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Requirement of Heme for Growth of *Bacteroides fragilis*

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Heme or protoporphyrin IX was required for growth of *Bacteroides fragilis* in a defined medium. The amount of heme necessary for half-maximal growth was 2 to 10 ng/ml (3.8 to 15 pmol/ml) among the *Bacteroides* species and strains tested. The growth rate, metabolic products from glucose fermentation, and cell yields were affected by the concentration of heme in the medium and by the length of time the culture was incubated. When heme was growth limiting (4 ng/ml), growth rates decreased by 50%, cultures started producing lactic and fumaric acids, and the cell yields declined. The cell yield for *B. fragilis* (ATCC 25285) at 24 h in medium containing 6.5 μg of heme per ml was 69 g (dry weight) of cells per mol of glucose compared to 16 g (dry weight) of cells per mol of glucose with 4 ng of heme per ml. *B. fragilis* was unable to grow in defined medium when a porphyrin precursor, δ-aminolevulenic acid or porphobilinogen, was added in place of heme.

Bacteroides fragilis, a gram-negative nonsporing rod, is the anaerobe most frequently isolated from clinical specimens (10). There has been some controversy over the necessity of adding heme to the growth medium for *B. fragilis*. In 1966, Quinto concluded that *Ristella pseudoinsolita* (*B. fragilis*) required heme (8). Varel and Bryant (11) included heme in their minimal medium with Casitone for growing *B. fragilis*; however, they said it was highly stimulatory but not required for growth. Varel and Bryant (11) did refer to unpublished work of D. R. Caldwell, which showed that many strains of *B. fragilis* failed to grow in this medium when Casitone was replaced with cysteine unless heme was added. More recently, Macy et al. (6) reported (in their study of the involvement of cytochromes in fumarate reduction by *B. fragilis*) that growth of the organism was stimulated by adding heme to a defined medium, but grew slowly in its absence, and produced fumarate and lactate instead of succinate. Work in our laboratory agreed with the major conclusions of Macy et al. (6) concerning the pathways for energy production in *B. fragilis*, but we found that heme was an absolute requirement for growth. *B. fragilis* could not be serially transferred in media carefully prepared to be free of all traces of heme. The work presented here was initiated to establish the heme requirement for growth of *B. fragilis* and to determine the quantitative level of this requirement.

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MATERIALS AND METHODS

Bacterial strains. Two strains of *B. fragilis* were used for most of the experiments, the reference strain (ATCC 25285) and the strain (ATCC 23745) used by Macy et al. (6). A subculture was obtained directly from Joan Macy, and the strain (ATCC 23745) was also obtained from the American Type Culture Collection. Reference strains of the *Bacteroides* deoxyribonucleic acid homology groups, some of which have now been elevated to species (2), were obtained from J. L. Johnson. Stock cultures were maintained under O_2-free CO_2 at room temperature in chopped-meat broth (4).

The inoculum for assaying the *B. fragilis* heme requirement was prepared by inoculating 10 ml of heme-free defined medium with 1 drop (0.03 to 0.05 ml) of a stock chopped-meat culture and incubating for 24 h at 37°C. Tubes containing different concentrations of heme in 10 ml of defined medium were inoculated with 1 drop (0.03 to 0.05 ml) of the first transfer into heme-free media.

Medium preparation. Special precautions were taken to assure that residual heme would not interfere with these experiments. Glassware was autoclaved, in a 10% (vol/vol) solution of 7X (Linbro Scientific Inc., Hamden, Conn.) for 15 min at 121°C, and rinsed in distilled water. Pipettes and glassware were also heated for 4 h at 450°C in an asher (Wilt-Laboratory Glass Blowing Inc., Asher, Latham, N.Y.). Only new rubber stoppers and rubber tubing were used, since we found that rubber stoppers used previously with any complex medium retained sufficient heme for growth of *B. fragilis*.

The defined medium was a modification of that of Varel and Bryant (11); it contained per liter: glucose, 5 g; $(NH_4)_2SO_4$, 1 g; KH_2PO_4, 0.9 g; NaCl, 0.9 g; $CaCl_2 \cdot 2H_2O$, 26.5 mg; $MgCl_2 \cdot 6H_2O$, 20 mg; $MnCl_2 \cdot 4H_2O$, 10 mg; $FeSO_4 \cdot 7H_2O$, 4 mg; $CoCl_2 \cdot 6H_2O$, 1 mg; resazurin, 1 mg; vitamin B_{12}, 10 μg; and the

pH was adjusted to 6.5 with NaOH. The medium was sterilized under O_2-free N_2 (121°C for 17 min). Solutions of cysteine-HCl (2.5%, wt/vol) and Na_2CO_3 (8%, wt/vol) were sterilized (121°C for 15 min) separately. Additions of 50 ml of Na_2CO_3 solution and 20 ml of cysteine solution were made aseptically under O_2-free CO_2.

The heme solution was prepared by adding 12.6 mg of hemin (Sigma Chemical Co., St. Louis, Mo.) to 40 ml of 0.01 M NaOH, and this suspension was stirred for 2 h. The undissolved heme was removed, and the solution was sterilized by membrane filtration (Millipore Corp., 0.22-μm pores). The final concentration of the heme solution was determined by preparation of a pyridine ferrochrome from a dilution of stock solution in NaOH (0.075 M, final concentration)-pyridine (25%, vol/vol, final concentration). The dithionite-reduced versus air-oxidized spectrum was observed, and the concentration of heme was calculated from absorbance at 557 − 540 nm ($\Delta A_{557-540}$), using $\epsilon = 20.7\ mM^{-1}\ cm^{-1}$ (3).

Porphobilinogen was dissolved in 1 N KOH, and δ-aminolevulenic acid was dissolved in distilled water. The solutions were filter sterilized (Millipore, 0.22-μm pores) and added aseptically to the defined medium. Protoporphyrin IX dimethyl ester was first hydrolyzed by refluxing in 1% (wt/vol) KOH in methanol (3) and was then diluted in sterile distilled water and added aseptically to the defined medium.

Growth rates and cell viability. Growth rates were estimated from the optical density at 650 nm (OD_{650}) of the cells. The percentage of viable cells was determined for strains ATCC 25285 and ATCC 23745 by diluting 24- and 48-h cultures in anaerobic dilution blanks (4) and plating the dilutions on brain heart infusion agar plates (containing 5 μg of heme per ml), which were incubated 48 h at 37°C in an anaerobic chamber.

Dry-weight determination. Twenty milliliters of culture was centrifuged at 40,000 × g for 15 min. The supernatant was carefully removed by aspiration and kept at −10°C until used for the determination of residual glucose and accumulation of acid products. The cells were washed twice with 20-ml portions of distilled water by centrifuging at 40,000 × g for 15 min. Pellets were suspended in 2 to 3 ml of distilled water and placed in tared petri dishes. The cells were then dried at 100°C to constant weight.

Glucose determination. Glucose was determined by the Glucostat (Worthington Biochemicals Corp., Freehold, N.J.) semimicro procedure.

Chromatography. Acid end products were determined by gas-liquid chromatography, using a Hewlett-Packard 5830A gas chromatograph with a flame ionization detector. Volatile fatty acids, extracted into ether according to Holdeman and Moore (4), were quantitated on a column (2 mm by 2 m) of 15% SP-1220/1% H_3PO_4 on acid-washed 100/200-mesh Chromosorb W (Supelco Inc., Bellefont, Pa.) with valeric acid as the internal recovery standard. Nonvolatile acids were quantitated as their methyl esters, prepared according to Holdeman and Moore (4). Methyl esters were separated on a column (4 mm by 2 m) of 25% Resoflex LAC-1-R-296 on acid-washed 30/60-mesh Chromosorb W (Supelco Inc.) with malonic acid as the internal standard.

RESULTS

Absolute heme requirement. The following reference strains did not grow when transferred serially in the minimal medium without heme: *B. fragilis* (ATCC 25285), *Bacteroides distasonis* (ATCC 8503), *Bacteroides eggerthii* (VPI B8-51), *Bacteroides thetaiotaomicron* (ATCC 29184), *Bacteroides vulgatus* (ATCC 8482), and *Bacteroides ovatus* (ATCC 8483). On the first transfer to heme-free medium, the organisms grew in 24 h to an optical density of approximately 1.0 (650 nm, 18-mm light path, Bausch & Lomb Spectronic 20). However, on the second transfer to heme-free medium, none grew. Bacteria in the first transfer remained viable, since they could be subcultured in medium containing heme. We obtained the same results with the strain (ATCC 23745) used by Macy et al. (6) as well as with a direct subculture provided by J. Macy.

We then determined the level of heme required for half-maximal growth. Plots of the optical density versus the log of the heme concentrations in the medium (Fig. 1 and 2) gave a definite relationship between the heme concentrations and optical density to the point when the cultures were reaching maximum turbidity. There was no further increase in optical density after 48 h, even after prolonged (6 days) incubation. The level required for half-maximal growth of *B. fragilis* ATCC 25285 was approximately 4 ng of heme per ml. Other species and strains of *Bacteroides* had similar requirements for half-maximal growth, from 2 to 10 ng of heme per ml.

End products of metabolism. We deter-

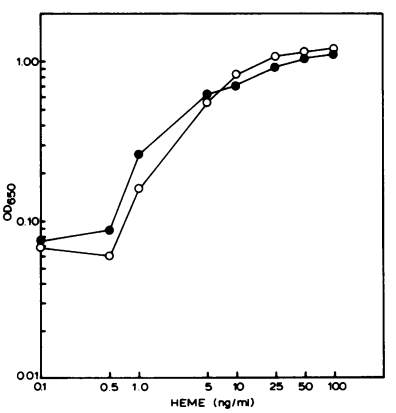

FIG. 1. *Growth (OD_{650}) of B. fragilis ATCC 25285 in defined medium containing various concentrations of heme at 24* (○) *and 48* (●) *h of incubation.*

mined the acids produced by the reference strain of *B. fragilis* (ATCC 25285) and strain ATCC 23745 when grown at a high-heme (6.5 μg/ml) and a low-heme (4 ng/ml) concentration in the defined medium containing 0.5% (wt/vol) glucose as carbon and energy source. Since the metabolic products of *B. fragilis* vary according to the length of incubation (7), they were determined at both 24 and 48 h. The relative production of acetic acid was slightly greater in the high-heme cultures (Table 1). The concentration of propionic acid was similar in all cultures at 24 h, but a definite increase was seen in high-heme cultures at 48 h. Lactic acid was detected in low-heme cultures, with more accumulating after 48 h. Fumaric acid also was detected only in low-heme cultures, and generally only after the extended period of incubation. More succinic acid was produced by high-heme cultures.

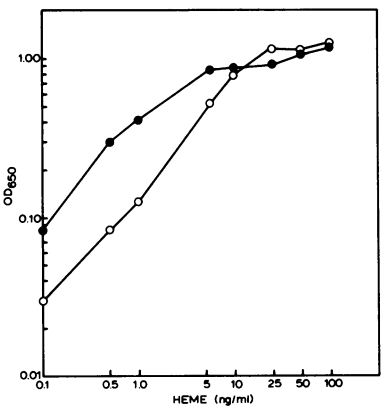

FIG. 2. *Growth (OD_{650}) of B. fragilis ATCC 23745 in defined medium containing various concentrations of heme at 24 (○) and 48 (●) h of incubation.*

Cell yields. The cell yields of two strains of *B. fragilis* grown at two different heme concentrations for 24 and 48 h are listed in Table 2. Cell yields were highest at 24 h, except for low-heme cultures of strain ATCC 25285. The most dramatic difference was between the 24-h low- and high-heme cultures of strain ATCC 25285, which gave values of 16 g (dry weight) of cells per mol of glucose and 69 g (dry weight) of cells per mol of glucose, respectively. Some of

TABLE 2. *Cell yields and adenosine 5'-triphosphate per mole of glucose fermented by B. fragilis grown for 24 and 48 h in defined medium with high- and low-heme concentrations*

Strain	Time (h)	Heme[a]	$Y_{glucose}$[b]	ATP/glucose[c]
25285	24	LH	16 ± 0.5	1.5
25285	24	HH	69 ± 18.0	6.6
23745	24	LH	42 ± 13.0	4.0
23745	24	HH	67 ± 4.0	6.3
25285	48	LH	31 ± 6.2	2.9
25285	48	HH	47 ± 4.8	4.5
23745	48	LH	32 ± 0.4	3.1
23745	48	HH	42 ± 0.3	4.0

[a] LH is 4 ng of heme and HH is 6.5 μg of heme per ml.
[b] Grams (dry weight) of cells per mole of glucose.
[c] Moles of adenosine 5'-triphosphate (ATP) per mole of glucose fermented calculated by dividing $Y_{glucose}$ by 10.5 (the standard Y_{ATP} value of cells).

TABLE 1. *Fermentation products of B. fragilis at 24 and 48 h of incubation in medium with either 6.5 μg (HH) or 4 ng (LH) of heme per ml*[a]

Product	ATCC 25285		ATCC 23745		ATCC 23745 (Macy et al. [6], expt 1)[b]	
	LH	HH	LH	HH	−Heme	+2 μg of heme per ml
24 h						
Acetate	40.0 ± 13.0	52.7 ± 5.6	41.4 ± 11.0	50.1 ± 2.0	29.8	40.9
Propionate	11.0 ± 3.9	13.3 ± 4.7	6.9 ± 2.5	12.9 ± 0.1	0.0	22.6
Lactate	1.4 ± 2.5	0.0	9.9 ± 6.2	0.0	68.6	7.0
Fumarate	0.0	0.0	0.0	0.0	97.8	2.7
Succinate	33.0 ± 13.0	46.0 ± 7.7	23.5 ± 5.2	41.5 ± 4.8	6.0	36.0
48 h						
Acetate	42.4 ± 8.2	58.1 ± 4.8	45.3 ± 10.0	60.7 ± 6.9		
Propionate	8.1 ± 2.7	24.0 ± 4.0	8.1 ± 6.8	23.4 ± 5.5		
Lactate	10.0 ± 5.3	0.0	23.4 ± 8.2	0.0		
Fumarate	9.1 ± 3.7	0.0	4.4 ± 4.4	0.0		
Succinate	21.3 ± 1.7	38.9 ± 3.4	22.5 ± 3.4	41.4 ± 5.2		

[a] Results are given in moles per 100 mol of glucose.
[b] Macy et al. did not present results as products of time.

the variance in the cell yields (Table 2) may be due to differences in the growth rates observed with the different concentrations of heme. The growth rate of strain ATCC 25285 with high heme was 0.38 generation per hour, whereas with low heme it was 0.24 generation per hour. The growth rate of strain ATCC 23745 with high heme was 0.5 generation per hour, and it was 0.31 generation per hour in low heme. The cells grown in medium containing high or low heme also died quickly after 24 h in the defined medium, with 20 to 99% of the cells dead at 48 h.

Porphyrin biosynthetic intermediates. δ-Aminolevulenic acid and porphobilinogen failed as replacements for heme in the defined medium. Protoporphyrin IX, however, did replace heme in the medium. Growth with protoporphyrin IX was comparable to that achieved with similar concentrations of heme.

DISCUSSION

Our data support most of the conclusions made by Macy et al. (6), but we do not agree that *B. fragilis* can grow without heme. They reported that *B. fragilis* did not require heme in the medium for growth and that glucose was metabolized via a fumarate–lactate-type fermentation when the organism was grown in medium lacking heme. We found that both a subculture of the organism used by Macy et al. (6) as well as the same collection strain (ATCC 23745) required heme for growth. The reference strain (ATCC 25285) for *B. fragilis* also required heme for growth, as did other strains previously classified as subspecies of *B. fragilis*. There were differences in growth responses to the amount of heme in the medium and the period of incubation for *B. fragilis* strains ATCC 23745 and ATCC 25285. Comparison of Fig. 1 and 2 suggests to us that ATCC 23745 may have a higher affinity for heme than ATCC 25285, and carry-over heme in the inoculum may have affected growth at the very low concentrations of heme in Fig. 1.

All traces of heme adhering to our glassware were eliminated by ashing, and only new rubber stoppers were used, eliminating the other major source of heme contamination. Possibly the reason Macy et al. (6) obtained growth without adding heme to the medium was contamination of their equipment. Rizza et al. (9) found that the concentration of protoheme in *Bacteroides melaninogenicus* grown on blood agar plates approached 43% of the dry weight of the cell, and, even though the organism required heme for growth, accumulated protoheme in the cells could support growth of the bacteria for at least eight generations. It seems possible that some of the initial growth of *B. fragilis* in heme medium by Macy et al. (6) was also due to residual heme in the cells. The fermentation products reported by Macy et al. (6) included 4.6 mol of succinate per 100 mol of glucose, which was about 15% of the amount of succinate reported for *B. fragilis* fermenting glucose in heme-containing medium. This is inconsistent with their statement that the cells were growing in the absence of heme, since they also stated that heme was required for the synthesis of a functional fumarate reductase. The other differences in fermentation products between our results with low-heme cultures and theirs in −hemin medium might be explained by the fact that fermentation of glucose does not require growing cells. Our results do tend to agree with the conclusion of Macy et al. (6) that pyruvate acts as an electron acceptor under low-heme concentrations, and, if we used even lower concentrations of heme in the medium, the levels of lactate and fumarate probably would increase and agree more with their results.

We obtained cell yields of 69 g (dry weight) of cells per mol of glucose for strain ATCC 25285 and 67 g (dry weight) of cells per mol of glucose for strain ATCC 23745 at 24 h in high-heme medium. These values were very close to the value of 66 g (dry weight) of cells per mol of glucose obtained for *Bacteroides ruminicola* by Howlett et al. (5). Our values decreased by one-third when the cells were incubated for another 24 h. Most or all of the glucose was exhausted in the first 24 h, followed by a large loss in cell viability; thus, the decrease in cell yields may have been due to cell lysis. Smaller cell yields were observed with low-heme cultures, especially the 24-h culture of ATCC 25285. This value (16 g [dry weight] of cells per mol of glucose) may have been due to the long generation time (4 h). The cell yields for the remaining low-heme cultures were near the theoretical maximum for substrate-level phosphorylation. The increases in cell yields and growth rates, and the change in fermentation products when the amount of heme in the medium was in excess, tend to support the hypothesis of Macy et al. (6) that cytochrome(s) and electron transport phosphorylation are involved in the glucose metabolism of *B. fragilis*.

B. fragilis was not capable of synthesizing porphyrins de novo and, like the heme-dependent strains of *B. ruminicola* (1), was unable to grow when δ-aminolevulenic acid or porphobilinogen was added as a substitute for heme. Protoporphyrin IX did replace the requirement for heme in the medium, as it did for the

heme-dependent strains of *B. ruminicola* (1). Apparently, *B. fragilis* has a ferrochelatase that inserts iron into the porphyrin ring. Thus, it appears that lack of a complete porphyrin biosynthetic pathway in *B. fragilis* necessitates the presence of heme or protoporphyrin IX in the culture medium for growth.

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