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Tetrapyrrole Utilization by *Bacteroides ruminicola*[1]

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Reduced versus oxidized difference spectra of whole cells and pyridine hemochromogens of heme-requiring isolates of *Bacteroides ruminicola* are altered when deuteroporphyrin or mesoporphyrin replaces protoheme as a growth factor. During growth in the presence of either deuteroporphyrin or mesoporphyrin, whole cells exhibit peaks at 545 to 547, 515 to 518, and 412 to 413 nm. Pyridine hemochromogen spectra confirm the presence of meso- or deuteroheme in cells grown in the presence of meso- or deuteroporphyrin. No evidence was found for the conversion of either meso- or deuteroporphyrin to protoheme. Cells grown in the presence of the manganese or magnesium chelates of protoheme form iron-containing hemes. Neither spontaneous decomposition of noniron metalloporphyrin chelates nor spontaneous formation of hemes from Fe^{2+} and metal-free porphyrins was detected. Protoheme-synthesizing isolates of *B. ruminicola* fail to use preformed metal-free porphyrins, but form both protoheme- and deuteroheme-containing cytochromes when grown in the presence of manganese deuteroheme. Versatility in tetrapyrrole utilization by *B. ruminicola* appears to reflect the ability of the organism to mediate the removal of nonferrous ions and to insert Fe^{2+} into the tetrapyrrole nucleus. The organism also forms functional *b*-type cytochromes with prosthetic groups other than protoheme.

Most strains of *Bacteroides ruminicola* require small concentrations (10 to 100 nM) of protoheme or related tetrapyrrolic compounds for growth (3), but the variety of tetrapyrroles and tetrapyrrolic compounds active as growth factors exceeds that for other heme-requiring microbes thus far studied (3, 7, 11). Active compounds include iron-free protoporphyrin IX, deuteroporphyrin IX, mesoporphyrin IX, and hematoporphyrin IX, the magnesium, manganese, and zinc analogs of protoheme, and the protoheme-containing heme proteins hemoglobin, catalase, and peroxidase (3).

Heme is involved in cytochrome synthesis of *B. ruminicola* since protoheme- or protoporphyrin IX-grown cells of heme-requiring isolates (*B. ruminicola* subsp. *ruminicola*) contain a cytochrome *b* and a carbon monoxide-binding pigment spectrally similar to cytochrome *o* (16). Spectrally identical pigments are also formed by isolates that synthesize protoheme from linear precursors by apparently classical reactions (*B. ruminicola* subsp. *brevis*) (3). It is likely that protoheme-requiring isolates of *B. ruminicola* use the protoheme in hemoglobin, catalase, and peroxidase in a manner identical to that for free protoheme (3), but the manner in which these organisms use noniron metalloporphyrin chelates and "nonbiological" iron-free porphyrins is not yet understood. This study shows that the ability of protoheme-requiring isolates of *B. ruminicola* to utilize noniron metalloporphyrin chelates results from the removal of nonferrous ions and their replacement by iron. The ability of the organism to use iron-free meso- and deuteroporphyrin results, at least in part, from the formation of functional *b*-type cytochromes with meso- or deuteroheme in place of protoheme. The *b*-type cytochrome in isolates that synthesize protoheme from linear precursors is unaffected by the presence of preformed metal-free porphyrins, but, in the presence of manganese deuteroheme, heme-independent isolates form both protoheme- and deuteroheme-containing *b*-type cytochromes. Metalloporphyrin chelates appear to influence the utilization of tetrapyrroles by *B. ruminocola*. The organism appears to be a useful model for the study of heme and cytochrome synthesis in bacteria.

MATERIALS AND METHODS

Organisms, media, and growth conditions. The organisms studied were protoheme-requiring *B. ruminicola* subsp. *ruminicola* GA20 and heme-synthesizing *B. ruminicola* subsp. *brevis* GA33. Strain

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GA20 was chosen as a representative of heme-requiring isolates in preference to the type strain (strain 23) because of its superior pelleting characteristics during harvesting of batch cultures. The organisms were grown in 4-liter thick-walled Erlenmeyer flasks, using the Hungate anaerobic technique (9) and the medium of Caldwell and Arcand (2) with protoheme and resazurin deleted. Appropriate hemes, metalloporphyrins, or porphyrins, all of greater than 95% (wt/wt) purity as determined by spectral evidence and obtained from Porphyrin Products (Logan, Utah), were added to media from stock solutions prepared in 0.1 M KOH–47.5% (vol/vol) ethyl alcohol as previously described (3). The concentration of tetrapyrrole compounds used varied slightly between experiments, but was between 100 and 200 nM. This concentration range supported abundant growth (optical density $\geqq$ 1.0; 1-cm light path) of all of the protoheme-requiring isolates.

Cell harvesting. Cells from stationary-phase cultures, as determined by optical density measurements, were harvested by centrifugation at 4°C for 20 to 40 min at 13,200 × *g*. Pelleted cells were washed twice in 50 mM phosphate buffer at pH 7.0 and suspended in 10- to 20-fold concentrations of the original. Concentrated cell suspensions contained between 2 and 25 mg of protein per ml as determined by the use of the methods and biuret reagent of Koch and Putnam (10). Harvested washed cells were stored in black rubber-stoppered Erlenmeyer flasks at −70°C under H_2. Storage under these conditions allowed the preservation of cytochromes for extended periods of time.

Difference spectra. Difference spectra were determined with a Beckman model DBG split-beam spectrophotometer equipped with a scale expansion accessory and a 25.4-cm linear logarithmic recorder. Manual slit settings were used to allow sufficient light to pass through the concentrated cell suspensions, but the peak locations obtained under these conditions were identical to those previously obtained with more optically exacting instruments (16). In many cases, spectra detection and the precise location of peaks required the use of the scale-expansion accessory.

Typical spectra were determined by placing 3.0-ml amounts of concentrated cell suspension into each of two glass-stoppered quartz cuvettes (1-cm light path). The cells in one cuvette were reduced by the addition of small amounts of ($\leqq$10 mg) of sodium dithionite and gassing under H_2. Oxidized cells were obtained by the addition of 0.05-ml amounts of NaOCl solution to a final concentration of 2.3 mM. Spectra were recorded by using the oxidized sample as a reference. Spectra of each type were determined at least three times.

Pyridine hemochromogens. Pyridine hemochromogens were determined as described by Falk (4). Amounts (4.2 ml) of washed concentrated cells were combined with 1.0 ml of pyridine and, after incubation at an ambient temperature for 5 to 10 min, 0.5-ml amounts of 1 N NaOH were added. Subsamples (2.8 ml) of the resulting mixture were rapidly transferred to glass-stoppered cuvettes. Hemochromogen reduction was accomplished by the addition of small quantities ($\leqq$3 mg) of sodium dithionite. Oxidized hemochromogens were obtained by the addition of 0.05-ml amounts of 3 mM aqueous $K_3Fe(CN)_6$ to give a final concentration of 53 μM.

RESULTS

Figure 1 shows typical spectra obtained when strain GA20 was grown in the presence of protoporphyrin IX, manganese protoheme, or iron-free deuteroporphyrin IX. Cells grown in the presence of protoporphyrin IX displayed a protoheme-containing *b*-type cytochrome with absorption peaks at 556 to 558 and 525 to 530 nm and a soret peak between 426 and 428 nm. The trough in the 450-nm region is indicative of flavoprotein. The results agree with previous results obtained for *B. ruminicola* subsp. *ruminicola* strain 23 (16). The spectrum obtained with cells grown in the presence of manganese protoheme was identical, with respect to peak locations, to that obtained for cells grown in the presence of protoporphyrin IX. Figure 2 shows

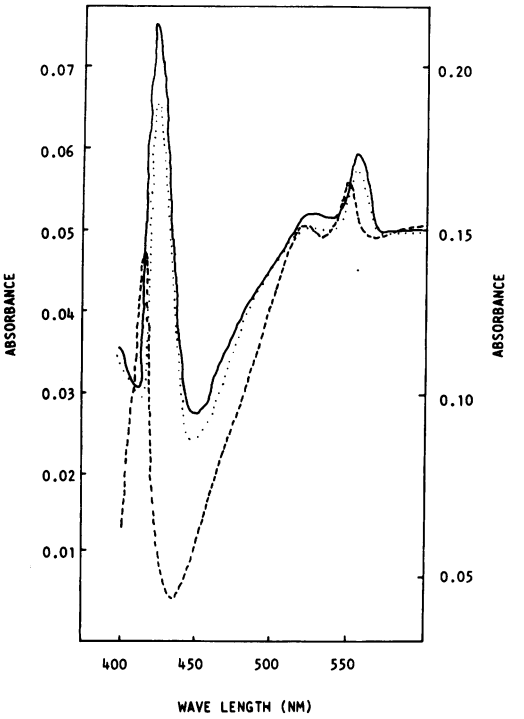

FIG. 1. *Difference spectra of B. ruminicola subsp. ruminicola GA20 grown in the presence of iron-free protoporphyrin* (——), *iron-free deuteroporphyrin* (-----), *and manganese protoheme* (.....). *The right-hand scale applies to protoporphyrin-grown cells, whereas the left-hand scale applies to the remaining spectra. The protoporphyrin cell suspension contained 22.0 mg of protein per ml, whereas the deuteroporphyrin and manganese protoheme suspensions contained 25.5 and 13.5 mg of protein per ml.*

the typical spectra obtained when sterile manganese protoheme was aseptically added to uninoculated medium and incubated for 72 h under conditions identical to those used with inoculated cultures. The spectrum for manganese protoheme immediately after the addition to medium and that obtained for 72 h, a time period that allows abundant growth of heme-requiring isolates of *B. ruminicola*, were essentially identical, indicating that little, if any, spontaneous breakdown of manganese protoheme occurs simply by the incubation of manganese protoheme at 37°C. These results combined with the spectrum of GA20 grown in the presence of manganese protoheme (Fig. 1) indicate that manganese protoheme utilization by strain GA20 involves organism-mediated manganese ion removal from the tetrapyrrole nucleus and insertion of iron (Fe^{2+}). Previous work has shown that, under the conditions used, spontaneous formation of hemes from Fe^{2+} and iron-free porphyrins does not occur (3).

Spectra identical, with regard to peak location, to those obtained with manganese protoheme were also observed for magnesium protoheme and zinc protoheme. Cells of strain GA20 grown under these conditions formed a protoheme-containing *b*-type cytochrome. As measured by peak height and area, no spontaneous degradation of magnesium protoheme occurred during a 72-h incubation period, but a substantial decomposition of zinc protoheme (approximately 23%) occurred under identical conditions. Mg^{2+} removal from tetrapyrroles is also apparently mediated by *B. ruminicola*, but it is likely that Zn^{2+} removal does not obligatorily require the organism.

The absorption peaks were shifted in cells of GA20 grown in the presence of deuteroporphyrin IX compared with those obtained with protoporphyrin IX-grown cells (Fig. 1). Peaks were observed at 545 to 547 and 515 to 518 nm, and a soret peak was observed at 412 to 413 nm. The results are those expected if the organism formed a deuteroheme-containing *b*-type cytochrome; i.e., the alpha and beta peaks are shifted as expected for deuteroheme, and the soret peak is shifted downward toward 400 nm. Identical spectra were obtained with cells grown in the presence of manganese deuteroheme or mesoporphyrin IX; i.e., peaks were obtained at 545 to 547 and 515 to 518 nm, and a soret peak was obtained at 412 to 413 nm.

Pyridine hemochromogen spectra of protoporphyrin- and deuteroporphyrin-grown cells of GA20 are shown in Fig. 3. Protoporphyrin-grown cells exhibited peaks at 557, 520, and 418 to 419 nm, whereas deuteroporphyrin-grown cells have pyridine hemochromogen spectra with peaks at 545 to 547, 512 to 513, and 406 to 407 nm.

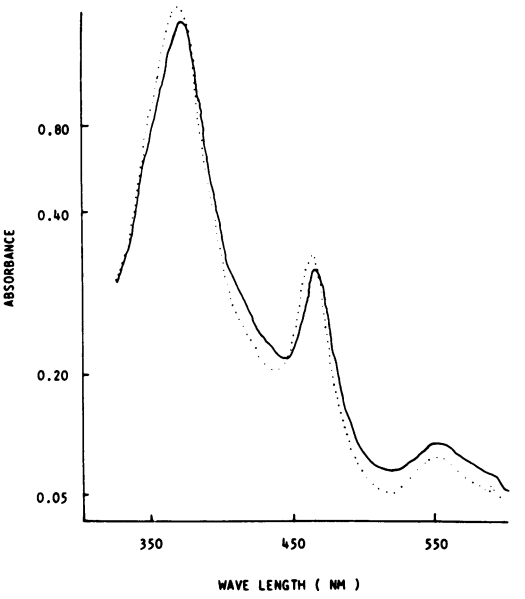

FIG. 2. *Spectra of manganese protoheme before (.....) and after (——) incubation for 72 h at 37°C in sterile uninoculated medium.*

Isolates of *B. ruminicola* subsp. *brevis* synthesize protoheme from linear precursors (3). However, it seemed possible that these isolates might use preformed porphyrins if they were supplied. To test this possibility, protoheme-synthesizing strain GA33 was grown in a medium devoid of tetrapyrroles, in a medium containing iron-free deuteroporphyrin, and in a medium containing manganese deuteroheme. Typical spectra obtained are shown in Fig. 4. The ability of the organism to synthesize protoheme from linear precursors is confirmed by the presence of a *b*-type cytochrome in cells grown without tetrapyrroles. The organism fails to use preformed metal-free porphyrins since the spectrum obtained with deuteroporphyrin-grown cells was identical to that for cells grown without tetrapyrroles. However, in the presence of manganese deuteroheme, GA33 forms two types of cytochromes, a protoheme type with a shoulder at 556 to 558 nm and a soret peak at 428 nm and a deuteroheme type with a peak at 545 to 547 nm and a soret peak at about 412 to 415 nm. Pyridine hemochromogen spectra confirm the presence of two cytochromes. In the alpha peak region, only one major peak at 558 nm is formed in the presence of deuteroporphyrin, whereas an additional peak at 545 to 547 nm is formed in manganese

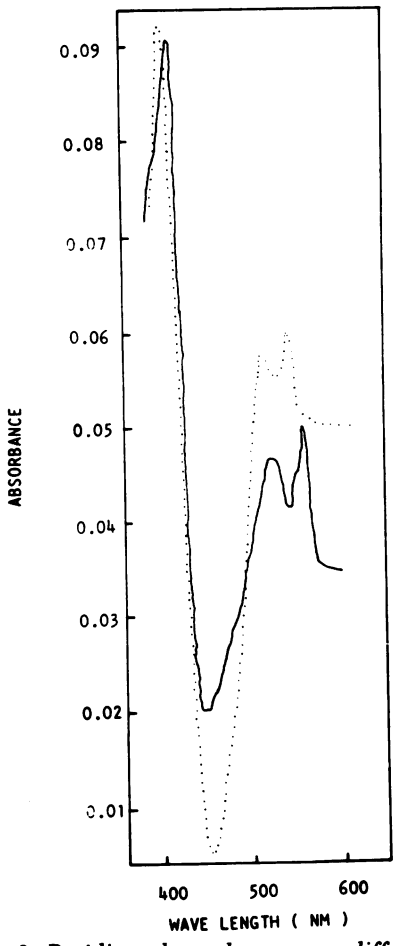

FIG. 3. *Pyridine hemochromogen difference spectra of B. ruminicola subsp. ruminicola GA20 grown in the presence of iron-free protoporphyrin* (——) *and iron-free deuteroporphyrin* (.....). *The cell suspension protein concentrations were the same as those indicated in the legend to Fig. 1.*

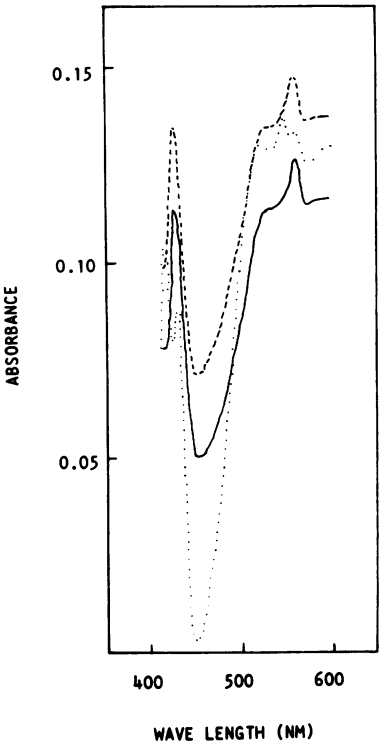

FIG. 4. *Difference spectra of protoheme-synthesizing B. ruminicola subsp. brevis GA33 grown without porphyrins* (——), *in the presence of metal-free deuteroporphyrin* (------), *and in the presence of manganese deuteroheme* (.....). *The cell suspension without porphyrin contained 7.9 mg of protein per ml, whereas the deuteroporphyrin and manganese deuteroheme suspensions contained 14.7 and 7.9 mg/ml.*

deuteroheme-grown cells (Fig. 5). The detection of the soret peaks of the hemochromogens was made difficult by the presence of large amounts of absorption in the soret region of the visible spectrum that was not due to cytochromes. However (data not shown), a peak at 419 to 420 nm could be detected in deuteroporphyrin-grown cells, and very small shoulders could be detected at 419 to 420 and 405 to 406 nm in manganese deuteroporphyrin-grown cells. The manganese-deuteroporphyrin chelate apparently exerts a regulatory effect on the uptake or utilization of deuteroporphyrin for deuteroheme and deuteroheme-containing cytochrome synthesis by *B. ruminicola* isolates that synthesize protoheme from linear precursors.

The functional nature of the *b*-type cytochromes formed by *B. ruminicola* in the presence of deuteroporphyrin was demonstrated by the finding that the cytochromes formed under these conditions were readily reduced by glucose when the spectrum of cells in the presence of 460 μM glucose under H_2 was compared with that obtained with cells maintained under H_2 alone. Also, the addition of 1 mM quinine to cells in the presence of glucose led to troughs at 547 and 412 nm and peaks at about 450 and 340 nm. These results are exactly those expected if quinine addition led to cytochrome and flavoprotein oxidation and the accumulation of reduced nicotinamide adenine dinucleotide. Analogous results were obtained with heme-independent strain GA33 grown in the presence of manganese deuteroheme. Double alpha peaks as well as double soret peaks were obtained with cells incubated with glucose under H_2 compared with cells under H_2 alone. A trough was observed for flavoprotein at 450 nm. The addition of quinine to cells in the presence

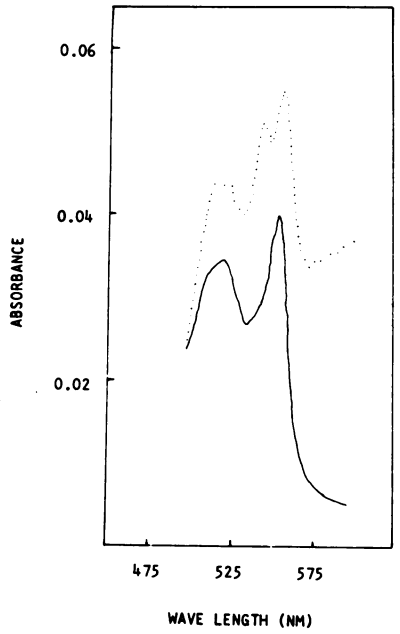

FIG. 5. *Pyridine hemochromogen difference spectra of B. ruminicola subsp. brevis GA33 grown in the presence of metal-free deuteroporphyrin (——) and manganese deuteroheme (.....). The cell suspension protein concentrations were the same as those indicated in the legend to Fig. 4.*

of glucose led to troughs at 557, 545, 428, and 412 nm and a peak at about 450 nm. Large increases in 340-nm absorption were also observed.

DISCUSSION

B. ruminicola is extremely versatile in the use of tetrapyrroles during growth. The ability of the organism to use a diversity of porphyrins and noniron metalloporphyrins reflects the ability of the organism to form cytochromes with unusual prosthetic groups. There is no evidence to suggest that the unusual tetrapyrroles are converted to protoheme. The only detectable cytochromes in heme-requiring isolates grown with unusual porphyrins or metalloporphyrins are those expected from the use of unusual tetrapyrroles.

The growth of heme-requiring isolates in the presence of unusual tetrapyrroles is reflective of the use of the unusual tetrapyrroles and not of the use of small amounts of contaminating porphyrins or metalloporphyrins, e.g., protoporphyrin or protoheme. If the latter had been present and used to any extent, protoheme-containing cytochromes would have been found in cells grown in the presence of metal-free deuteroporphyrin or mesoporphyrin. Furthermore, in most cases, the purity of the compounds used (95 to 98% by weight) precludes substantial growth due to contaminating tetrapyrroles if, in fact, growth would have occurred at all (3).

The functional nature of the unusual cytochromes in heme-requiring and heme-independent isolates of *B. ruminicola* grown in the presence of unusual tetrapyrroles is indicated by the fact that the unusual cytochromes are reduced by glucose metabolism and that quinine, an inhibitor of electron flow, interferes with electron flow in *B. ruminicola*. It is of special interest that both cytochromes are reduced by glucose and reoxidized by quinine in the case of protoheme-synthesizing strain GA33 grown in the presence of manganese deuteroheme. Under these conditions, both the protoheme-containing and deuteroheme-containing cytochromes are functional simultaneously.

The ability of *B. ruminicola* to use a wide variety of noniron metalloporphyrins reflects the ability of the organism to mediate metal ion removal and to insert iron, but the mechanisms by which these processes are accomplished remain to be determined. It is possible, although unlikely, that the metals are removed non-specifically as the result of poising of the medium E_h by the metabolism of the organism. This possibility appears unlikely since it is well documented that Mn^{2+} is removed from the tetrapyrrole nucleus only with great difficulty (14). An additional possibility is that the metalloporphyrins are reduced to porphyrinogens (4; Bruce F. Burnham, personal communication), but this possibility is also somewhat unlikely since previous work shows that coproporphyrinogen and uroporphyrinogen are growth factors for heme-requiring isolates, whereas both coproporphyrin and uroporphyrin are inactive (3). *B. ruminicola* may lack the ability to reduce porphyrins to porphyrinogens, unless protoporphyrin, deuteroporphyrin, and mesoporphyrin are handled differently from coproporphyrin and uroporphyrin (3). A third intriguing possibility is that *B. ruminicola* contains unusual metalloporphyrin chelatases. Further study is required to determine the precise mechanisms for the removal of nonferrous ions and the insertion of Fe^{2+}.

To our knowledge, this study is the first report of functional *b*-type cytochromes in bacteria with prosthetic groups other than protoheme, although various functionally active non-protoheme-containing heme proteins have been prepared from other sources (1, 6, 13, 15, 17). It would be interesting to isolate cytochromes formed by *B. ruminicola* in the presence of deuteroporphyrin and mesoporphyrin

and to compare their properties with those of protoheme-grown cells.

The formation of both deuteroheme-containing and protoheme-containing cytochromes by strain GA33 in the presence of manganese deuteroheme compared with the formation of only a protoheme cytochrome *b* when the organism is grown in metal-free deuteroporphyrin indicates that the Mn^{2+} tetrapyrrole chelate influences the use of the deuteroporphyrin ring structure for heme and cytochrome synthesis in isolates that synthesize protoheme from linear precursors. It is possible that the presence of Mn^{2+} in the tetrapyrrole nucleus influences tetrapyrrole uptake. It is also possible that synthetic mechanisms are influenced. We are currently initiating studies to determine whether other metalloporphyrin chelates with deuteroporphyrin and other non-biological tetrapyrroles exert similar effects and also to determine whether other rumen microbes that contain *b*-type cytochromes are similarly affected. It is likely that the metalloporphyrin chelates or a metabolite(s) formed from them is an active rather than the free metal ion since free Mn^{2+} ions were present in significant quantities in all media.

Reports of microbes known to require heme are relatively rare (2, 3, 4, 11), and studies of the modes of utilization of heme-replacing substances are even scarcer (3, 7, 8). It would be interesting to study the specificity for tetrapyrroles and their mode of utilization by *B. ruminicola* in parallel with other heme-requiring organisms. *Haemophilus influenzae* (7, 8), *Corynebacterium diphtheriae* (12), *B. fragilis* (3), and *B. melaninogenicus* (5) appear especially useful for study. It is highly likely that *B. ruminicola* may serve as an excellent model for the study of heme and cytochrome synthesis and their control in bacteria and other life forms.

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