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Biochemical Characteristics of *Edwardsiella ictaluri*†

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A total of 119 isolates of *Edwardsiella ictaluri* collected over the last 7 years from several states were biochemically characterized. Although not very reactive biochemically, this bacterium shows a high degree of homogeneity. Differences were found only in the production of gas from formate or from glucose at 37°C and in the production of hydrogen sulfide as detected by lead acetate paper. Analysis of *E. ictaluri* by year or geographic area indicated that some differences existed, but no clear-cut biotypic variations were found. All isolates studied were capable of degrading chondroitin sulfate, a major component of cartilage, which may be an important virulence factor in the formation of the hole-in-the-head lesion characteristic of infected fish.

Edwardsiella ictaluri was first isolated in 1976 as the etiologic agent in acute mortalities in channel catfish. This disease condition has been termed enteric septicemia of catfish. Enteric septicemia of catfish is predominantly a disease of channel catfish, usually occurring in the early summer and autumn months when water temperatures range from 25 to 30°C. In less than 10 years, *E. ictaluri* has become the leading cause of bacterial mortality in catfish in the Mississippi delta, resulting in losses of about $4 million in 1984 (T. Wellborn, personal communication).

E. ictaluri was initially described by Hawke (1a), and 16 isolates were biochemically characterized. The genus and species designation was later based on the DNA-DNA homology of five isolates (2). Thus, the biochemical characterization and taxonomic position of this bacterium are based on a limited number of isolates. For this reason a study was undertaken to characterize a large number of isolates biochemically, allowing for the comparison of isolates for biotypic variation based on year or geographic area of isolation.

MATERIALS AND METHODS

Isolates. A total of 119 isolates of *E. ictaluri* was collected and studied. Several isolates were from the authors' stock collection whereas others were generously supplied by others. This pool of isolates was collected during an 8-year period. They were primarily associated with clinical outbreaks in Alabama (12 isolates), Georgia (13 isolates), Mississippi (88 isolates), and other areas (6 isolates).

Biochemical characterization. A total of 86 biochemical characteristics were examined. Basic biochemical tests were performed with triple sugar iron, Simmons citrate, Christensen's urea, malonate, methyl red (MR), Voges-Proskauer (VP), phenylalanine, lysine, arginine, ornithine, and sulfide-indole-motility (SIM) media.

Carbohydrate and gas production were assayed by incorporating specific carbohydrates into a purple broth base with an inverted Durham's tube. Glycerol fermentation was tested with O/F basal medium (BBL Microbiology Systems, Cockeysville, Md).

Extracellular enzymes were detected with a plate assay technique (4–6). The substrates tested were cellulose, starch, esculin, gelatin, casein, fibrinogen, elastin, lecithin, Tween 20, 40, 60, 80, and 85, pectin, alginate, collagen, DNA, heat-killed bacteria (i.e., *Staphylococcus aureus*, *Aeromonas hydrophila*, *Escherichia coli*), chitin, and bovine erythrocytes. *E. ictaluri* isolates were point inoculated onto each plate medium, and the reactions were evaluated as a zone ratio after 5 days of incubation (5). Degradation of chondroitin sulfate and hyaluronic acid was tested by a tube method (3).

The ability of *E. ictaluri* to grow under certain conditions was determined with MacConkey, salmonella-shigella, brilliant green, and cetrimide agars, by growth ability at 42°C, and on agar containing 1 to 3% NaCl.

Determinations of acid and gas production from glucose, motility in 0.4% agar (4), hemolysis (2% bovine blood agar), MR-VP reactions, and hydrogen sulfide (H_2S) production (SIM tube with lead acetate strip) were performed at 20, 30, and 37°C. All other tests were incubated at 30°C.

Quantitative data were obtained from carbohydrate gas production (height of the gas column in Durham's tube after 5 days) (6).

Statistical analysis. For analysis by year of isolation, isolates from 1977 through 1981 were grouped together. Tukey's test for multiple comparisons among proportions was used to determine differences in qualitative (i.e., positive or negative) data between geographic area or year of isolation. The Scheffe test for multiple comparisons was used to analyze quantitative data. The tests were selected because of the high disparity in members between groupings.

RESULTS

The biochemical characteristics of *E. ictaluri* are summarized in Table 1. The typical triple sugar iron agar reaction was alkaline over acid with or without gas production (when present, only slight) and no H_2S. All isolates were negative for citrate, urea, malonate, VP, phenylalanine, indole, arginine dihydrolase, cytochrome oxidase, and β-galactosidase. Conversely, all isolates were positive for MR, nitrate reductase, lysine decarboxylase, ornithine decarboxylase, and catalase.

The homogeneity of *E. ictaluri* was further illustrated by the carbohydrate fermentation pattern. The six-carbon sugars glucose, fructose, galactose, and mannose were fermented with the production of gas. Glucose was fermented by all isolates at 20, 30, and 37°C. Gas was produced from glucose by all isolates at 20 and 30°C; gas was produced by

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TABLE 1. Biochemical and enzymatic characteristics of *E. ictaluri*

Characteristic or substrate tested	No. tested	% Positive	Characteristic or substrate tested	No. tested	% Positive
Simmons citrate	119	0.0	Adonitol	112	0.0
Urea	119	0.0	Arabinose	115	0.0
Malonate	110	0.0	Cellobiose	113	0.0
Phenylalanine	119	0.0	Cellulose	115	0.0
Indole	119	0.0	Dulcitol	115	0.0
Lysine	119	100.0	Esculin	118	0.0
Arginine	119	0.0	Fromate (gas)	103	80.6
Ornithine	119	100.0	Fructose	118	100.0
Nitrate	119	100.0	Galactose	118	100.0
β-Galactosidase	119	0.0	Gluconate	116	0.0
Oxidase	119	0.0	Glucose (acid)	117	100.0
Catalase	119	100.0	Gas (20°C)	104	100.0
MR (20°C)	100	100.0	Gas (30°C)	110	100.0
VP (20°C)	100	0.0	Gas (37°C)	104	53.8
MR (37°C)	100	100.0	Glycerol	118	100.0
VP (37°C)	100	0.0	Inositol	113	0.0
Gelatin	116	0.0	Inulin	113	0.0
Casein	116	0.0	Lactose	118	0.0
Elastin	116	0.0	Maltose	115	100.0
Fibrinogen	116	0.0	Mannitol	117	0.0
Lecithin	116	0.0	Mannose	114	100.0
Tween 20	44	0.0	Raffinose	116	0.0
Tween 40	44	0.0	Rhamnose	116	0.0
Tween 60	44	0.0	Ribose	115	100.0
Tween 80	116	0.0	Salicin	116	0.0
Tween 85	116	0.0	Sorbitol	109	0.0
Chondroitin SO_4	100	100.0	Sorbose	110	0.0
Hyaluronic acid	50	0.0	Starch	116	0.0
Pectin	50	0.0	Sucrose	114	0.0
Alginate	50	0.0	Trehalose	113	0.0
Collagen	116	0.0	Xylose	113	0.0
DNA	116	0.0			
Chitin	116	0.0	Motility (20°C)	117	0.0
Bacteriolysis	50	0.0	Motility (30°C)	117	0.0
MacConkey agar	119	100.0	Motility (37°C)	117	0.0
Salmonella-shigella agar	96	99.0			
Brilliant green agar	94	100.0	H_2S production (20°C)	112	0.0
Cetrimide	116	0.0	SIM (30°C)	112	0.0
Growth at 42°C	119	0.0	SIM (37°C)	112	0.0
NaCl (1.0%)	93	100.0			
NaCl (1.5%)	93	90.3	H_2S production (20°C)	109	13.8
NaCl (2.0%)	93	0.0	Paper (30°C)	109	86.2
NaCl (3.0%)	93	0.0	Paper (37°C)	109	38.9
Hemolysis (20°C)	105	97.1			
Hemolysis (30°C)	105	97.1			
Hemolysis (37°C)	105	96.2			

53.8% of all isolates at 37°C. Gas production was greatest at 30°C. When present at 37°C, gas production was reduced by approximately 75%. Ribose was the only five-carbon sugar fermented. Of the sugar alcohols, only glycerol was weakly fermented. Maltose was the only disaccharide or complex carbohydrate fermented or degraded.

All *E. ictaluri* lacked proteases, lipases, esterases, pectinase, alginase, collagenase, chitinase, and hyaluronidase as measured with the substrates listed in Table 1. However, all isolates were able to degrade chondroitin sulfate.

Additional characteristics included the ability to grow on MacConkey, salmonella-shigella, and brilliant green agars but not at 42°C or on cetrimide agar or 2% NaCl. *E. ictaluri* were nonmotile as measured by the plate method at 20, 30, and 27°C. Almost all isolates were hemolytic (97%), regardless of temperature. Hydrogen sulfide was not observed in SIM medium at any temperature. However, by using lead acetate paper, H_2S was produced by 13.8% of the isolates at 20°C, by 86.2% of the isolates at 30°C, and by 38.9% of the isolates at 37°C. *E. ictaluri* isolates were essentially biochemically identical, except for gas production from glucose at 37°C and from formate and the presence of H_2S detected by lead acetate paper.

The isolates were analyzed for biotypic differences based on geographic area and year of isolation. Qualitative differences in isolates based on year of isolation are listed in Table 2. The production of gas from formate was significantly higher in the 1982 and 1984 isolates. Gas production from glucose at 37°C was highest in 1982 and lowest in 1984. Differences in H_2S production detected by lead acetate paper were seen at 20 and 37°C but not at 30°C.

When the same data were analyzed by geographic area (Table 2), statistically significant variations were found in the production of gas from formate, gas from glucose at 37°C, and in H_2S production at 20°C.

TABLE 2. Biochemical differences between isolates of *E. ictaluri* based on geographic area and year of isolation

Biotypic variable	% of isolates producing				
	Gas from formate	Gas from glucose	H_2S on lead acetate paper		
			20°C	30°C	37°C
1981	44.4[a,b]	72.7[b]	36.4[b,c]	72.7	45.4
1982	95.8[c,d]	90.5[b,c]	18.2[c]	95.4	22.7
1983	33.3[a,b]	68.0[a,b,d]	0.0[a,b,d]	83.3	66.7[a,b]
1984	96.2[c,d]	25.5[a,c,d]	13.5[c,d]	86.5	30.8[c]
Alabama	44.4[e,f]	16.7[e,f,g]	72.7[e,f,g]	81.8	36.4
Georgia	66.7[f,g,h]	66.7[f,h]	30.0[f,g,h]	80.0	50.0
Mississippi	85.0[e,f]	58.0[f,h]	4.7[f,h]	88.2	39.3
Other areas	100.0[e,g,h]	0.0[e,g,h]	0.0[e,h]	66.7	0.0

[a] Differs significantly ($P < 0.05$) from 1982.
[b] Differs significantly ($P < 0.05$) from 1984.
[c] Differs significantly ($P < 0.05$) from 1983.
[d] Differs significantly ($P < 0.05$) from 1981.
[e] Differs significantly ($P < 0.05$) from Georgia.
[f] Differs significantly ($P < 0.05$) from other areas.
[g] Differs significantly ($P < 0.05$) from Mississippi.
[h] Differs significantly ($P < 0.05$) from Alabama.

DISCUSSION

E. ictaluri was placed in the genus *Edwardsiella* within the family *Enterobacteriaceae* based on biochemical characterization and DNA-DNA homology (2). This microorganism shares many common features with another member of this genus, *E. tarda*. Both species produce disease and mortality in fish. *E. ictaluri* has a narrow host range (catfish) and produces septicemic disease, whereas *E. tarda* has a less specific range and produces hepatic or renal necrosis or both with putrefaction in several species of fish. Biochemically, the two species show numerous common reactions (W. D. Waltman, Ph.D. dissertation, University of Georgia, Athens, 1985). However, some differences include production of indole, production of H_2S in SIM media, growth at 42°C, and motility at 37°C by *E. tarda* but not *E. ictaluri*.

Biochemically *E. ictaluri* is a very homogenous microorganism. It is not very reactive, fermenting only a few carbohydrates and lacking most extracellular enzymes. The biochemical results of this study greatly augment and parallel the original description of Hawke (1a) with few exceptions. *E. ictaluri* was nonmotile at temperatures of 20 to 37°C on semisolid (0.4%) motility agar which has been found useful for *A. hydrophila* (4) and *E. tarda* (Waltman, Ph.D. dissertation). *E. ictaluri*, however, have peritrichous flagella when examined by flagellum staining and are weakly motile in SIM media after 5 to 7 days at 20 or 30°C. These findings agree with those of Hawke, in that he described *E. ictaluri* as being weakly motile at 22°C by peritrichous flagella but not motile at 37°C (1a).

The production of gas from glucose at 37°C represents another difference from the original description of Hawke (1a). We found that 53.8% of our isolates produced gas at 37°C. The amount of gas produced was only about 25% of that produced at 30°C. The difference from the original description (1a) probably reflects the use of slightly more sensitive detection media in our study. The production of gas produced from glucose at 37°C should probably not be used as one of the characteristics differentiating *E. ictaluri* and *E. tarda* because gas production varies with the basal media used.

The production of H_2S was not evident in triple sugar iron or SIM media, but the use of the more sensitive lead acetate paper indicated production of H_2S at 20°C (13.76%), 30°C (86.24%), and 37°C (38.89%). This finding in *E. ictaluri* further indicated its biochemically close relationship with *E. tarda*. However, the species could still have been distinguished by the presence of H_2S (blackening) in SIM media.

E. ictaluri weakly fermented glycerol. When a purple broth base with glycerol was used, many negative reactions resulted. This parallels the findings of Hawke, as he lists the reaction for glycerol as positive-negative (1a). However, if a more sensitive basal medium such as O/F is used and supplemented with glycerol, then all of the isolates produce acid from glycerol (Waltman, Ph.D. dissertation).

Most *E. ictaluri* demonstrated hemolytic activity over a wide range of temperatures, but the hemolysis was distinctive and occurred as a narrow zone adjacent to the colony. This type of hemolysis has been described in *E. tarda* as a cell-bound type of hemolysin (7; Waltman, Ph.D. dissertation).

The ability of *E. ictaluri* to grow on various isolation media such as MacConkey and salmonella-shigella shows that it is fairly resistant to bile salts. It is, however, moderately sensitive to concentrations of NaCl (approximately 2%), a factor which should be considered in growing this bacterium and one which may also be advantageous in the control of disease.

E. ictaluri lacks proteases, lipases, esterases, and most other extracellular enzymes. However, it does have the ability to degrade chondroitin sulfate, a major component of cartilage. This capability has been proposed (E. B. Shotts, V. S. Blazer, and W. D. Waltman, J. Fish. Res. Board Can., in press) as a major factor in the formation of the hole-in-the-head lesion seen in many catfish infected with *E. ictaluri*. Histopathological examination of this lesion clearly shows diffuse granulomatous inflammation of the olfactory lobe, intracellular microorganisms, and destruction of the cartilagenous material in this area (1).

Of the numerous biochemical tests used, the only ones that varied among the isolates studied were production of gas from formate, production of gas from glucose at 37°C, and production of H_2S as detected by lead acetate paper. Analysis of these reactions by geographic area or year of isolation showed some significant differences but established no general trend. Therefore we feel that there are no clear-cut biotypic differences among the *E. ictaluri* studied. Further documentation of the homogeneity of *E. ictaluri* was provided by a study of the isoenzyme patterns of 37 isolates for 25 enzymes, in which the results clearly demonstrated that all *E. ictaluri* were biochemically identical (W. D. Waltman, W. B. Schill, S. R. Phelps, and E. B. Shotts, unpublished data).

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