Plasmid Homologies in Edwardsiella ictaluri†

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Plasmids from all available non-channel catfish isolates of Edwardsiella ictaluri were classified by gel electrophoresis and hybridization methods. All isolates, regardless of source, contained classes of homologous plasmids with similar sizes.

Enteric septicemia, caused by Edwardsiella ictaluri (4), is a major disease of cultured channel catfish (Ictalurus punctatus) (9). This disease can be the cause of significant expense to a catfish producer due to loss of fish and the high cost of the antibiotics necessary for its control. Therefore, it is of great interest to achieve an early and accurate diagnosis so that controls may be employed quickly.

One promising detection scheme makes use of the fact that all isolates of this species from the Mississippi delta catfish production region tested to date had the same plasmid profile (8, 10, 14). It was also observed that all of the plasmids present in the bacteria would hybridize to each other (14). Therefore, one of the bacterial plasmids could be used as a nucleic acid probe to indicate the presence of the bacteria in a fish.

While it was originally seen that E. ictaluri infected few fish other than ictalurids (11), there have been reports of E. ictaluri-like bacteria occurring in natural infections of several different fish types worldwide. These fish are green knifefish (Eigmounia virescens) (7), danio (Danio devario) (15), rosy barb (Puntius crouchouius) (5), walking catfish (Clarisr battrichus) (6), white catfish (Ictalurus catus) (10), and harlequin tetra (Rosbaria heterophora) (C. Starlipes, personal communication).

The bacterial isolates have similar biochemical characteristics to each other and to E. ictaluri (5, 6), but no direct genetic comparisons have been made. In an attempt to further characterize the relatedness among these different isolates, plasmid profiles were examined and plasmid-to-plasmid hybridizations were carried out for all known non-channel catfish isolates.

Isolates of E. ictaluri from channel catfish were obtained from the College of Veterinary Medicine, Mississippi State University. The isolates from walking catfish were obtained from W. A. Rogers (Auburn University). All other isolates were obtained from J. Newton (Louisiana State University). The initial locations of isolation were as follows: green knifefish, South America; danio, Florida; walking catfish, Thailand (three isolates); harlequin tetra, Australia (originally Indonesia; held in quarantine in Australia before release for sale); and white catfish, Maryland and Mississippi (two isolates).

The method of Davis et al. (1) was used to purify plasmids from each isolate. Purity was determined by agarose gel electrophoresis (14). In order to prepare plasmid probes, 5 μg of E. ictaluri isolate DBES-2 plasmids were electrophoresed in a 0.8% low-melting-point agarose gel (FMC Corp.). After visualization, the upper supercoiled plasmid (Fig. 1, lane 2, 5.6 kilobases [kb]) or both supercoiled plasmids were excised from the gel, weighed to estimate the gel volume, and had water added at 3 ml/g of gel. The sample was heated to 100°C for 5 min to melt the agarose and denature the DNA. DNA was labeled with [α-32P]dCTP (3,000 Ci/mol, ICN) by the random-priming method of Feinberg and Vogelstein (2, 3).

Gels containing plasmid DNA were Southern blotted (13) to Zeta-Probe (Bio-Rad Laboratories), a nylon membrane. The alkaline transfer method of Reed and Mann (12) was used. The membrane was rinsed in 2× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), air dried, and stored in a heat-sealed plastic bag.

Prehybridization and hybridization were carried out in 325-ml Styrene acrylonitrile boxes (Nalgene) with close-fitting lids and rubber bands to prevent evaporation. A prehybridization solution of 4× SSPE (1× SSPE is 0.72 M NaCl, 0.04 M Na2PO4 [pH 7.7], 4 mM sodium dodecyl sulfate [SDS]-0.5 mg of salmon sperm DNA per ml-0.5% [wt/vol] Blotto (Carnation powdered milk) was added at a rate of 1 ml of solution per 4 cm2 of membrane. Prehybridization was carried out for at least 4 h at 65°C with gentle agitation. This solution was removed prior to beginning hybridization. An identical solution plus 10% polyethylene glycol was used for hybridization. The probe was added, and hybridization was carried out at 65°C overnight with gentle agitation.

After hybridization, the solution with probe was pipetted off and the membrane was rinsed in 2× SSC. Successive 15-min room temperature washes in 100 ml of 2× SSC-0.1% SDS, 100 ml of 0.5× SSC-0.1% SDS, and 100 ml of 0.1× SSC-0.1% SDS and a final wash with 100 ml of 0.1× SSC-1% SDS at 50°C for 30 min were then carried out with gentle agitation.

Membranes were blotted dry, wrapped in plastic wrap, and autoradiographed on Kodak XR film with a DuPont Cronex Hi-plus intensifying screen at −80°C for 12 h to 3 days. The film was then developed and analyzed.

Plasmids from each isolate are shown in Fig. 1. This gel shows each plasmid in two different conformations: supercoiled plasmids, which migrated farther in the gel, and presumably relaxed circular plasmids, which did not migrate as far. Lanes 2, 8, 10, and 11 had large quantities of chromosomal DNA above all of the plasmids. In lane 2 is a standard channel catfish isolate, which contained two plasmids of 5.6 kb (band A) and 4.7 kb (band B). Other lanes contained plasmids of 4 and 3 kb. DNA from isolates in lanes 3253
FIG. 1. Electropherogram of purified plasmid DNA from E. ictaluri isolates. Lane 1, Kilobase ladder; lane 2, channel catfish isolate; lanes 3, 4, and 5, walking catfish isolates; lane 6, Maryland white catfish isolate; lanes 7 and 8, Mississippi white catfish isolates; lane 9, danio isolate; lane 10, green knifefish isolate; lane 11, harlequin tetra isolate; lane 12, supercoiled kilobase ladder. The sizes indicated in the figure are from this ladder. A and B indicate the upper (5.7 kb) and lower (4.6 kb) supercoiled plasmids in the channel catfish isolate.

3 to 8 showed bands located near chromosomal DNA. These would be plasmids greater than 20 kb long.

Figure 2 shows an autoradiogram resulting from hybridization of the upper supercoiled band (band A, 5.6 kb) from the channel catfish isolate (lane 2 of Fig. 1) to a Southern blot of the gel in Fig. 1. This probe clearly hybridized to every 5.6-kb band on the gel. Every isolate but the Maryland white catfish isolate in lane 6 had this plasmid. Hybridization was also seen to the relaxed circular form of this plasmid in all but lane 6.

Figure 3 shows the results of hybridizing a probe consisting of a mixture of the A and B plasmids of the channel catfish isolate to gels that contained plasmids from isolates found in lanes 2 (channel catfish), 6 (Maryland white catfish), 9 (danio), and 10 (green knifefish) of Fig. 1 and 2. Hybridization was seen to all forms of the plasmid present in each lane.

The work of Newton et al. (10) showed that several isolates of E. ictaluri from different fish species had different plasmids profiles from isolates found in channel catfish from the Mississippi delta. This finding was confirmed and extended in the results shown in Fig. 1. Plasmids from every known isolate of E. ictaluri are displayed.

There was a definite pattern to the plasmids. By the criterion of size alone, there were only five different small to medium plasmids. Of these, the plasmids of 4, 4.7, and 5.6 kb were the most common. Every isolate examined had one or two of these plasmids, and the 4 kb plasmid was never seen with the 4.7-kb plasmid. In two cases, we saw two other plasmids; one isolate had a 3-kb plasmid, and one had an 8.7-kb plasmid. In several isolates we saw large plasmids.

FIG. 2. Autoradiogram from the hybridization of a plasmid probe constructed from band A (upper band) of the channel catfish isolate. The gel shown in Fig. 1 was initially blotted and then hybridized. The lanes are identical to those in Fig. 1.

but it was not possible to compare their sizes because of the lack of resolution of the gel in this size range. The large plasmid in Fig. 1, lane 8, may be the relaxed circular form of the 8.7-kb plasmid. The Maryland white catfish isolate was unique in that it had only one small plasmid and was the only one lacking the 5.6-kb plasmid.

Size correlations may reflect identity or may simply be fortuitous. A better idea of relationships can be achieved by hybridizations. When a purified form of the 5.6-kb channel catfish plasmid was used as a hybridization probe, every other 5.6-kb plasmid was complementary. The conditions of hybridization and washing were very stringent, and so this homology should be relatively extensive. It seems clear that all the 5.6-kb plasmids are closely related and may be identical.

Previous work (14) suggested that all plasmids from channel catfish isolates shared homologies. This is in disagreement with the results presented above, in which only one band was hybridized. It is likely that the earlier work used an impure preparation of the plasmid as a probe. Both the 4.7- and 5.6-kb bands may have been present. This hypothesis is supported by the results shown in Fig. 3. Here, a mixed probe of both these plasmids was used, and there was hybridization to all small and medium-sized plasmids. It is especially interesting that the single small band in the lane 6 isolate showed complementarity, as did the 4-kb band in the lane 10 isolate. All other 4-kb bands were also complementary to this mixed probe (data not shown). Since these plasmids were not hybridized by the 5.6-kb band, they must be homologous to the 4.7-kb plasmid.

Therefore, it appears that all E. ictaluri isolates contain similar small plasmids. A family of 4-kb or 4.7-kb plasmids that are homologous are found in every isolate. Restriction map comparisons of these two plasmid sizes may reveal that the smaller one has simply lost a segment of DNA contained in the larger one. Every isolate except the Maryland white catfish isolate contained the 5.6-kb plasmid. Again, these are likely to be very similar since all hybridized strongly under stringent conditions.

The extra band seen in the lane 10 isolate seemed to hybridize slightly in both Fig. 2 and 3. This level of hybridization could represent nonspecific binding, but the stringency of the conditions argues against that. The extra band in the lane 8 isolate seemed to hybridize with the 5.6-kb probe, but the level of labeling present in Fig. 2 seems very low considering the intensity of the band in Fig. 1. This is probably nonspecific binding. No hybridization was seen to any large plasmids or chromosomal DNA.

The presence of these homologous families of plasmids in E. ictaluri isolates from around the world raises two important points. First, what is the role of these extrachromosomal pieces of DNA? They seem to be highly conserved in E. ictaluri strains infecting various fish in diverse geographic locations. Are they essential for bacterial growth? Do they
code for virulence factors or antibiotic resistance factors? It would be interesting to study the relative resistance and virulence of the different isolates in a channel catfish host. The isolate from the Maryland white catfish would be of special interest because of its lack of one of the small plasmids. The second point is a more practical one. The fact that every isolate of E. ictaluri thus far observed has some plasmid that hybridizes to one of the small plasmids of the channel catfish isolate means that a mixed probe consisting of both plasmids would readily identify any such isolate as E. ictaluri. Speyerer and Boyle (14) showed that a mixed probe does not hybridize to plasmids or chromosomal DNA of other bacteria, including the related E. tarda. Therefore, such a probe would be an E. ictaluri-specific probe. The antigenic characteristics of the various isolates are still not certain, but Kasornchandra et al. (6) have shown that the walking catfish isolates may represent a different serological type. Therefore, an antibody test for the bacteria may not yield definitive results.

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LITERATURE CITED