive *c*-type cytochromes. In addition, the spectrum of all strains revealed an absorption maximum or shoulder at 422 to 425 nm. This is not a characteristic of the *c*-type cytochromes found in the soluble fraction. We fractionated the hemoprotein(s) in the soluble fraction of a mutant deficient in cyt *c*, and preliminary spectral data of a partially purified fraction (Fig. 3, curve 3) suggest that the 422- to 425-nm absorption is due to a hemoglobinlike protein. Appleby (2) described a hemoglobinlike protein in cultured cells of strain 505, but it had peaks in the (reduced plus CO) minus reduced spectrum at 417, 540, and 569 nm, whereas P-422 has absorption maxima at approximately 422, 529, and 574 nm. Appleby's preparation contained a significant amount of cyt *c* which may have influenced the (reduced plus CO) minus reduced spectrum. This compound may be regulated by oxygen as it is not

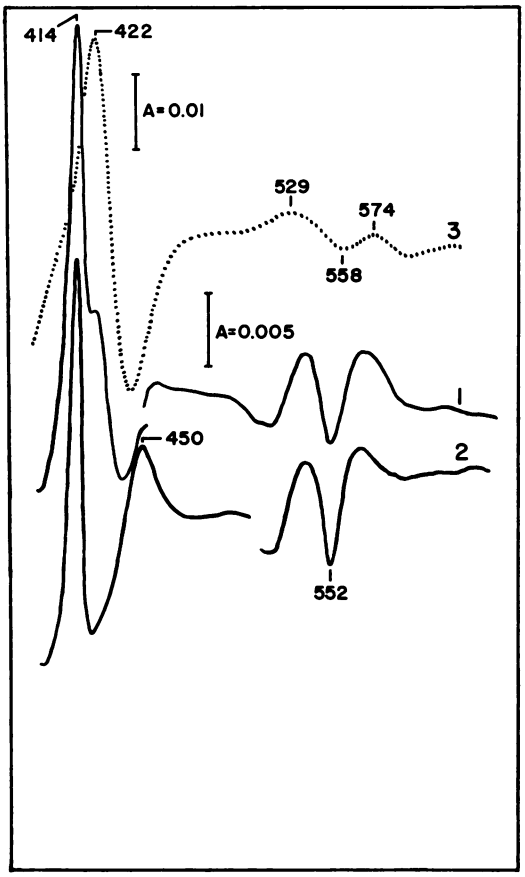

FIG. 3. (Reduced plus CO) minus reduced difference spectra of soluble fractions of USDA 130 and of partially purified P-422. 1, Cultured cells; 2, bacteroids; 3, partially purified P-422. The soluble fraction of cultured cells of a mutant deficient in *c*-type cytochromes (NPK63 [19]) was partially purified by ammonium sulfate fractionation (45 to 75% saturated fraction) followed by fractionation on a DE-52 ion-exchange column. The P-422 was absorbed on the column in 0.01 M phosphate buffer (pH 7.5) and eluted with 0.1 M NaCl in 0.01 M phosphate.

found in significant amounts in bacteroids of many strains (see below).

Hemoprotein content of bacteroids. The hemoprotein content of bacteroids of 18 strains of *B. japonicum* is shown in Table 2. There are several differences compared with cultured cells.

(i) cyt aa_3. The small $\lambda_{max_{432}}$ and large $\lambda_{min_{446}}$ in the (reduced plus CO) minus reduced spectrum of the particulate fraction of cultured cells (Fig. 2, spectrum 1) are due to cyt aa_3. In bacteroids, this spectrum is modified due to the lower concentration of aa_3 and the presence of P-450 (Fig. 2, spectrum 2). Note that the P-450 peak is at 456 nm. This is due to the influence of cyt aa_3. In strains which do not have aa_3 (DNA homology groups I and Ia), the λ_{max} of P-450 is at 450 nm. The level of cyt aa_3 was lower in all strains compared with aerobically grown cells. Only very low or trace amounts were found in strains belonging to DNA homology groups I and Ia (Table 2). Significant amounts were retained by strains of DNA homology group II. Thus, retention of this terminal oxidase in bacteroids appears to be a phenotype of this group.

(ii) cyt *b* and *c*. The membrane-bound cyt *b* and *c* were found at higher levels in bacteroids than in cultured cells in every strain except USDA 130 (Table 2). The average was 52% greater for cyt *b* and 115% for cyt *c*, although the range of variation was large; e.g., strains USDA 58 and USDA 110 were over 100% greater, whereas strains 8° and USDA 130 were only slightly higher. In general, the soluble *c*-type cytochromes were higher in bacteroids, the CO-reactive soluble *c*-type by over 100% on average.

(iii) cyt P-450. P-450 is an unusual cytochrome which resembles the cyt P-450 found in some other microorganisms, plants, and liver microsomes. In other organisms this cytochrome is involved in the hydroxylation of xenobiotics, but in rhizobia no catalytic function has been found (5). P-450 is not synthesized to any significant extent under aerobic cultural conditions but is found in the soluble supernatant fraction of all strains of bacteroids. It is found also in the membrane fraction in most but not all strains. The levels recorded for P-450 in the particulate fraction of DNA homology group II strains are somewhat low owing to the influence of cyt aa_3 on the spectrum (see above). We did not attempt to correct for the aa_3 contribution. The total amount of P-450 in the cell varied by over sixfold among the strains.

(iv) Hemoprotein P-422. Hemoprotein P-422 was found in the soluble fraction of all strains of cultured cells (Table 1) but was observed in bacteroids of only a few strains, all of which belong to DNA homology group I. No significant amounts were found in strains of DNA homology group Ia or II. This may be a phenotype of this group, and if so, it is the first reported which differentiates groups I and Ia other than serology.

(v) cyt *o*. The (reduced plus CO) minus reduced difference spectrum of the membrane fraction of all strains had a minimum or shoulder on the minimum at about 560 nm which is attributed to cyt *o* (Fig. 2). Appleby (1) reported that bacteroids of strain 505 did not contain cyt *o*. From the spectral data in some strains, it is sometimes difficult to detect cyt *o* since the CO-reactive *c*-type cytochromes dominate the spectra (e.g., Fig. 2, spectrum 1). However, the *c*-type cytochromes react slowly with CO at pH 6.8 and lower, whereas cyt *o* reacts more rapidly. Thus, we were able to detect cyt *o* in all strains. Because of the contribution of cyt *c*, the data presented for cyt *o* should be regarded as semiquantitative, especially in strains with the lower levels. The average cyt *o* content of all strains in bacteroids was similar to that of cultured cells, but the strains of DNA homology group I had a lower cyt *o* content, whereas the strains of group II had a higher amount.

DISCUSSION

There have been a few reports on the cytochrome composition of cultured cells and bacteroids, but this is the first which describes sufficient strains so that phenotypes can be assigned to the *B. japonicum* DNA homology groups. cyt aa_3 and *o* are the major terminal oxidases of cultured *Rhizobium* and *Bradyrhizobium* cells (see O'Brian and Maier [22] for a review). Several reports have shown that cyt aa_3 is not found in bacteroids of specific strains, and this had almost become accepted as a characteristic of *Rhizobium* and *Bradyrhizobium* bacteroids as compared with cultured cells. In this study, we showed that loss of cyt aa_3 is a characteristic of DNA homology group I and Ia strains but not of group II strains; these strains retain a significant amount of this cytochrome. cyt aa_3 also has been reported in bacteroids of *Rhizobium phaseoli* (24) and in *Rhizobium* sp. strain NGR231 (4), the symbiont of *Parasponia* species. If the affinity of cyt aa_3 for O_2 in rhizobia is similar to that in

other organisms, the level of O_2 normally found in nodules is too low for cyt aa_3 to function effectively. Therefore, it is unlikely that cyt aa_3 has a significant role in O_2 metabolism in bacteroids even in the DNA homology group II strains. This conclusion is supported by the isolation of mutants deficient in cyt aa_3 which were symbiotically effective (19, 20). A recent report has described the isolation of a mutant of *R. phaseoli* which had a higher level of cyt aa_3 in bacteroids than the wild-type parent (24). This mutant reduced more acetylene in nodules from young plants (but not older plants) than the wild type. This mutant was isolated after mutagenesis with a chemical mutagen. Since multiple mutations and deletions in DNA may result from chemical mutagenesis, it is possible that the cytochrome mutation was not responsible for the symbiotic phenotype.

Appleby (1) failed to observe cyt *o* in bacteroids of *B. japonicum* 505, and others have assumed the absence of cyt *o* in bacteroids (16, 23), although the spectra they published could indicate the presence of cyt *o*. O'Brian and Maier (21) clearly demonstrated the presence of cyt *o* in bacteroids of strain SR; Appleby et al. (4) reported it in bacteroids of NGR231; and Soberon et al. (24) found it in *R. phaseoli*. Our interpretation of the spectra we obtained is that cyt *o* was present in bacteroids of all strains including strain 505. However, there is no concrete evidence that it is functional in bacteroids. The mean cyt *o* levels in bacteroids of all strains were about the same as in cultured cells. In general, however, the levels in bacteroids of group II are higher than in cultured cells, while the levels in bacteroids of group I are lower.

Rhizobia contain an array of other CO-reactive hemoproteins. CO reactivity of a hemoprotein is indicative of O_2 binding and that the protein potentially is an oxidase. To date, the oxidase(s) which is functional in bacteroids has not been identified. Bergersen and Turner (8) measured the rate of O_2 consumption as a function of O_2 concentration and proposed that there may be four oxidases with different affinities for O_2 in bacteroids. In particles, in addition to cyt aa_3 and *o*, the CO-reactive pigments are particle-bound cyt c_{552}, P-450, and possibly P-420; soluble proteins P-450 and P-422; and one or two soluble *c*-type cytochromes, c_{552} and c_{554}. The relationship between the particle-bound and soluble *c*-type cytochromes is not known. In addition, a flavoprotein oxidase has been identified in bacteroid membranes of *B. japonicum* (3) and shown to be involved in H_2 oxidation (21).

Daniel and Appleby (11) identified both cyt c_{552} (13 kilodaltons) and c_{554} (85 kilodaltons) in the soluble fraction of strain 505 by fractionation on Sephadex G-100. Our spectral measurements do not differentiate between these two cytochromes. In recent experiments, we have isolated cyt c_{554} (15.5 kilodaltons) from strain 61A89 (= USDA 110) (R. E. Tully, M. J. Sadowsky, and D. L. Keister, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, N99, p. 301), but it is not identical to the one Daniel and Appleby (11) identified. We are currently isolating and characterizing this protein. We have developed an antibody to c_{554} so that in future work we will be able to quantitate c_{554}.

P-450 is found only in trace amounts in aerobically cultured cells and appears to be synthesized under reduced oxygen tensions (11). It was found in all strains of bacteroids, distributed between the soluble and particulate protein fractions. The total content varied over sixfold, from a low of 25 nmol of protein per mg in strain USDA 83 to 157 nmol/mg in strains 61A50 and USDA 110. Inhibitor experiments suggested that P-450 is important in respiration under low oxygen tensions (6, 7, 17), but it did not appear to be an oxidase. Thus, it was proposed that P-450 serves as a carrier of O_2 to an oxidase or peroxidase, with the process being efficiently linked to ATP production (6). This is an attractive hypothesis, especially if P-450 is a periplasmic protein, since it could facilitate the diffusion of O_2 under very low O_2 tensions, similar to leghemoglobin function in the plant nodule cell.

A hemoprotein which had properties of a hemoglobin was isolated from among the soluble proteins in extracts from cultured cells (2). This protein only slowly autooxidized and therefore is not likely to function as an oxidase. We identified a hemoprotein, which we labeled P-422, with absorption characteristics resembling those of leghemoglobin. P-422 (Fig. 3) was isolated from a mutant deficient in *c*-type cytochromes (19), so that *c*-type hemoproteins would not contribute to the spectrum of the partially purified protein. The spectra of the soluble fraction of all strains of cultured cells of *B. japonicum* had an absorption maximum or shoulder at 422 to 425 nm in the (reduced plus CO) minus reduced difference spectrum. While we have not yet isolated P-422 from wild-type cells, it is probable that the absorption at 422 to 425 nm in the soluble fractions is due to this protein. We believe that P-422 is probably a protein similar to the one which Appleby identified as *Rhizobium* hemoglobin. Appleby did not detect this protein in bacteroids (1) or cells grown under anaerobic conditions (11). Our results support this observation for most strains, but strains of DNA homology group I appear to retain P-422 in bacteroids. Kretovich et al. (16) found a protein in the soluble fraction of *Bradyrhizobium* sp. from lupine nodules which had an absorption maximum at 421 nm in the (reduced plus CO) minus reduced difference spectrum. It was not characterized sufficiently to determine its properties. Further work is in progress to characterize P-422.

Appleby (1) observed a CO-reactive component in the particulate fraction of bacteroids which he called P-420. The (reduced plus CO) minus reduced spectrum of the particulate fraction of bacteroids has a shoulder at 422 nm (Fig. 2, spectrum 2). Most but not all of the strains had this characteristic. No similar shoulder was observed in cultured cells except for USDA 110 and 115. It is not likely to be due to P-422 since P-422 was not found in the soluble fraction of bacteroids of most strains. The nature of P-420 is obscure.

Appleby (1, 2) identified a pigment (P-428) present in small amounts in the soluble fraction of bacteroid and cultured cell extracts. This pigment was not further characterized nor mentioned in a subsequent publication (11). In our experiments, a very small shoulder in the (reduced plus CO) minus reduced difference spectra at 428 to 430 nm was observed in the soluble extract of three strains: 505, 5633, and USDA 110. It was present in such small amounts that characterization would be difficult.

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