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Trophic Links between the Acetogen *Clostridium glycolicum* KHa and the Fermentative Anaerobe *Bacteroides xylanolyticus* KHb, Isolated from Hawaiian Forest Soil[▽]

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Isolate KH was obtained from Hawaiian forest soil and found to be composed of two functionally linked anaerobes, KHa and KHb. Gene analyses (16S rRNA, *fhs*, *cooS*) identified KHa as an acetogenic strain of *Clostridium glycolicum* and KHb as *Bacteroides xylanolyticus*. KHb fermented xylan and other saccharides that KHa could not utilize and formed products (e.g., ethanol and H_2) that supported the acetogenic growth of KHa.

Acetate can be the most abundant organic acid in soil extracts (9, 33). The capacity of aerated soils to form acetate is likely due to transient fermentative activities that can occur when O_2 becomes limited and might be dominated by facultative aerobes (e.g., *Enterobacteriaceae*) (6, 17, 26, 35). However, aerated soils also contain acetogenic bacteria (11, 16, 17, 24, 35), acetate-forming anaerobes that utilize the O_2-sensitive acetyl coenzyme A (acetyl-CoA) Wood-Ljungdahl pathway as a terminal electron-accepting process (7, 8). Although the occurrence of acetogens in aerated soils might be considered paradoxical, certain species of acetogens can cope with small amounts of O_2 by (i) reductive processes that remove O_2 and its toxic by-products, (ii) utilizing alternative terminal electron-accepting processes that are less sensitive to O_2 than is the acetyl-CoA pathway, or (iii) forming symbiotic associations with aerotolerant O_2-consuming anaerobes (1, 2, 3, 11, 12, 15, 18, 29). Most acetogens have been isolated from anoxic habitats, such as gastrointestinal tracts or sediments, and relatively few acetogens have been isolated from aerated soils (8). The presence of acetogens in aerated Hawaiian soils has been implicated by the H_2-dependent augmentation of acetate formation under anoxic conditions (19), and the main objective of the present study was to isolate an acetogen from Hawaiian forest soil and determine its physiological response to O_2.

Analytical procedures. Gases, organic acids, alcohols, saccharides, pH, and optical densities were measured as described elsewhere (17). The pressures of culture tubes and bottles were determined prior to each gas measurement and taken into consideration for calculating amounts of gases. Gas samples from culture tubes and bottles were analyzed, and the total amount of a gas (e.g., H_2) in a tube or bottle was determined by taking into consideration the volume of the gas phase and also the theoretic amount of the gas that was dissolved in the liquid phase (note that the amount of H_2 dissolved in the liquid phase was insignificant due to its low solubility). The micromoles of H_2 per tube or bottle were converted to millimolar of H_2 by taking into account the volume of the liquid phase, thus making it possible to compare the relative (not absolute) concentrations of all products formed from a particular substrate. For example, for a 28-ml culture tube with a 10-ml liquid phase, 50 μmol H_2 in the tube would equal 5 mM H_2 when weighted against the liquid phase. Concentrations were corrected for the changing liquid-to-volume ratio due to liquid sampling.

fhs (encoding formyltetrahydrofolate synthetase) and 16S rRNA gene sequences were amplified and evaluated according to published protocols (14) with the primers FTHFSf and FTHFSr (21) and 27f and 1492r (20), respectively. *fhs* sequences of *Sporomusa* and *Moorella* were amplified with the newly developed primers fhs610f (GTWGCHTCIGARRTIA TGGC) and fhs1249r (CYRCCYTTHGCCCANAC). Sequences of *cooS* (encodes the carbon monoxide dehydrogenase subunit of acetyl-CoA synthase) were amplified with the newly developed primers cooS805f (AARSCMCARTGTGGTTT TGG) and cooS2623rw (TTTTSTKMCATCCAYTCTGG).

Isolation of KHa and KHb. For initial enrichment, soil from Koke'e State Park (Kaua'i, HI; for site description, see reference 19) was diluted 1:10 in H_2-supplemented yeast extract medium (5) containing vitamins, minerals, and trace metals and lacking reducing agents so as to increase the likelihood of obtaining an acetogen with at least a minimal tolerance to O_2. Culture tubes were incubated horizontally at 30°C and were not shaken. The acetogenic culture KH (for Kaua'i, HI) was obtained by streaking enrichments on solidified yeast extract medium (H_2-CO_2 gas phase), transferring colonies to liquid yeast extract medium, and then restreaking two times. KH was initially thought to be a pure acetogen by its being composed of very similar-looking rods that formed short chains and occasional spores and by its ability to convert numerous substrates primarily to acetate under anoxic conditions. However, KH also formed acetate in response to xylan and raffinose, saccharides that are not normal substrates for known acetogens (8), and it was suspected that KH might contain more than one

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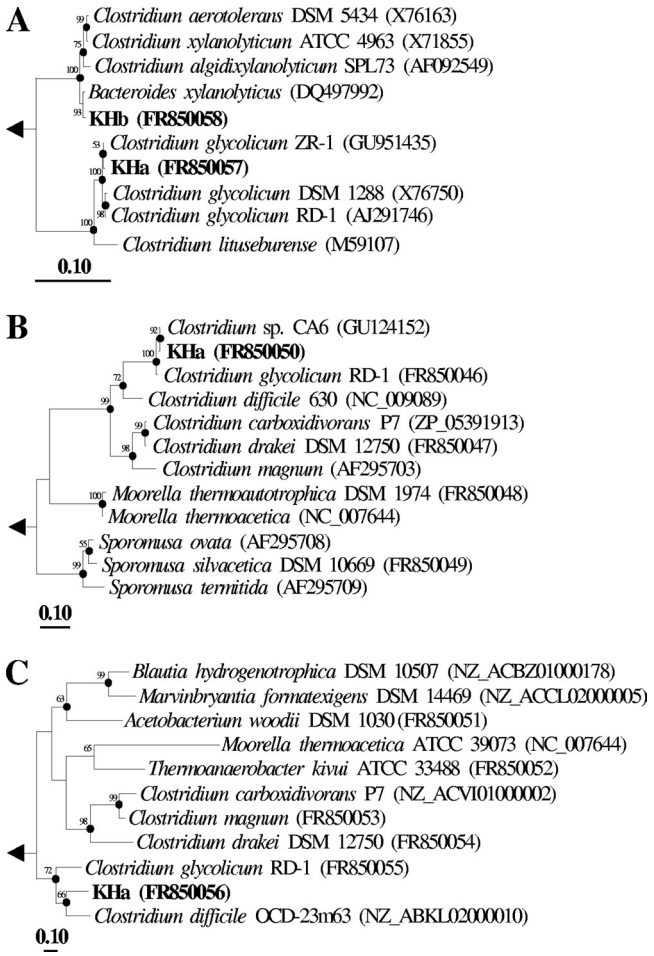

FIG. 1. Phylogenic neighbor-joining trees of 16S rRNA gene sequences of KHa, KHb, and reference sequences (A), *in silico*-translated amino acid sequences encoded by *fhs* of KHa and reference sequences (B), and *in silico*-translated amino acid sequences encoded by *cooS* of KHa and reference sequences (C). Values next to the branches represent the percentages of replicate trees (>50%) in which the associated taxa clustered together in the bootstrap test (1,000 bootstraps). Dots at nodes indicate the confirmation of tree topology by maximum likelihood and maximum parsimony calculations with the same data set. Bar indicates a 0.1 estimated change per nucleic acid or amino acid. The 16S rRNA gene sequence of *Methanopyrus kandleri* (M59932), the *fhs*-encoded amino acid sequences of *Methanocorpusculum labreanum* (CP000559), and the *cooS*-encoded amino acid sequence of *Archaeoglobus fulgidus* (NC_000917) were used as outgroups.

organism, one being an acetogen and another being a saccharide-utilizing anaerobe that formed products that could be utilized by the acetogen. KHa and KHb were then obtained from the highest growth-positive dilutions of yeast extract medium supplemented with H_2 and raffinose, respectively. KHa and KHb were then obtained from isolated colonies on solidified media; they will be made available upon request.

Morphology, physiology, and phylogeny of KHa. KHa was a spore-forming rod and tended to lyse, thus making it difficult to determine maximum optical densities. Cells were approximately 1.2 μm wide and 4 to 5.2 μm long and often occurred in pairs. The optimum temperature and pH for growth on glucose were 37°C and 8.7, respectively. Growth occurred from 15 to 37°C and pH 5.8 to 9.6 but not at 10°C and 45°C or at pH 5.5 and 9.9. H_2-CO_2, formate, ethanol, lactate, pyruvate, glucose, xylose, fructose, maltose, citrate, 1-propanol, *n*-butanol, and yeast extract supported the anaerobic growth of KHa. Under anoxic conditions, acetate was the sole product from H_2-CO_2 (stoichiometries approximated 4 mol H_2 consumed per mol acetate produced) and was the main product with other substrates, with small amounts of butyrate, ethanol, lactate, and H_2 also being occasionally formed. Cellulose, xylan, cellobiose, saccharose, raffinose, oxalate, *N*-acetylglucosamine, galactose, mannose, succinate, butyrate, ethylene glycol, methanol, fumarate, vanillate, ferulate, and CO did not support growth or enhance acetate production above that of control cultures lacking substrate.

The substrate-product profile as well as the optimal growth conditions of KHa differed from those of fermentative *Clostridium glycolicum* strains (4, 10) but were similar to those of acetogenic *C. glycolicum* RD-1 (18). KHa had a 99.1% 16S rRNA gene sequence similarity (1,300 bp) to that of the type strain of *C. glycolicum*, a 97.1% *fhs* sequence similarity (315 amino acids) to that of *C. glycolicum* RD-1, and a 72.8% *cooS* sequence similarity (554 amino acids) to that of *C. glycolicum* RD-1 (Fig. 1). These combined phenotypic and phylogenetic properties demonstrated that KHa was a new acetogenic strain of *C. glycolicum*.

KHa grew on glucose in medium containing up to 3% oxygen (O_2) in the gas phase (Table 1). Up to 1.5% O_2 in the gas phase was consumed. Exposing glucose cultures of KHa to O_2 resulted in a shift of the product profile from acetate as the main product without O_2 to the enhanced production of lactate and ethanol in response to O_2. The acetogen *C. glycolicum* RD-1 undergoes a similar O_2-dependent metabolic shift (18), reinforcing the identification of KHa as an acetogenic strain of

TABLE 1. Effect of O_2 on the glucose-dependent product profile of KHa[a]

% of initial O_2	Amt of glucose consumed (mM)	Max OD_{660}	Amt of product (mM)					% recovery	
			Acetate	Ethanol	Lactate	Butyrate	H_2	Carbon	Reductant
0	4.6	0.45	13.1	0.0	0.0	0.3	0.1	99	100
0.5	5.4	0.52	13.0	0.0	0.5	0.1	0.1	86	87
1.0	5.9	0.35	11.4	0.1	0.7	0.0	0.2	71	72
2.0	5.0	0.42	12.2	0.6	0.6	0.0	0.2	91	94
3.0	4.5	0.46	6.8	1.3	1.8	0.0	0.2	80	85
4.0	0.0	0.00	0.0	0.0	0.0	0.0	0.0	NA	NA

[a] The medium was yeast extract medium lacking reducing agents (5) supplemented with approximately 5 mM glucose. Values were corrected with values from control cultures lacking glucose and O_2 (i.e., 2.4 mM acetate and 0.1 mM H_2). Values are means from two replicates. No products and no growth were detected with higher concentrations of O_2. NA, not applicable.

TABLE 2. Effect of O_2 on the glucose-dependent product profile of KHb[a]

% of initial O_2	Amt of glucose consumed (mM)	Max OD_{660}	Amt of product (mM)[b]					% recovery	
			Acetate	Ethanol	Lactate	Formate	H_2	Carbon	Reductant
0	5.2	0.53	2.6	5.5	0.1	0.6	6.6	55	82
0.5	4.9	0.56	3.2	6.6	0.1	0.8	5.1	70	100
1	5.0	0.50	3.3	6.7	0.2	0.9	3.7	72	99
2	4.9	0.52	3.8	8.4	0.3	1.3	2.3	90	121
3	3.9	0.51	3.6	5.3	0.4	1.1	1.9	86	110
4	4.6	0.39	3.8	4.9	0.6	1.4	1.7	75	93
5	5.1	0.49	5.4	5.3	0.9	1.4	1.6	83	101
6[c]	5.1	0.47	4.6	4.5	0.7	1.4	1.6	71	86
10	0.0	0.00	0.0	0.0	0.0	0.0	0.0	NA	NA

[a] The medium was yeast extract medium lacking reducing agents (5) supplemented with approximately 5 mM glucose. Values were corrected with values from control cultures lacking glucose and O_2 (0.4 mM acetate, 0.3 mM formate, 0.6 mM H_2). Values are means from two replicates. NA, not applicable.
[b] It is likely that KHb also produced CO_2.
[c] Growth and activity were observed with only one of the two replicates. Thus, the values for this cultivation condition are from the single growth-positive culture.

C. glycolicum. An increased salt concentration of up to 20 g NaCl $liter^{-1}$ also resulted in an increased production of ethanol (up to 1.6 mM) from 5 mM fructose. The capacity to produce ethanol is a property of other clostridial acetogens (e.g., *Clostridium carboxidivorans*, *Clostridium ljungdahlii*, and "*Clostridium ragsdalei*" [quotation marks indicate that the organism has not been validated]) (22, 25, 27, 34).

Morphology, physiology, and phylogeny of KHb. KHb was a non-spore-forming rod. As with KHa, KHb tended to lyse, thus making it difficult to determine maximum optical densities. Cells were approximately 1.0 μm wide and 2.6 to 3.2 μm long and often occurred in pairs or short chains. The optimum temperature and pH for growth on glucose were 37°C and 6.9, respectively. Growth occurred from 15 to 37°C and pH 5.5 to 8.2 but not at 10°C and 45°C or at pH 5.1 and 8.7. Xylan (provided as a suspension prepared from autoclaved powder, a suspension prepared from UV-irradiated powder, or an autoclaved suspension, all of which yielded similar product profiles), raffinose, cellobiose, saccharose, maltose, galactose, glucose, xylose, mannose, *N*-acetylglucosamine, citrate, fumarate, pyruvate, ethylene glycol, and yeast extract supported the growth of KHb, as determined by an increase in optical density or the production of products above control cultures lacking substrate. Acetate, ethanol, lactate, formate, and H_2 were formed as end products (Table 2). Although CO_2 was not determined, it is likely that CO_2 was also produced by KHb, a matter that would partly explain the relatively low recovery of carbon (Table 2). Cellulose, ethanol, 1-propanol, *n*-butanol, oxalate, lactate, succinate, butyrate, methanol, vanillate, ferulate, and CO did not support growth.

The morphology and substrate-product profile of KHb were very similar to those of the type strain of *Bacteroides xylanolyticus* (28). KHb had a 99.4% 16S rRNA gene sequence (1,336 bp) similarity to that of the type strain of *B. xylanolyticus* (Fig. 1). No PCR signal was obtained for *fhs* and *cooS* with KHb. These combined phenotypic and genotypic properties demonstrated that KHb was a new strain of fermentative anaerobe *B. xylanolyticus*. KHb grew on glucose in medium containing up to 6% O_2 in the gas phase (Table 2). Up to 4% O_2 in the gas phase was consumed. Exposure to O_2 resulted in a modest alteration of the product profile of KHb, with lactate and formate increasing and ethanol and H_2 decreasing in response to large amounts of O_2. The capacity of KHb to utilize diverse saccharides and the moderate aerotolerance of KHb are properties consistent with those of other *Bacteroides* species (13, 23, 30–32).

Trophic interaction of *C. glycolicum* KHa and *B. xylanolyticus* KHb. KHb fermented substrates that were not used by KHa and formed products that supported the growth of KHa. For example, raffinose, a trisaccharide, was not utilized by KHa but was growth supportive for KHb, yielding large amounts of ethanol and H_2 (Table 3). In contrast, cocultures of KHa and KHb converted raffinose to acetate (Table 3). This trophic interaction also occurred with cocultures of KHa and KHb on xylan. As noted above, KHa did not utilize xylan. Xylan cultures of KHb produced ethanol, H_2, and acetate (Fig. 2A). Cocultures of KHa and KHb on xylan yielded primarily acetate, with ethanol and H_2 being minimal (Fig. 2B). The apparent capacity of cocultures to convert xylan to acetate is

TABLE 3. Raffinose-dependent product profiles of KHa, KHb, and cocultures of KHa and KHb[a]

Organism	Substrate consumed (mM)	Max OD_{660}	Amt of product (mM)[b]			% recovery	
			Acetate	Ethanol	H_2	Carbon	Reductant
KHa	Raffinose (0)	0.15[c]	0.0	0.0	0.0	NA	NA
KHb	Raffinose (1.7)	0.60	3.1	9.4	5.8	82	122
KHa/KHb	Raffinose (1.7)	0.81	14.2	0.0	0.2	93	93

[a] The medium was yeast extract medium lacking reducing agents (5) supplemented with approximately 1.7 mM raffinose. Values were corrected with values from control cultures lacking raffinose (for KHa: 4 mM acetate, 1 mM butyrate; for KHb: 0.8 mM acetate, 0.6 mM H_2). Values are means from two replicates. NA, not applicable.
[b] It is likely that KHb also produced CO_2.
[c] The OD_{660} was due to growth on yeast extract.

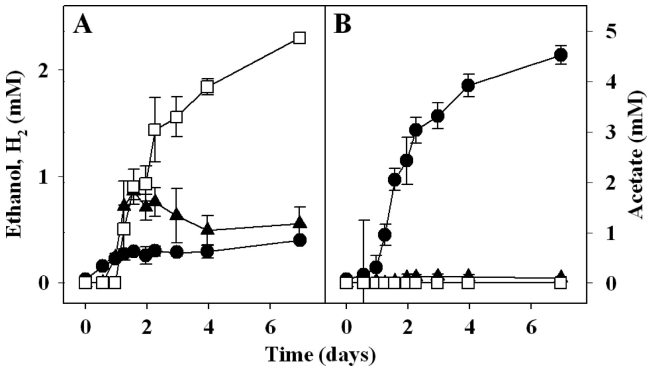

FIG. 2. Xylan-dependent product profiles of KHb (A) and cocultures of KHa and KHb (B). Values were corrected with values obtained from control cultures (i.e., KHb for panel A and cocultures of KHa and KHb for panel B) lacking xylan. Medium was yeast extract medium (5). Xylan was provided at a final concentration of approximately 0.1% (wt/vol); the xylan stock solution was a sterile anoxic suspension prepared from autoclaved xylan powder. Error bars show the variance of triplicate cultures; symbols are placed at mean values (the error bar is smaller than the size of the symbol where no bar is apparent). Symbols: filled circles, acetate; empty squares, ethanol; filled triangles, H_2.

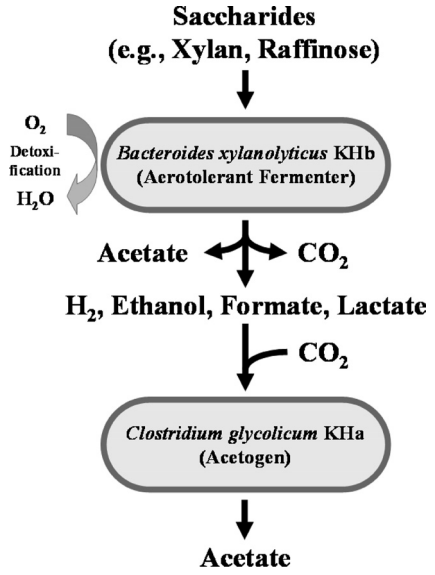

FIG. 3. Hypothetical model for the trophic interaction of *Bacteroides xylanolyticus* KHb and *Clostridium glycolicum* KHa.

noteworthy, given the commercial interest in using acetogens to convert plant biomass to useful chemicals (ZeaChem, Inc.).

These collective results suggested that products of KHb (e.g., ethanol and H_2) were converted to acetate by KHa in cocultures of KHa and KHb (Fig. 3). The production of lactate, formate, and H_2 by an aerotolerant fermentative bacterium and subsequent utilization by an acetogen have been observed with two other commensal cocultures, namely, *Thermicanus aegyptius* and *Moorella thermoacetica* (11) and *Clostridium intestinale* and *Sporomusa rhizae* (12). In the present study, ethanol was also found to be a functional link between an aerotolerant fermenter (*B. xylanolyticus* KHb) and an acetogen (*C. glycolicum* KHa) (Fig. 3). This proposed interaction does not preclude the capacity of KHa to consume monosaccharides released during the hydrolysis of polysaccharides by KHb.

Acetogens are classically considered to be obligate anaerobes (7). KHa tolerated minimal amounts of O_2 (Table 1), a characteristic shared with other acetogens (e.g., *Sporomusa silvacetica*, *C. glycolicum* RD-1) (2, 3, 12, 15, 18). However, the fermentative partner KHb tolerated and consumed larger amounts of O_2 than did the acetogen KHa (Tables 1 and 2), a pattern also observed with the aforementioned commensal partnerships (11, 12). As with the parent culture KH, the aforementioned partnerships were also initially isolated as a presumably pure culture that was subsequently found to be composed of two functionally linked bacteria. Although the isolation of an acetogen together with an aerotolerant fermenter might be considered a laboratory phenomenon, the accidental isolation of three such partnerships illustrates a type of interaction that might occur *in situ* between so-called obligate anaerobes and aerotolerant fermentative organisms. In the case of acetogens in habitats subject to large fluctuations of O_2 (e.g., soil), it would seem beneficial to be associated with O_2-consuming aerotolerant fermentative organisms that convert nonacetogenic substrates to products that can subsequently support acetogenic growth.

Nucleotide sequence accession numbers. Nucleotide sequences are available from the EMBL nucleotide sequence database under accession numbers FR850046 to FR850058.

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