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Characterization and Ecology of Carboxymethylcellulase-Producing Anaerobic Bacterial Communities Associated with the Intestinal Tract of the Pinfish, *Lagodon rhomboides*

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Carboxymethylcellulase (CMCase)-producing obligate anaerobes were isolated from the intestinal tract contents but not the feeding habitat of seagrass-consuming pinfish. Taxonomic characterization of these CMCase-producing strains revealed four taxonomic clusters; three were clostridial and one was of unknown taxonomic affinity. Our results demonstrated that the CMCase-producing obligate anaerobe community from pinfish differed from functionally similar microbial communities in terrestrial herbivores.

A symbiotic cellulolytic microbial community that assists in the digestion of plant polysaccharides refractory to host-derived digestive enzymes has been established to exist in terrestrial organisms (2, 4, 6, 9, 27). However, direct evidence for microbially assisted degradation of dietary plant matter among marine organisms, particularly fishes, remains poorly documented (8, 11, 12, 15). Although cellulase activity has been detected in the digestive tract contents of selected fish species (10, 18, 29), attempts to isolate cellulase-producing microbial symbionts from fishes have been unsuccessful (1, 3, 16, 26, 29) until recently (7).

In a previous study, we documented the occurrence of a carboxymethylcellulase (CMCase)-producing microbial community and established that the CMCase component of the microbial community fluctuated temporally but did so independently of the protease-producing component of the community (7). However, in the absence of taxonomic information for these CMCase-producing isolates, we were unable to determine the ecology of the CMCase-producing microbial community. In this report, we describe the characterization and distribution of CMCase-producing anaerobic bacteria from the feeding habitat and intestinal tract contents of pinfish. We provide evidence for a CMCase-producing microbial community composed of four distinct taxonomic clusters that is present in the intestinal tract contents but not the feeding habitat of pinfish.

A total of 550 anaerobic bacterial strains, 200 from environmental samples and 50 each from the intestinal tract contents of seven different pinfish, were isolated and screened for CMCase activity by the Congo red assay described previously (7, 25). The 200 environmental strains revealed no detectable CMCase activity. In contrast, 36 of the 350 (10.3%) obligate anaerobes recovered from the intestinal tract contents of seagrass-consuming pinfish expressed CMCase activity. The absence of CMCase-producing obligate anaerobes from the sampled food sources and feeding habitat of pinfish was unexpected but may be explained if the CMCase-producing microbial community of the pinfish is exclusively endosymbiotic or is present in environmental sites that were not sampled in this study.

To further understand the taxonomic relationships among CMCase-producing strains, a comparison of the morphological, physiological, and biochemical properties of each of these 36 strains was conducted. Biochemical tests, including catalase, nitrate reductase, esculin hydrolysis, and urease as well as carbohydrate utilization profiles, were determined by using the BBL Minitek numerical identification system for anaerobes (Becton Dickinson, Cockeysville, Md.). Biochemical test results were interpreted as specified by the manufacturer and compared with the results obtained with control strains, including *Clostridium perfringens* ATCC 13124, *Bacteroides ovatus* ATCC 8483, and *Eubacterium aerofaciens* ATCC 25986. All biochemical tests were repeated at least twice to ensure that culture conditions provided reliable evidence for assigning a value in the presence/absence matrix (Table 1). Volatile fatty acid, alcohol, and organic acid profiles were determined by methods detailed in the Virginia Polytechnic Institute *Anaerobe Laboratory Manual* (5) following cultivation of isolates on peptone-yeast extract-glucose medium at 37°C for 48 to 72 h. Taxonomic relatedness of strains was based on the Euclidean distance between strains established by pairwise comparison of a presence/absence matrix constructed from the biochemical, physiological, and morphological traits of each strain (Table 1). By using the hierarchical clustering strategy of SYSTAT statistical analysis software (17, 30), these distances were used to group strains into similar clusters by means of an average linkage method of unweighted pairs (UMPGA).

Taxonomic characterization of the 36 CMCase-producing obligate anaerobes revealed four clusters (I to IV) on the basis of a similarity coefficient of 0.7 or greater by the unweighted average pair-group method (17) (Fig. 1). Cluster I strains (strains 26 to 36) were characterized as gram-negative, nonmotile, non-spore-forming cocci, each of which exhibited virtually identical gas chromatographic (GC) end product profiles, dominated by acetate with much lower but detectable levels of formate, propionate, and butyrate (Table 1). Of these 11 strains, none produced nitrate reductase or urease activities, nor were they able to ferment arabinose, glycerol, or salicin. However, all cluster I strains fermented glucose and mannose. Three separate pairs of strains (strains 26 and 29, 27 and 31, and 32 and 34) were identical for all the characteristics tested, indicating multiple isolates of the same strain (Table 1) (Fig. 1). Interestingly, the individual members of these identical pairs were isolated from different fish, providing evidence for the dissemination of presumably identical cluster I strains among

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TABLE 1. Characterization of bacterial strains

Strain	Shape	Motility	Spore formation	Result[a]												
				A	F	P	B	V	IB	IV	IC	C	LA	SU	FU	PY
1	Rod	+	+	+	+	+	+	−	−	−	−	−	+	+	−	−
2	Rod	+	+	+	+	+	+	−	−	−	−	−	+	+	−	−
3	Rod	+	+	+	+	+	+	−	−	−	−	−	+	+	−	−
4	Rod	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
5	Rod	+	+	+	+	+	+	−	−	−	−	−	+	+	−	−
6	Rod	+	+	+	+	+	+	−	−	−	−	−	+	+	−	−
7	Rod	+	+	+	+	+	+	−	−	−	−	−	+	+	−	−
8	Rod	+	+	+	+	+	+	−	−	−	−	−	+	+	−	−
9	Rod	+	+	+	+	+	+	−	+	+	−	−	+	−	+	−
10	Rod	+	+	+	+	+	+	−	+	+	−	−	+	+	−	−
11	Rod	+	+	+	+	+	+	−	−	−	−	−	+	+	−	−
12	Rod	+	+	+	+	+	+	+	−	−	−	−	+	+	−	−
13	Rod	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
14	Rod	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
15	Rod	+	+	+	+	+	+	+	+	+	+	−	+	+	−	−
16	Rod	+	+	+	+	+	+	+	+	+	−	−	+	+	−	−
17	Rod	+	+	+	+	+	+	−	−	−	−	−	+	+	−	−
18	Rod	+	+	+	+	+	+	−	−	−	−	−	+	+	−	−
19	Rod	+	+	+	+	+	+	−	−	−	−	−	+	+	−	−
20	Rod	+	+	+	+	+	+	−	−	−	−	−	+	+	−	−
21	Rod	+	+	+	+	+	+	−	−	−	−	−	+	+	−	−
22	Rod	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+
23	Rod	+	+	+	+	+	+	−	+	+	−	−	+	+	−	−
24	Rod	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
25	Rod	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+
26	Coccus	−	−	+	+	+	+	−	−	−	−	−	+	+	+	−
27	Coccus	−	−	+	+	+	+	−	−	−	−	−	+	+	+	−
28	Coccus	−	−	+	+	+	+	−	−	−	−	−	+	+	+	+
29	Coccus	−	−	+	+	+	+	−	−	−	−	−	+	+	+	−
30	Coccus	−	−	+	+	+	+	−	−	−	−	−	+	+	+	+
31	Coccus	−	−	+	+	+	+	−	−	−	−	−	+	+	+	−
32	Coccus	−	−	+	+	+	+	−	−	−	−	−	+	+	+	−
33	Coccus	−	−	+	+	+	+	−	−	−	−	−	+	+	+	−
34	Coccus	−	−	+	+	+	+	−	−	−	−	−	+	+	+	−
35	Coccus	−	−	+	+	+	+	−	−	−	−	−	+	+	+	−
36	Coccus	−	−	+	+	+	+	−	−	−	−	−	+	+	+	−

[a] A, acetate; F, formate; P, propionate; B, butyrate; V, valerate; IB, isobutyrate; IV, isovalerate; IC, isocaproate; C, caproate; LA, lactate; SU, succinate; FU, fumarate; PY, pyruvate; MA, malonate; ES, esculin; NR, nitrate reductase; IN, indole; GL, glucose; AR, arabinose; GY, glycerol; LC, lactose; ML, maltose; MN, mannitol; RH, rhamnose; SA, salicin; SC, sucrose; TR, trehalose; XY, xylose; CT, catalase; CE, cellobiose; MN, mannose; RA, raffinose; SO, sorbose; UR, urease.

pinfish. Although strains assigned to cluster I shared many characteristics with extant obligate anaerobes, their overall level of divergence from extant taxa precluded their taxonomic assignment.

The remaining 25 strains were all gram-positive or gram-variable, anaerobic, endospore-forming, non-sulfate-reducing, rod-shaped bacteria; these characteristics warrant the inclusion of these strains in the genus *Clostridium*. Although morphologically similar to one another, these 25 strains were distinguishable on the basis of differences in GC end products and carbohydrate utilization profiles (Table 1). Characteristics common to all cluster II strains (strains 1, 2, 6 to 8, 11, 17, 18, and 21) included a GC end product profile restricted to formate, acetate, propionate, and butyrate; production of acid from glucose, maltose, arabinose, salicin, cellobiose, and mannose; and an absence of nitrate reductase, catalase, and/or urease activities (Table 1). On the other hand, of the CMCase-producing strains, cluster III strains (strains 4, 9, 13 to 15, 22, 24, and 25) produced the most complex GC end product profiles, including all the volatile fatty acids detected in clusters I and II as well as isocaproate, caproate, and in two instances, heptanoate. Moreover, cluster III strains produced either pyruvate or malonate, neither of which was detected in cluster II strains, in addition to lactate and succinate. Cluster IV strains (strains 3, 10, 16, 19, and 23) were most readily differentiated from the other clostridial strains on the basis of the production of proportionately higher levels of butyrate and isovalerate with low or undetectable levels of valerate, isocaproate, or caproate. There was insufficient evidence to support a precise relationship between cluster II, III, and IV strains and extant species within the genus *Clostridium*. It is possible that clusters II, III, and IV correspond to species within this genus; however, further taxonomic characterization is required before definitive assignments of these strains can be substantiated. Preliminary DNA sequence analysis of PCR-amplified 16S ribosomal DNA from representatives of clusters II, III, and IV revealed no greater than 93% DNA sequence relatedness to extant species within the genus *Clostridium* (17a). These results suggest that the clostridial strains isolated from the pinfish intestinal tract may represent novel species. The likely discovery of novel CMCase-producing anaerobic bacterial species is not unexpected because most taxonomically characterized anaerobes have been isolated from either terrestrial or freshwater aquatic habitats (13–15, 19–24, 26).

Previous characterization of obligate anaerobes from the gastrointestinal tracts of fishes provided evidence that *Eubacterium*, *Fusobacterium*, and *Bacteroides*, referred to as *Bacte-*

TABLE 1—*Continued*

Result[a]																				
MA	ES	NR	IN	GL	AR	GY	LC	ML	MN	RH	SA	SC	TR	XY	CT	CE	MN	RA	SO	UR
−	+	−	−	+	−	−	−	+	−	−	+	−	+	−	−	+	+	−	−	−
−	+	−	+	+	−	−	−	+	−	−	+	+	+	−	−	+	+	−	−	−
−	+	−	+	+	−	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−
+	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
−	+	+	−	+	+	−	+	+	+	+	+	+	+	+	+	+	+	−	+	−
−	+	−	−	+	−	+	+	+	−	−	+	+	+	−	−	+	+	−	−	−
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+	+	+	+	−	−	+	+	+	−	−	+	+	−	−	−	+	+	−	−	−
−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
−	−	−	+	+	−	−	−	+	−	−	−	−	+	−	−	−	+	−	−	−
−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
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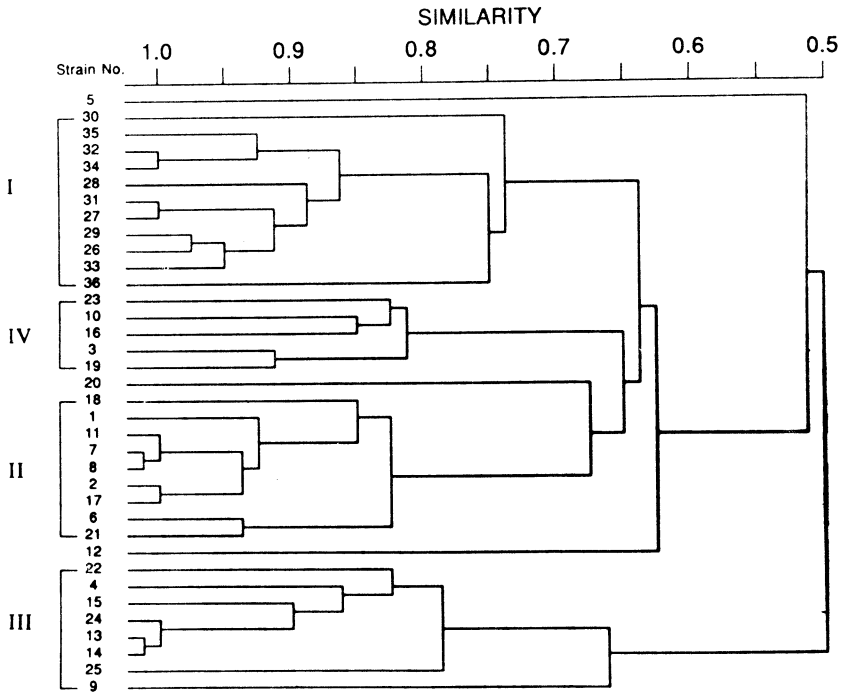

FIG. 1. Phenogram obtained by the unweighted average pair-group method of clustering classification on the basis of morphological, physiological, and biochemical characteristics of each strain (Table 1). Roman numerals I to IV represent the four major clusters of the tree.

roides types A and B, were the most abundant genera and that clostridia were present in such small numbers that enrichment was required for their isolation (14, 15, 19, 21–24, 26). Although our data do not provide information for the abundance of all bacterial species in the intestinal tract of pinfish, clostridial CMCase-producing anaerobes are a readily detectable component of the intestinal microbial community in our sampled population. These results suggest that the intestinal microbial community of freshwater fishes differs from that of the pinfish, the only marine fish characterized so far. It is also important to point out that the CMCase-producing microbial community of seagrass-consuming pinfish differs from the cellulolytic communities associated with terrestrial herbivores (2, 4, 27, 28), suggesting that physiological differences in the host as well as the environment influence the composition of the CMCase-producing intestinal anaerobic bacterial community. Characterization of the factors responsible for regulating the composition of the intestinal tract microbial community is essential if we are to understand the importance of these bacterial strains in the digestion of plant material by pinfish and other marine herbivorous fishes.

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