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Development of a Defined Minimal Medium for the Growth of *Edwardsiella ictaluri*†

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In this report, a complete defined medium and a minimally defined medium are described for *Edwardsiella ictaluri*. The complete defined medium consists of 46 individual components, including a basal salt solution, glucose, magnesium sulfate, iron sulfate, six trace metals, four nucleotides, 10 vitamins, and 19 amino acids. This medium supports growth in broth and on solid media. Optimal growth at 30°C was obtained at pH 7.0, and at an osmolality of 390 mosmol/kg of H_2O, with a glucose concentration of 4 g/liter. The defined minimal medium reduces the 46 components of the complete medium to eight essential components, including the basal salt solution, glucose, magnesium sulfate, pantothenic acid, and niacinamide. In addition, specific amino acids that depend on the specific requirements of the individual strains of *E. ictaluri* are added.

Edwardsiella ictaluri was first isolated from diseased channel catfish in 1976 and is the causative agent of enteric septicemia of catfish, the most serious disease affecting commercial catfish production (7). The organism is a weakly motile, gram-negative rod with peritrichous flagella and is cytochrome oxidase, indole, and H_2S negative (8, 17). Growth is relatively slow and generally requires a rich, complex medium. Although a selective medium for the isolation of *E. ictaluri* has been described previously (21), no work has been conducted to define the nutritional requirements for this important pathogen of farm-raised catfish.

Complete defined media contain complex mixtures of bacterial nutrients, while minimal defined media contain only those nutrients essential to the growth of a given species. These media have been used for the examination of microbial physiology (11), nutrition (12, 20), elicitation and accumulation of toxins (15), determination of growth requirements, and the development and characterization of auxotrophic mutants (6, 9). In addition, because the expression of virulence factors is often regulated by environmental conditions (13), defined media have been used to examine nutritional control of the expression of bacterial virulence mechanisms (2, 3, 19). At present, major virulence factors and pathogenic mechanisms for *E. ictaluri* have not been identified, possibly because of the current dependence on complex media for propagation of the organism. The development of a defined minimal medium that supports the growth of *E. ictaluri* is essential to the design of reproducible studies relating to biochemical, physiological, and genetic variation within the species and to subsequent studies on virulence and pathogenesis. This study was undertaken to determine the minimal nutritional requirements of *E. ictaluri* by first developing a complete defined medium, from which minimal requirements could be determined, ultimately leading to the development of a minimal defined medium that supports growth of *E. ictaluri.*

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MATERIALS AND METHODS

Bacterial strains. All *E. ictaluri* strains in this study were originally isolated from moribund channel catfish. Strains 89-9, 90-476, 91-581, 91-638, 92-266, 93-146, 93-154, 93-170, 93-264, and 93-297 were isolated at the Aquatic Animal Diagnostic Laboratory, Louisiana State University School of Veterinary Medicine, strains 587-671 and 587-673 were isolated at the Mississippi State Cooperative Extension Service Diagnostic Lab in Stoneville, strain 589-521 was isolated at the South Carolina Aquatic Diagnostic Lab, Clemson, and strain 83-189 was isolated at the Auburn University Diagnostic Lab, Auburn, Ala. Stock cultures were grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) in a rotary shaker at 30°C and stored frozen in BHI broth with 20% glycerol at −70°C.

Chemicals and growth media. All chemicals were of analytical grade unless stated otherwise. Amino acids, vitamins, purines and pyrimidines, sugars, and inorganic salts were purchased in the highest grade available from Sigma Chemical Co. (St. Louis, Mo.). Sodium chloride, dibasic sodium phosphate, ammonium chloride, and glucose were purchased from EM Sciences (Gibbstown, N.J.). Calcium chloride and monobasic potassium phosphate were obtained from Mallinckrodt (Chesterfield, Mo.).

Preparation of glassware. Glassware was soaked in a 5% (vol/vol) solution of Roccal II (National Laboratories, Montvale, N.J.) overnight, rinsed once in distilled water, immersed overnight in 10% nitric acid, rinsed six times in distilled water, dried in an oven at 60°C, and autoclaved.

Growth conditions and assessment of growth. Starter cultures were inoculated from single colony isolates grown from frozen BHI broth-glycerol stock cultures into glass test tubes containing 5 ml of complete defined medium (see recipe below) and grown overnight at 30°C on a Cel-Grow tissue culture rotator (Lab-Line Instruments, Inc., Melrose Park, Ill.). Starter cultures for optimization of osmolality, pH, carbon source, and carbon concentration were inoculated at a 1:50 dilution into 50 ml of test medium in 250-ml Klett flasks (Kontes, Vineland, N.J.), incubated in a rotary shaker (New Brunswick Scientific Co., Inc., Edison, N.J.) at 30°C, and assayed for growth on a Klett-Summerson model 800-3 photoelectric colorimeter with a green filter (Klett Manufacturing Co., Inc., Long Island, N.Y.). Starter cultures for minimalization of minerals, vitamins, and amino acids were inoculated at a 1:50 dilution into 5 ml of test medium in glass test tubes, grown at 30°C on a Cel-Grow tissue culture rotator, and assayed for growth as described above. To ensure elimination of carryover nutrients, cultures grown in complete defined medium were passaged twice at 24-h intervals, while cultures grown in minimal defined medium were passaged at 48-h intervals.

Defined medium optimization. Preliminary work in our lab indicated that M9 salts (18) supplemented with glucose and Casamino Acids (Difco) supported growth of *E. ictaluri*. Subsequent replacement of the Casamino Acids with vitamins, purines and pyrimidines, trace minerals, and amino acids resulted in a complete defined medium that was used to optimize osmolality, carbon source and concentration, and pH as described below. Water for all media was deionized and double distilled prior to use. Stock solutions were prepared as described in Table 1. Stock solutions were filter sterilized individually with a 0.2-μm-pore-size filter and stored at 20°C for not more than 30 days. Medium components were prepared separately as the following: (i) basal salts, (ii) carbohydrates, (iii) minerals, (iv) vitamins, (v) purines and pyrimidines, and (vi) amino acids. Data for the complete defined medium optimization are the means of triplicate cultures repeated twice and recorded at 12 and 24 h.

Optimal osmolality was determined in Klett flasks containing 50 ml of com-

TABLE 1. Preparation and composition of defined[a] and defined minimal[b] media for *E. ictaluri*

Ingredient	Stock solution concn	Final medium concn [mg/liter (mM)]
M9 salts		
$Na_2HPO_4 \cdot 7H_2O$	60 g/liter	15,000 (56)
KH_2PO_4	30 g/liter	7,500 (55)
NH_4Cl	10 g/liter	2,500 (47)
NaCl	5 g/liter	1,250 (21)
Glucose	200 g/liter	4,000 (22)
$MgSO_4 \cdot 7H_2O$	240 g/liter	240 (0.970)
$FeSO_4 \cdot 7H_2O$	5.0 g/liter	5 (0.018)
Minerals		
$MnSO_4 \cdot H_2O$	1.0 g/liter	5 (0.029)
$CaCl_2 \cdot 2H_2O$	3.0 g/liter	15 (0.012)
$ZnSO_4 \cdot 7H_2O$	1.0 g/liter	5 (0.017)
$CuSO_4 \cdot 5H_2O$	0.02 g/liter	0.1 (4.0×10^{-4})
$CoCl_2 \cdot 6H_2O$	0.02 g/liter	0.1 (4.0×10^{-4})
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.02 g/liter	0.1 (8.0×10^{-5})
Vitamins		
p-Aminobenzoic acid	0.04 g/liter	0.1 (7.3×10^{-4})
Niacinamide	0.4 g/liter	1.0 (8.2×10^{-3})
DL-Pantothenic acid, calcium salt	0.2 g/liter	0.5 (2.1×10^{-3})
Pyridoxal HCl	0.12 g/liter	0.3 (1.5×10^{-3})
Pyridoxamine $(HCl)_2$	0.12 g/liter	0.3 (1.2×10^{-3})
Pyridoxine HCl	0.4 g/liter	1.0 (4.9×10^{-3})
Riboflavin	0.2 g/liter	0.5 (1.3×10^{-3})
Thiamine HCl	0.2	0.5 (1.5×10^{-3})
d-Biotin	—[c]	0.001 (4.1×10^{-6})
Folic acid	—	0.01 (2.3×10^{-5})
Purines and pyrimidines[d]		
Adenine sulfate $\cdot H_2O$	1.0 g/liter	10 (0.054)
Uracil	1.0 g/liter	10 (0.089)
Guanine HCl $\cdot H_2O$	1.0 g/liter	10 (0.054)
Xanthine	1.0 g/liter	10 (0.066)
Amino acids[e]		
L-Alanine	400 mg (dry wt)	200 (2.2)
L-Arginine	484 mg (dry wt)	242 (1.4)
L-Asparagine	800 mg (dry wt)	400 (3.0)
L-Aspartic acid	200 mg (dry wt)	100 (0.75)
L-Cysteine	100 mg (dry wt)	50 (0.41)
L-Glutamic acid	600 mg (dry wt)	300 (2.0)
Glycine	200 mg (dry wt)	100 (1.3)
L-Histidine	124 mg (dry wt)	62 (0.39)
L-Isoleucine	500 mg (dry wt)	250 (1.9)
L-Leucine	500 mg (dry wt)	250 (1.9)
L-Lysine	500 mg (dry wt)	250 (1.4)
DL-Methionine	200 mg (dry wt)	100 (0.67)
L-Phenylalanine	200 mg (dry wt)	100 (0.61)
L-Proline	200 mg (dry wt)	100 (0.87)
L-Serine	100 mg (dry wt)	50 (0.48)
L-Threonine	400 mg (dry wt)	200 (1.7)
L-Tryptophan	80 mg (dry wt)	40 (0.20)
L-Tyrosine	200 mg (dry wt)	100 (0.55)
L-Valine	500 mg (dry wt)	250 (2.1)

[a] Defined medium was made by combining 250 ml of M9 salts, 20 ml of glucose solution, 1 ml of $MgSO_4$ solution, 1 ml of $FeSO_4$ solution, 5 ml of trace minerals, 20 ml of each purine and pyrimidine, 3 ml of vitamins, and 3.1 g of the amino acid mixture dissolved in 500 ml of distilled water. The volume was brought to 1 liter with warm distilled water, and the pH was adjusted to 7.0. The medium was filter sterilized with a 0.45-μm-pore-size filter.

[b] Defined minimal medium was made by combining 250 ml of M9 salts, 20 ml of glucose solution, 1 ml of $MgSO_4$ solution, 3 ml of vitamins containing only niacinamide and pantothenic acid, and the appropriate amino acids for the strain dissolved in 500 ml of distilled water. The volume was brought to 1 liter with warm distilled water, and the pH was adjusted to 7.0. The medium was filter sterilized with a 0.45-μm-pore-size filter.

[c] —, biotin and folic acid were prepared in concentrations of 1 mg/ml. Biotin was solubilized by warming, and folic acid was solubilized by the addition of NH_4OH. From these solutions, 0.02 ml of biotin and 0.2 ml of folic acid were added to a 50-ml solution of the remaining vitamins, brought to a final volume of 60 ml, and filter sterilized.

[d] Separate xanthine and guanine stock solutions of 1 mg/ml were made by adding concentrated HCl drop by drop until dissolved. Adenine and uracil were dissolved separately in warm water to the same concentration.

[e] Amino acids were combined dry in the indicated amounts, ground in a mortar and pestle, and stored at 20°C. A total of 3.1 g of the amino acid mixture was added per 1,000 ml of medium.

plete defined medium (pH 7.0) with concentrations ranging from 120 to 700 mosmol/kg of H_2O. Osmolality was varied by changing the concentration of the M9 salt solution and confirmed with a model 3DII DiGi-Matic osmometer (Advanced Instruments, Needham Heights, Mass.).

Carbon source utilization was determined for fructose, glucose, galactose, glycerol, maltose, mannose, ribose, and sucrose at a concentration of 4 g/liter. Although all sources except sucrose provided for saturated growth of *E. ictaluri*, glucose was selected for determination of optimal concentration by inoculating complete defined medium at pH 7 containing concentrations of 0, 4, 20, 50, and 100 g/liter.

Optimal pH was determined by dividing a batch of complete defined medium into six aliquots and adjusting the pH to 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0 by adding either 1 N NaOH or 1 N HCl. The pH of the medium was measured prior to inoculation (initial) and after 24 h of incubation (final).

The optimized complete defined medium was then used in a series of deletion and add-back experiments to determine the minimal essential nutrients required for growth of *E. ictaluri*. Initial deletion of entire nutrient groups, including 10 vitamins, eight minerals, two purines and two pyrimidines, or 19 amino acids, was followed by deletions of individual components when the group was determined to be necessary for growth. Subsequently, each nutrient that yielded no growth when deleted was individually restored to the medium to confirm that it was essential for growth.

Determination of growth curves. Growth of *E. ictaluri* in complete broth medium with 19 amino acids was compared with growth in minimal medium with 19 amino acids and with growth in minimal medium containing only the specific amino acids required for growth of each particular strain (see below). Cultures were grown in 250-ml Klett flasks at 30°C, and cell density was measured in Klett units for a 144-h time period. Samples for the first 24 h were taken at 2-h intervals, and subsequent samples were taken every 12 h.

Statistical analysis. Data were analyzed by analysis of variance. For effects found to be significant, paired comparisons were evaluated by Tukey's procedure (Statistix Version 4.1; Analytical Software, Tallahassee, Fla.).

RESULTS

Optimal osmolality for growth was observed at 260 and at 390 mosmol/kg (Table 2). An osmotic concentration of 570 mosmol/kg resulted in a slight reduction in bacterial growth, while growth was suboptimum at concentrations of 120 and 690 mosmol/kg. Although bacterial growth levels in salt concentrations of 260 and 390 mosmol/kg were equivalent, 390 mosmol/kg was selected for subsequent experiments. Optimal pH for growth was observed at pH 7.0 (Table 3). Subsequent testing of a variety of carbon sources, including fructose, galactose, glucose, glycerol, maltose, mannose, ribose, and sucrose, indicated that there was little significant difference in growth between many of them after 24 h (Table 4). Glucose was selected for determination of optimal concentration by evaluating *E. ictaluri* cultures grown with various glucose concentrations, and because growth in 4 g of glucose per liter was significantly higher at 12 and 24 h of incubation than any other glucose concentration, the concentration of 4 g/liter was chosen for subsequent experiments (Table 5).

Initial determination of vitamin, purine and pyrimidine, mineral, and amino acid requirements utilized a defined medium that was made deficient in the individual nutrient groups. Results indicated that deletion of the nucleotides did not affect growth, deletion of the vitamins or amino acids completely abrogated growth, and deletion of the minerals reduced final Klett readings by about 50%.

Subsequent deletion of individual vitamins indicated that pantothenic acid and niacinamide were essential for growth (Table 6). Testing of four strains in triplicate tubes of defined medium containing only pantothenic acid and niacinamide re-

TABLE 2. Effect of osmolality on the growth of *E. ictaluri* in complete defined medium

mosmol/kg of H_2O	Klett units[a]	
	12 h	24 h
120	229 ± 16.8B	235 ± 11.3C
260	363 ± 3.8A	383 ± 2.6A
390	364 ± 7.0A	371 ± 6.0A
570	206 ± 8.7B	325 ± 1.5B
690	73 ± 14.5C	212 ± 9.5C

[a] Data represent mean values of triplicate flasks (± standard deviations). Within columns, means followed by the same letter are not significantly different ($P < 0.01$).

sulted in mean growth of 362 ± 21.3 Klett units (mean ± standard deviation), while mean growth in medium containing all 10 of the vitamins was 361 ± 6.1 Klett units. Mineral deletion experiments indicated that magnesium was the only mineral component required for maximal growth (Table 7).

At this point, a medium containing basal salts, pantothenic acid, niacinamide, magnesium, and all 19 amino acids (MM19) supported excellent growth for 10 strains of *E. ictaluri*. Subsequent deletion studies with individual amino acids indicated that minimal amino acid requirements varied depending on the strain of *E. ictaluri* (Table 8). For purposes of defining a minimal medium, amino acids that reduced growth to less than 200 Klett units in studies with MM19 less individual amino acids (MM19-1) were used in add-back studies with the 10 individual strains. These results, reported in Table 9, indicated that the final amino acid content of the minimal medium is strain dependent. The preparation and composition of the complete defined and defined minimal media are presented in Table 1.

Growth on solid media. Colonies of *E. ictaluri* produced on BHI broth are smooth, circular, and slightly convex (7). Colony morphology on the complete defined medium and on MM19 was similar to that on BHI broth after 48 to 72 h, although the colonies on MM19 were smaller. Growth was not observed for any strain on solid minimal media with minimal amino acids.

Determination of growth curves. In general, all *E. ictaluri* strains tested reached peak cell density between 12 and 24 h of incubation in complete broth with 19 amino acids. When bacteria were grown in MM19 broth, growth and cell yields were reduced only slightly, but 48 h was required to reach peak cell density. Use of minimal medium broth containing the specific amino acids required by the individual strains resulted in detectable growth after about 48 h of incubation and peak cell density at about 96 h. For individual strains that grew well in minimal medium broth, growth was similar to that in the MM19 broth.

TABLE 3. Effect of pH on the growth of *E. ictaluri* in complete defined medium for 24 h

Initial pH	Final pH	Klett units
4.0	3.9 ± 0.006	6 ± 1.7D
5.0	4.2 ± 0.013	119 ± 3.4C
6.0	4.6 ± 0.007	298 ± 5.0B
7.0	5.1 ± 0.011	488 ± 4.7A
8.0	7.2 ± 0.095	163 ± 29.6C
9.0	8.8 ± 0.052	4 ± 2.9D

[a] Data represent mean values of triplicate flasks (± standard deviations). Within columns, means followed by the same letter are not significantly different ($P < 0.01$).

TABLE 4. Examination of various carbon sources supporting the 24-h growth of two *E. ictaluri* strains in complete defined medium

Sugar	Klett units[a]	
	Strain 93-154	Strain 93-146
Fructose	430 ± 45.9A	478 ± 7.6A
Galactose	460 ± 25.6A	487 ± 7.5A
Glucose	472 ± 43.1A	426 ± 2.9AB
Glycerol	471 ± 17.9A	475 ± 11.1A
Mannose	274 ± 14.5B	475 ± 22.9A
Maltose	493 ± 17.5A	472 ± 10.8A
Ribose	494 ± 16.5A	499 ± 8.1A
Sucrose	241 ± 12.0B	225 ± 50.6C
None	242 ± 1.5B	351 ± 33.5B

[a] Data represent mean values of triplicate flasks of duplicated experiments (± standard deviations). Within columns, means followed by the same letter are not significantly different ($P < 0.01$).

DISCUSSION

This report describes a complete defined medium and a minimal defined medium that support sustained growth of *E. ictaluri*. The complete defined medium, used as a base medium to develop the minimal medium, consisted of 46 individual components. The minimal medium reduces those 46 ingredients to eight essential components plus the specific amino acids required for the strain in question.

Optimal growth of *E. ictaluri* was recorded for osmolalities of 260 to 390 mosmol/kg of water, with reduced growth both above and below that range. This is in agreement with preliminary determinations in our laboratory that indicate that BHI broth, which supports excellent growth of *E. ictaluri*, has an osmolality of 360 mosmol/kg of water. Furthermore, previous work by Waltman et al. (24) and Plumb and Vinitnantharat (17) demonstrated that *E. ictaluri* is able to grow in BHI broth supplemented with up to 1.5% NaCl but not in NaCl concentrations greater than 1.5%. Plumb and Vinitnantharat (17) also showed that growth was reduced even in 0.5% NaCl compared with growth in BHI broth without NaCl. As determined in our lab, BHI broth cultures supplemented with 0.5% NaCl and 1.0% NaCl have osmolalities of 521 and 715 mosmol/kg of water, respectively.

High-density growth was observed within a narrow pH range of 7.0 to 7.5 in the defined medium. A 61% reduction in growth was observed in media at pH 6.0, with a 23 and 33% reduction in growth at pH 5.0 and 8.0, respectively. There was no growth of *E. ictaluri* in culture media at either pH 4.0 or 9.0. These data are consistent with those previously reported for BHI broth. The optimum pH range for *E. ictaluri* growth in BHI broth, as reported by Plumb and Vinitnantharat (17), was

TABLE 5. Effect of glucose concentration on the growth of *E. ictaluri* in complete defined medium

Glucose concn (g/liter)	Klett units[a]	
	12 h	24 h
0	153 ± 32.5C	192 ± 4.5D
4	426 ± 3.5A	461 ± 5.1A
20	338 ± 11.5B	420 ± 3.5B
50	97 ± 32.0C	289 ± 12.7C
100	15 ± 2.5D	15 ± 2.6E

[a] Data represent mean values of triplicate flasks (± standard deviations). Within columns, means followed by the same letter are not significantly different ($P < 0.01$).

TABLE 6. Growth of *E. ictaluri* in a defined medium deficient in a specific vitamin

Deleted vitamin	Klett units[a]
Thiamine	335 ± 17.6B
Pyridoxine	326 ± 4.5B
Pyridoxal	324 ± 5.4B
Pyridoxamine	320 ± 2.4B
Pantothenic acid	27 ± 0.82A
Riboflavin	324 ± 7.4B
Niacinamide	8 ± 1.6A
PABA	367 ± 9.5B
Biotin	327 ± 5.7B
Folic acid	335 ± 8.2B
None	361 ± 6.1B

[a] Data represent mean values of triplicate tubes at 24 h of growth (± standard deviations). Means followed by the same letter are not significantly different ($P < 0.01$).

between 7.0 and 7.5 pH units, with reduced growth at pH 6.0 and 8.0.

Although wild-type *E. ictaluri* is a facultative anaerobe, it is capable of fermenting only a limited number of carbohydrates, with only glucose, fructose, galactose, mannose, and ribose reported consistently (7, 8, 24). There are, however, variable reports concerning the ability of *E. ictaluri* to utilize glycerol. Using purple broth base, Waltman et al. (24) showed that 118 of 118 strains tested were able to grow in glycerol, while Plumb and Vinitnantharat (17), who also used purple broth base, reported that 40 of 40 strains were negative for glycerol. Our results indicate that excellent growth of *E. ictaluri* was obtained with glycerol as a carbon source. It should also be noted that *E. ictaluri* will grow weakly in defined media without sugar supplementation, presumably by using alternative carbon sources, such as amino acids, as does *Flexibacter columnaris* (22).

Variable results were obtained in this study when individual amino acids were omitted from the initial 19-amino-acid mix, with results dependent on the strain used. As indicated in Table 9, a medium composed of minimal medium supplemented with the individual amino acids that had previously given reduced growth when omitted from the various media of the MM19-1 studies did not result in a minimally defined medium for any strain examined. Absolute requirements for certain individual amino acids varied from zero to six. For example, as shown in Table 8, strains 587-671 and 89-9 appear to require only cysteine supplementation, but growth in minimal medium plus cysteine resulted in less than optimal growth of 169 Klett units for strain 587-671 and no growth for strain 89-9 (Table 9). On the other hand, strain 91-638, which also appeared to require cysteine, grew well in medium without any amino acid supplementation. Some strains, such as 83-189 and 90-476, responded with excellent growth in media when only the amino acids indicated as essential in the MM19-1 studies were supplied. Strain 92-266 showed stricter amino acid requirements than did any other strain tested. This strain failed to grow when as many as nine amino acids were added back. On the other hand, the requirement for nucleotides, vitamins, and trace metals was not strain specific.

The degree of auxotrophy in *E. ictaluri* is somewhat surprising, although variable, strain-dependent amino acid or vitamin requirements are not uncommon in bacteria (4, 12, 25). *E. ictaluri* is reported to be extremely stable and homogeneous in its biochemical reactivity (24), plasmid profile (10, 14, 23), and serological relatedness (1, 16, 17). Recent results indicate that *E. ictaluri* possesses one or two restriction endonucleases that

TABLE 7. Growth of *E. ictaluri* in a defined medium deficient in a specific mineral

Deleted mineral	Klett units[a]
Magnesium	147 ± 57.0A
Iron	395 ± 49.7B
Cobalt	425 ± 49.1B
Calcium	432 ± 54.0B
Copper	432 ± 46.0B
Molybdenum	424 ± 62.9B
Manganese	433 ± 41.6B
Zinc	431 ± 41.6B
No supplement	165 ± 63.0A

[a] Data represent mean values of seven tubes at 24 h of growth (± standard deviations). Means followed by the same letter are not significantly different ($P < 0.01$).

TABLE 8. Growth of *E. ictaluri* in defined media deficient in a specific amino acid at 24 h

Deleted amino acid	Klett units of strain:									
	83-189	587-671	89-9	90-476	91-581	91-638	92-266	93-146	93-264	93-297
Alanine	488	490	450	545	387	420	337	403	350	268
Arginine	430	444	380	475	74	400	260	360	316	394
Asparagine	470	490	460	535	414	436	325	421	390	349
Aspartic acid	490	447	405	502	94	405	296	371	337	285
Cysteine	35	132	66	125	10	178	41	148	34	24
Glutamic acid	495	520	475	550	228	476	272	454	368	350
Glycine	420	380	330	455	105	349	234	344	300	190
Histidine	470	471	475	525	275	409	352	402	342	307
Isoleucine	460	447	430	523	226	428	254	400	349	354
Leucine	485	473	425	525	200	415	282	399	316	207
Lysine	480	500	495	570	318	410	232	415	345	327
Methionine	348	375	315	410	85	318	178	385	228	176
Phenylalanine	372	460	266	375	5	440	40	180	37	23
Proline	460	480	485	505	330	405	292	368	334	260
Serine	460	458	410	520	134	300	305	45	125	12
Threonine	392	414	337	470	254	364	209	325	295	268
Tryptophan	430	420	355	460	232	400	330	377	302	304
Tyrosine	405	450	345	520	235	374	111	232	300	99
Valine	465	470	470	523	167	395	186	158	325	330

TABLE 9. Effect of supplementation with various amino acids on the growth of 10 strains of *E. ictaluri* in a defined minimal medium

Supplemented amino acid(s)[a]	Klett units of strain[b]:									
	83-189	587-671	89-9	90-476	91-581	91-638	92-266	93-146	93-264	93-297
None						439		147	93	102
C	347	169	NG[c]	355	NG	370	NG	219	170	140
S						395		136	113	123
C, F	268	107	31	250	NG	378	NG	260	223	170
C, F, S						240		205	128	98
C, F, V	303	33	NG	144	NG		NG			
C, F, S, V						262		192	171	103
C, F, V, M, Y	260	150	NG	244	NG		NG			
C, F, V, M, Y, L, P, S, D	404	390	390	395	230		NG			
C, F, S, M, Y, G						335		200	159	210

[a] C, cysteine; S, serine; F, phenylalanine; V, valine; M, methionine; Y, tyrosine; L, leucine; P, proline; D, aspartic acid; G, glycine.

[b] Values at 96 h of growth are given. Combinations where no values are given were not evaluated.

[c] NG, no growth.

may act to restrict the intrusion of foreign DNA (26). This could explain the homogeneity of the major phenotypic traits and also account for the persistence of minor phenotypic mutations.

The possible toxic effects of various amino acids in *E. ictaluri* are not clear, although some amino acid toxicity was observed with *E. ictaluri* when essential amino acids were reintroduced in various combinations. For example, when valine was added in combination with cysteine and phenylalanine at concentrations described in Table 1, a threefold drop in growth occurred in the cysteine-requiring strains. However, when a number of other amino acids, such as methionine and tyrosine or methionine, tyrosine, leucine, proline, or serine, were added to valine, cysteine, and phenylalanine, the growth level was restored to that obtained with cysteine and phenylalanine alone. Further work is required to determine the synergistic or antagonistic effects of the amino acid combinations utilized by various strains of *E. ictaluri*.

Combination toxicity for amino acids was first observed by Gladstone (5) for *Bacillus anthracis*. The amino acids that abrogate these toxic effects were themselves toxic when added singly. The author also showed that certain amino acids are necessary for growth but not essential since growth still occurs, although at a lower rate, when these amino acids are absent. Reduction of growth rate may indicate that the organism can synthesize some amino acids when they are not added to the medium (5).

Culture of *E. ictaluri* generally requires the use of complex or enriched media for growth. Although a medium for the selective isolation of *E. ictaluri* that is useful in the isolation of *E. ictaluri* from mixed flora in environmental reservoirs and clinical sources was described previously (21), it is also of a complex, undefined formulation. The minimal medium reported here is well defined and should be preferable to complex media for conducting future physiological, nutritional, and pathogenicity studies on *E. ictaluri.*

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