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Plasmid Profiling of *Vibrio salmonicida* for Epidemiological Studies of Cold-Water Vibriosis in Atlantic Salmon (*Salmo salar*) and Cod (*Gadus morhua*)

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In 1988, a new plasmid profile was observed for *Vibrio salmonicida* isolated from cod (*Gadus morhua*) and Atlantic salmon (*Salmo salar*) in fish farms in northern Norway. This new plasmid profile, which consisted of plasmids of 61, 21, 3.4, and 2.8 megadaltons, is 1 of 11 plasmid profiles which have so far been observed for *V. salmonicida*. Plasmid profiling and plasmid DNA hybridization were used in epidemiological studies of cold-water vibriosis. Our results indicate that *V. salmonicida* was transmitted from Atlantic salmon to cod and vice versa. The 61-megadalton plasmid was found exclusively in *V. salmonicida* strains originating from northern Norway, which is the only area in which this plasmid has ever been observed. Plasmid DNA hybridization and restriction endonuclease analysis show that the plasmid DNA of *V. salmonicida* remained stable throughout a 7-year survey.

Vibrio salmonicida, which is the causative agent of cold-water vibriosis (6), has until recently been isolated only from salmonids. In autumn 1988, however, the bacterium was isolated from diseased Arcto-Norwegian cod fry (*Gadus morhua*) (12). The main clinical symptoms of cod fry infected with *V. salmonicida* were cataract, hemorrhages in the head region (occasionally in the eyes), and swollen spleen (O. M. Rødseth, personal communication). These symptoms are similar to those observed when cod are infected with *Vibrio anguillarum* (vibriosis) (2, 3). The diseased cod fry, however, did not show the extensive internal hemorrhages which are characteristic of Atlantic salmon (*Salmo salar*) suffering from cold-water vibriosis (4, 5).

Arcto-Norwegian cod fry and Atlantic salmon were kept in neighboring floating-net pens in a fish farm in northern Norway (county of Finnmark). Initially, an outbreak of cold-water vibriosis was confirmed among the Atlantic salmon. About 1.5 months later, cod fry became diseased, with mortality reaching approximately 50% within 3 to 4 weeks (12). In the present work, *V. salmonicida* isolates from both diseased cod fry and Atlantic salmon in the disease outbreaks described above were examined. To date, plasmid profiles are the only tool which can be used to differentiate clearly between strains of *V. salmonicida* (15, 16). One purpose of this study was to use plasmid profiling and plasmid DNA hybridization to test the hypothesis that *V. salmonicida* was transmitted between Atlantic salmon and cod. Another purpose was to investigate the geographic distribution of *V. salmonicida* strains harboring a 61-megadalton (MDa) plasmid, which had previously been associated only with diseased Atlantic salmon in northern Norway in the period 1982 through 1983 (15). In spite of examining more than 250 strains of *V. salmonicida* from outbreaks all along the Norwegian coast during the period July 1986 through July 1987, Sørum et al. (15) did not detect the 61-MDa plasmid; neither did Wiik et al. (16) observe this plasmid in 31 Norwegian strains of *V. salmonicida* isolated between 1983 and 1987. Except for the plasmid profiles including the 61-MDa plasmid, none of the other plasmid profiles of *V. salmonicida* seem to be limited to a certain geographic area (15, 16).

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MATERIALS AND METHODS

Bacterial strains. Ten strains from diseased cod and 130 strains from diseased Atlantic salmon reared along the major part of the Norwegian coast from July 1987 to January 1989 were analyzed. Each strain was isolated from an individually infected fish. Details about selected isolates are given in Table 1. *V. salmonicida* A/2181/82 L1R, 41.839/83 L2R, NCMB 2262, and HI 9724 were used as controls in the plasmid screening. Identification of the strains was accomplished by testing for the *V. salmonicida*-specific surface layer antigen VS-P1 by using monoclonal antibodies (clone 2B5) in an enzyme-linked immunosorbent assay (8).

Plasmid screening. *V. salmonicida* strains were grown on 5% cattle blood agar with 2% NaCl at 15°C for 2 days before being cultivated in 5 ml of Luria broth with 2% NaCl at 15°C for 2 days in a roller drum. The plasmid isolation procedure of Kado and Liu (13) was used. In addition, the screening methods of Birnboim and Doly (1) and Wiik et al. (16) were performed as controls on some of the results.

Plasmid DNA purification. Plasmid DNAs from selected strains of *V. salmonicida* were isolated from 2-liter cultures of Luria broth with 2% NaCl by using a large-scale sodium dodecyl sulfate method (14). Plasmid DNA was purified in a cesium cloride-ethidium bromide (CsCl-EtBr) gradient; the EtBr was then extracted with isopropanol, and the salt was removed by dialysis against TE buffer (14).

Restriction endonuclease characterization. Purified plasmids from strains HI 11366-2 and A/2181/82 L1R were digested with restriction endonucleases *Hin*dIII and *Pst*I (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) according to the instructions of the manufacturer.

Agarose gel electrophoresis. Electrophoresis was generally performed in 1% agarose gels with Tris-borate-EDTA buffer

TABLE 1. Details about selected strains of *V. salmonicida*

Strain	Source[a]	Host	Plasmid profile (MDa)	Farm no.	Geographic origin (county) and yr of isolation
A/2181/82 L1R	NVI	*S. salar*	61, 21		Troms, 1982
41.839/83 L2R	NVI	*S. salar*	61, 21		Troms, 1983
NCMB 2262	NCMB	*S. salar*	21, 3.4		Hordaland, 1983
HI 9724	IMR	*S. salar*	21, 3.4, 2.8		Hordaland, 1987
2933/88 L-7	NVI	*S. salar*	61, 21, 3.4, 2.8	1	Finnmark, 1988
F.nr. 171	NVI	*G. morhua*	61, 21, 3.4, 2.8	1	Finnmark, 1988
HI 11366-1	IMR	*G. morhua*	21, 3.4	1	Finnmark, 1988
HI 11366-2	IMR	*G. morhua*	61, 21, 3.4, 2.8	1	Finnmark, 1988
HI 11366-5	IMR	*G. morhua*	61, 21, 3.4, 2.8	1	Finnmark, 1988
HI 11366-6	IMR	*G. morhua*	61, 21, 3.4, 2.8	1	Finnmark, 1988
12.1.89	NVI	*S. salar*	61, 21, 3.4, 2.8	1	Finnmark, 1989
88 1104. 60	NVI	*G. morhua*	61, 21, 3.4, 2.8	2	Nordland, 1988
89 S1	FHLS	*S. salar*	61, 21, 3.4, 2.8	3	Nordland, 1988
89 S2	FHLS	*S. salar*	61, 21, 3.4, 2.8	3	Nordland, 1988
LFI 1201	FORUT	*G. morhua*	61, 21, 3.4, 2.8	4	Finnmark, 1988
LFI 1226	FORUT	*G. morhua*	61, 21, 3.4, 2.8	4	Finnmark, 1988
LFI 1236	FORUT	*G. morhua*	61, 21, 3.4, 2.8	5	Finnmark, 1988
LFI 1237	FORUT	*G. morhua*	61, 21, 3.4, 2.8	5	Finnmark, 1988
89012601.04	RVLH	*S. salar*	61, 21, 3.4, 2.8	6	Finnmark, 1989
870903629 L1R	NVI	*S. salar*	61, 21	7	Troms, 1987

[a] NVI, National Veterinary Institute, P.O. Box 8156 Dep., N-0033 Oslo 1, Norway; IMR, Division of Aquaculture, Institute of Marine Research, P.O. Box 1870, N-5024 Nordnes, Norway; NCMB, National Collection of Marine Bacteria, Aberdeen, Scotland; FORUT, Foundation of Applied Research at the University of Tromsø, Tromsø, Norway; RVLH, The Regional Veterinary Laboratory of Harstad, N-9400 Harstad, Norway; FHLS, Food Hygienic Laboratory of Sortland, N-8400 Sortland, Norway.

(89 mM Tris, 89 mM boric acid, 2.5 mM EDTA [pH 8.0]) at 120 V (42 mA) for 3 h at room temperature in a vertical gel apparatus.

Blotting procedures. The transfer of DNA from agarose gels to GeneScreen nylon membranes (Dupont, NEN Research Products, Boston, Mass.) was performed by Southern blotting (14).

Probe DNA preparation and hybridization. The 21-MDa plasmid in a *V. salmonicida* strain harboring this plasmid only (A/1046/82 L1R) and the 61-MDa plasmid in strain HI 11366-2 were used as the DNA probes. The plasmid DNAs were isolated and purified as described above. The 61-MDa plasmid was separated from the remaining plasmids by running the purified plasmid DNA in a 1% horizontal agarose gel containing EtBr. After electrophoretic separation of the four plasmids of strain HI 11366-2, the areas in front of and behind the band containing the 61-MDa plasmid were removed with a scalpel under UV illumination. Low-melting-temperature agarose (SeaPlaque; FMC BioProducts, Rockland, Maine) containing EtBr was poured into the empty squares of the gel. Electrophoresis was continued until the 61-MDa plasmid band migrated into the low-melting-temperature agarose block. The band was cut out of the low-melting-temperature agarose and used directly as the probe in hybridization experiments. The DNA probes were labeled with ^{32}P using the random primer DNA labeling system (Bethesda Research Laboratories). The GeneScreen nylon membranes (Dupont, NEN Research Products) were prehybridized for 3 h and then hybridized overnight in a mixture of 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]), 0.1% sodium dodecyl sulfate, 1 mM EDTA, and 1× Denhardt solution at 42°C (14). The membranes were washed in 5× SSC–0.1% sodium dodecyl sulfate at 65°C and prepared for exposure with X-ray films overnight. Deprobing of the membranes was done with an excess of 0.4 M sodium hydroxide at 42°C for 30 min with gentle agitation. The equilibration was performed in 0.1× SSC–0.5% sodium dodecyl sulfate–0.2 M Tris hydrochloride (pH 7.5) at 42°C for 30 min with shaking.

Biochemical tests. Biochemical analyses of the HI 11366-2 and HI 11366-1 strains from cod (Table 1) were performed as described by Wiik et al. (16).

RESULTS

Identification. All the strains included in the present study had the surface layer antigen VS-P1, which is specific for *V. salmonicida* (8).

Plasmid profiles. The results obtained by the different plasmid screening methods (1, 13, 16) were in accordance with each other. The coexistence of four plasmids of 61, 21, 3.4, and 2.8 MDa was found in 9 of 10 *V. salmonicida* isolates from cod. The 10th strain (HI 11366-1) harbored only the 21- and 3.4-MDa plasmids (Table 1) (Fig. 1).

The geographic locations of the fish farms are shown in Fig. 2. Five isolates from cod originated from farm 1 (Table 1). The other isolates originated from diseased cod from farms 2, 4, and 5, which had received cod from farm 1. The shipping of cod from farm 1 to farm 2 was performed about 2 weeks before the cod in farm 1 became diseased. The distance between farms 1 and 2 is about 400 km.

The 130 remaining *V. salmonicida* strains were isolated from Atlantic salmon from the greater part of the Norwegian coast from July 1987 to January 1989. These strains represent the major part of the outbreaks of cold-water vibriosis in that period. Two of the strains were isolated from farm 1.

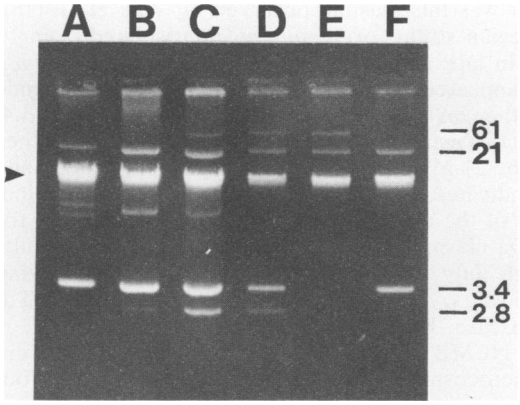

FIG. 1. Demonstration of plasmid profiles in selected strains of *V. salmonicida* by the method of Wiik et al. (16). Lanes: A, NCMB 2262; B, HI 9724; C, HI 11366-5; D, 2933/88 L-7; E, A/2181/82 L1R; F, HI 11366-1. Molecular masses (in megadaltons) are indicated. The molecular masses of the plasmids of the control strains NCMB 2262, HI 9724, and A/2181/82 L1R have been determined elsewhere (15, 16). Arrowhead indicates chromosomal bands.

One strain was isolated from a salmon before the outbreak among the cod occurred, and the other strain was isolated from a diseased salmon after the outbreak. The two isolates had plasmid profiles identical to those of the nine isolates from cod described above.

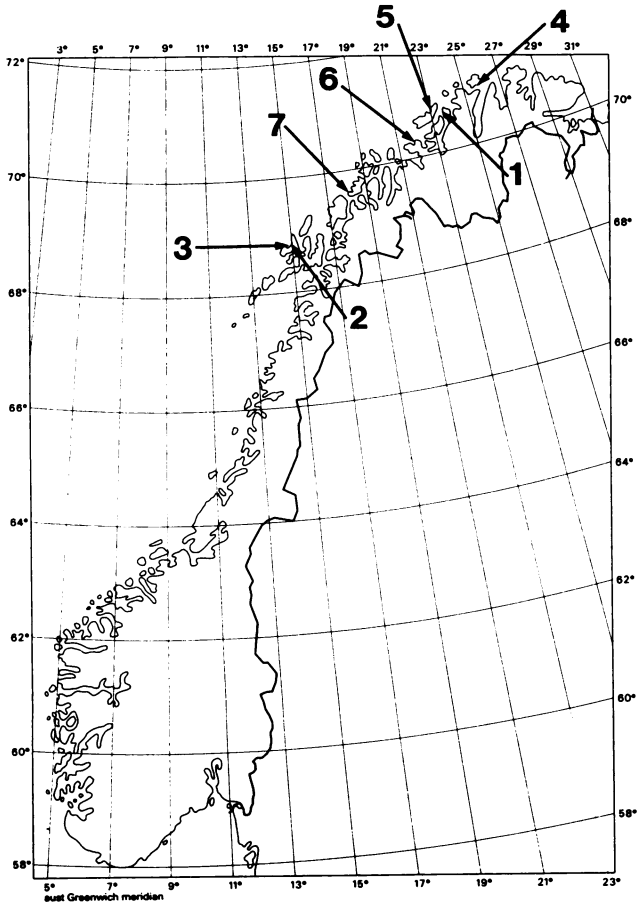

FIG. 2. Geographic distribution of the fish farms mentioned in the text and in Table 1. The numerals refer to farms 1 to 7.

TABLE 2. Distribution of different plasmid profiles of *V. salmonicida* along the Norwegian coast[a]

Plasmid profile (MDa)	No. of examined strains from:			
	Northern Norway	Mid-Norway	Western Norway	Unknown source
61, 21, 3.4, 2.8	14			
61, 21	1			
21, 3.4, 2.8	20	1	3	8
21, 3.4	13	38	24	6
21	3		6	3

[a] The isolates are from outbreaks of cold-water vibriosis in the period July 1987 to January 1989.

At farm 3, two strains with the same unique plasmid profile as that found in isolates from cod were isolated from Atlantic salmon attacked by cold-water vibriosis. This outbreak appeared after confirmation of cold-water vibriosis among the cod in farm 2. Farm 3 is situated about 5 km from farm 2 (Fig. 2). Water currents in this area lead directly from farm 2 to farm 3. In addition to the strains which could be related to the disease outbreak among cod, the unique plasmid profile was found in a strain isolated from Atlantic salmon in farm 6 in late January 1989, i.e., 4 months after the detection of disease among cod in farm 1.

The 61-MDa plasmid was also detected in one strain of *V. salmonicida* isolated from diseased Atlantic salmon in 1987 (farm 7). This strain had only the 21-MDa plasmid in addition to the 61-MDa plasmid.

The rest of the investigated strains harbored the 21-, 3.4-, and 2.8-MDa plasmids in three different combinations (Table 2). The coexistence of the 21- and 3.4-MDa plasmids was the dominating profile (97.4%) found in mid-Norway in the period July 1987 to January 1989.

In addition to the present and previously reported plasmid profiles (15, 16), a major part of the *V. salmonicida* strains harbor a 170-MDa R plasmid (H. Sørum, M. C. Roberts, K. Fossum, and J. H. Crosa, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, A-74, p. 13). This R plasmid will not be discussed in the present paper.

Restriction endonuclease characterization. Plasmid DNAs from strain HI 11366-2 from cod in farm 1 and strain A/2181/82 L1R isolated from Atlantic salmon in 1982 were compared after separate digestion with the restriction enzymes *Hin*dIII and *Pst*I (data not shown). The digests had to be interpreted on the basis of HI 11366-2 having the 3.4- and 2.8-MDa plasmids in addition to the plasmids in A/2181/82 L1R (61 and 21 MDa). The 21-MDa plasmids in the two strains had identical restriction endonuclease cleavage patterns. The pattern of the 61-MDa plasmid in strain HI 11366-2 was partly masked by the DNA from the 3.4-MDa plasmid. The 61-MDa plasmid exists in comparatively low copy number. The few bands of the 61-MDa plasmids which could be compared had identical molecular masses.

Hybridization experiments. The DNAs of the 21-MDa plasmids in all the strains tested were homologous. The 21-MDa plasmid, however, did not show homology to the 61-MDa plasmid in any of the investigated strains. The 21-MDa plasmid did seem to have sequences in common with the 2.8-MDa plasmids in strains from both Atlantic salmon and cod but did not show any homology with the 3.4-MDa plasmid.

The 61-MDa plasmid DNA probe was contaminated with small amounts of the 21-, 3.4-, and probably the 2.8-MDa plasmids. This probe hybridized with the 61-, 21-, 3.4-, and

2.8-MDa plasmids in all strains tested, including strains from a great part of the Norwegian coast. The 61-MDa plasmid from HI 11366-2 hybridized with all the other 61-MDa plasmids tested. The 61-MDa plasmids of *V. salmonicida* strains from diseased Atlantic salmon and cod in farm 1 appear to be homologous. The 61-MDa plasmids of cod from farms 1 and 2 also seem to be homologous. The 61-MDa plasmids isolated from cod and Atlantic salmon in 1988 and 1989 seem to be the same as the 61-MDa plasmid harbored by strains from Atlantic salmon in 1982 and 1983 (strains A/2181/82 L1R and 41.839/83 L2R).

Biochemical properties. The biochemical reaction patterns of strains from cod having four and two plasmids were identical to each other and to previously reported results for *V. salmonicida* (16).

DISCUSSION

The only characteristic which makes it possible to differentiate clearly between strains of *V. salmonicida* is plasmid profile. Otherwise, *V. salmonicida* is a very homogeneous species (11, 16, 17). As many as 11 different plasmid profiles have been observed for *V. salmonicida* (15, 16; H. Sørum, unpublished data). This makes plasmid profiling a valuable tool in epidemiological studies of cold-water vibriosis. *V. salmonicida* isolates from Atlantic salmon and cod in farm 1, from cod in farms 2, 4, and 5, and from Atlantic salmon in farm 3 had identical plasmid profiles. In autumn 1988, the coexistence of the 61-, 21-, 3.4-, and 2.8-MDa plasmids was restricted to *V. salmonicida* isolates from these five farms. The 61-, 21-, 3.4-, and 2.8-MDa plasmids in isolates from Atlantic salmon in farm 1 hybridized with the respective plasmids in isolates from cod. The unique, identical plasmid DNA in *V. salmonicida* isolates from cod and Atlantic salmon indicates that both fish species were infected by the same bacterial strain. On the condition that the latency period is approximately the same for Atlantic salmon and cod, the relative dates for the outbreaks of cold-water vibriosis together with the identical plasmid DNA indicate that a transmission of *V. salmonicida* from Atlantic salmon to cod in farm 1 occurred. Because of the preceding outbreaks among Atlantic salmon, the cod were exposed to an extensive infection pressure. Since the latency period for cod has not yet been established, however, independent infection of cod and Atlantic salmon or the transmission of the infection from cod to salmon in farm 1 cannot be ruled out completely. The cod from farms 2, 4, and 5 originated from farm 1. The outbreak of cold-water vibriosis among Atlantic salmon in farm 3 is very interesting. Farm 3 is situated about 5 km west of farm 2. The water currents in this area lead directly from farm 2 to farm 3. In other outbreaks of cold-water vibriosis in this area, the unique plasmid profile has not been found in the bacterial isolates. This indicates that *V. salmonicida* was transmitted from the cod in farm 2 to the Atlantic salmon in farm 3. The fact that a *V. salmonicida* strain isolated from Atlantic salmon in late January 1989 from farm 6 harbored the 61-, 21-, 3.4-, and 2.8-MDa plasmids indicates that strains having this new plasmid profile are becoming more widespread.

The 61-MDa plasmid has been restricted to *V. salmonicida* isolates from northern Norway. It was observed for the first time in a plasmid screening of *V. salmonicida* strains in the period 1982 through 1983 (15). In spite of examining more than 250 *V. salmonicida* isolates from the majority of outbreaks all along the Norwegian coast from July 1986 to July 1987, Sørum et al. (15) did not observe the 61-MDa plasmid; neither was this plasmid observed by Wiik et al. (16) in 31 Norwegian strains of *V. salmonicida* isolated from 1983 to 1987. In late 1987, the 61-MDa plasmid of *V. salmonicida* again appeared in the same area as it did in 1982 and 1983. From then on it was observed together with the 21-, 3.4-, and 2.8-MDa plasmids in the new profile and also together with only the 21-MDa plasmid as mainly seen in 1982 and 1983. This indicates that the 61-MDa plasmid reappeared independently of the cod-related plasmid profile. The fact that the 61-MDa plasmid was not observed in the period July 1986 through July 1987 indicates that bacteria harboring this plasmid survived in the environment without causing detectable disease. Hoff (10) has recently reported that *V. salmonicida* NCMB 2262 survived for more than a year in seawater microcosms. This bacterium has also been found in seawater samples from both fish farms and waters far away from fish farms (10 to 15 bacteria per ml) by using an immunofluorescence technique (9; B. Husevåg, K. A. Hoff, and Ø. Enger, personal communications). By this technique (9), large numbers of *V. salmonicida* have been found in sediments (10^5 to 10^6 bacteria per ml of wet sediments) under the pens in fish farms previously affected by cold-water vibriosis and also in a farm in which this disease had not occurred (7). Our results, together with the high survival potential in seawater and sediments, indicate that *V. salmonicida* is endemic in seawater and behaves as an opportunistic fish pathogen.

One of the five isolates from cod in farm 1 had a divergent plasmid profile consisting of only the 21- and 3.4-MDa plasmids. This strain was isolated from a cod which was smaller and had the clinical symptoms of a more prolonged disease than the remaining cod from farm 1 which were investigated. The occurrence of two different plasmid profiles in the same outbreak was unexpected. Previously, only one plasmid profile has been observed in *V. salmonicida* isolates from several individual Atlantic salmon in the same outbreak as well as in isolates from different organs in the same individual salmon (H. Sørum, unpublished data). Therefore, the individual cod having the divergent plasmid profile does not seem to represent this outbreak.

The DNA hybridization experiments and restriction endonuclease cleavage analysis demonstrated that the 61- and 21-MDa plasmids of *V. salmonicida* isolates from cod in 1988 were homologous to the respective plasmids in isolates from Atlantic salmon in 1982 and 1983. These results indicate that the plasmid DNA of *V. salmonicida* is very stable over time. The 61-, 21-, 3.4-, and 2.8-MDa plasmids in a strain from cod hybridized with respective plasmids in strains originating from widely distributed fish farms along the Norwegian coast. This shows that the composition of a plasmid of a certain molecular mass does not vary obviously from one area to another.

The fact that the 61-MDa plasmid has only been observed in northern Norway is very interesting. The northern part of our coast may be comparatively favorable for *V. salmonicida* strains harboring the 61-MDa plasmid (i.e., water temperatures are low in this area). The first report ever of cold-water vibriosis was from Finnmark (the northernmost county of Norway) in 1977. Since 1977, there have always been outbreaks of the disease in Finnmark county regardless of the situation in other parts of the country (C. W. Koren, personal communication). Northern Norway is the part of the country in which we have found the greatest diversity of plasmid profiles. In fact, all the 11 different plasmid profiles which have so far been observed are represented in strains from northern Norway. It may be asked whether northern

Norway is a more favorable area for *V. salmonicida* than is the rest of the country.

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