APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1992, p. 1980–1985
0099-2240/92/061980-06$02.00/0

Vol. 58, No. 6

Development of a Gene Transfer System for Curing of Plasmids in the Marine Fish Pathogen *Vibrio salmonicida*

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Received 28 October 1991/Accepted 23 March 1992

All reported natural isolates of the marine fish pathogen *Vibrio salmonicida* contain plasmids, and in another marine fish pathogen, *Vibrio anguillarum*, it has been shown that a plasmid is important for expression of virulence by the organism. To study the function of the plasmids in *V. salmonicida*, we developed a gene transfer system based on the plasmid RSF1010 replicon. The gene transfer system was used to construct a plasmid-free strain, and this strain was found to behave similarly to the wild type in a fish pathogenicity test based on intraperitoneal injection of the bacteria. We were unable to detect any other phenotypic differences between the two strains. It could therefore be concluded that at least in the *V. salmonicida* strain tested, extrachromosomal DNA is not required for expression of virulence.

Several bacterial species belonging to the genus *Vibrio* are known to be fish pathogens, and one of these, *Vibrio anguillarum*, has caused serious losses worldwide in fish farms (16). A plasmid-associated virulence factor has been identified in this bacterium, and cells containing the plasmid (designated pJM1) can grow in the presence of the iron-sequestering protein transferrin at concentrations that do not permit growth of the corresponding plasmid-free strain. In addition, it has been shown that curing of the plasmid results in a drastic reduction of the virulence of the organism (3).

Another fish pathogenic *Vibrio* species, *Vibrio salmonicida*, appeared for the first time in Norwegian salmonid farms around the island of Hitra in 1979 (7). In the following years, this organism caused serious losses in fish farms along the western and northern coastline of Norway and in Scotland (2, 6). *V. salmonicida* has (similarly to *V. anguillarum*) been analyzed with respect to its content of extrachromosomal DNA, and these experiments showed that all natural isolates contained plasmids. The majority of the strains contained two, three, or four different plasmids, while a few strains contained only one (15, 18). The apparently universal presence of extrachromosomal DNA in *V. salmonicida*, and the fact that a plasmid is essential in *V. anguillarum* virulence, might indicate that a plasmid is essential in *V. salmonicida* virulence. We describe in this article a plasmid incompatibility method which could be used for plasmid curing in this organism. Atlantic salmon were infected by intraperitoneal injection of one of these plasmid-free derivatives, and the development of disease was compared with that of a similar experiment involving the corresponding plasmid-containing strain. The results of the experiments show that both strains behave similarly with respect to their ability to induce disease in Atlantic salmon, and, in addition, we were not able to detect any other differences in the phenotypes of the two strains.

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MATERIALS AND METHODS

Bacterial strains and plasmids. One *Escherichia coli* strain, DH1 (*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*), was used in the experiments (11). The *V. salmonicida* strains used were TEO 83.001 (18), LFI 024, LFI 031 (14), and LFI 029. The latter strain was isolated at a fish farm in northern Norway in 1984. In addition, we isolated and used a spontaneous streptomycin-resistant derivative of TEO 83.001, and the new derivative was designated TEO 83.001S. Plasmids and their selective markers are as follows: pRK2013, ColE1 replicon and Km^r (9); pBR322, ColE1 replicon, Ap^r, and Tc^r (1); pAL200, RSF1010 replicon containing the promoterless *cat* gene, Tc^r (A. Greener, unpublished data); RP4, IncP1, Ap^r, Tc^r, and Km^r (4).

Growth of bacteria, construction of pPV14, and conjugative matings. Cells were grown with shaking in LB (12) at 37°C (*E. coli*) or in Difco marine broth (MB) at 14°C (*V. salmonicida*). The attempts to develop a system for conjugal plasmid transfer from *E. coli* were initially unsuccessful, and the reason for this was that we were not aware of the temperature requirements for the transfer process (see below). Before this point became clear, we were concerned that the antibiotic resistance markers might not be expressed in *V. salmonicida*, and we therefore constructed a plasmid derivative (pPV14) in which the gene encoding chloramphenicol resistance was expressed from a *V. salmonicida* promoter. This was done by cloning (in a *Bam*HI site) random *Sau*3AI-generated fragments from total *V. salmonicida* DNA 5′ of the promoterless *cat* gene in plasmid pAL200. Plasmid pPV14 was selected on the basis of its high level of expression of chloramphenicol resistance in *E. coli*, and further analysis showed that pPV14 contained a 100- to 150-bp DNA insert in the *Bam*HI site.

Matings were performed by harvesting exponentially growing cells by centrifugation and then resuspending them in MB. Cell growth was monitored by optical density measurements, and the growth medium used for *V. salmonicida* was centrifuged prior to inoculation to remove particles that interfered with the optical density measurements. Plasmid pRK2013 was used as the mobilizing plasmid for the two pAL200 derivatives (pPV14 and pPV14.16), and the matings

were performed triparentally by mixing DH1(pRK2013), DH1(pPV14/pPV14.16), and TEO 83.001S. Approximately 10^9 cells of each organism was filtered onto a membrane (Millipore, catalog no. HAWP02500), and the membrane was incubated on agar medium (MB supplemented with 1.5% agar) for 2 to 5 h at 22 to 25°C. These temperatures were too high to allow growth of *V. salmonicida* but proved to be necessary for plasmid transfer to take place. The likely reason for this is that *E. coli* is inefficient as a donor at the temperatures optimal for growth of *V. salmonicida* (7). After the incubation, the cells were resuspended in MB and grown overnight at 14°C. The cells were finally plated on MB agar medium supplemented with 500 μg of streptomycin per ml plus 1 μg of chloramphenicol per ml and incubated for 2 to 3 days at 14°C. Further analysis of the phenotypes of the transconjugants showed that they also expressed tetracycline resistance in *V. salmonicida*.

Recombinant DNA techniques. The isolation of plasmids from *V. salmonicida* was performed as described previously for *Acetobacter xylinum* (17). The isolation of plasmids from *E. coli*, agarose gel electrophoresis, cloning, transformation, labelling of DNA by ^{32}P nick translation, and Southern hybridization (stringent conditions) were performed by standard methods (12). Heteroduplex formation and electron microscopy of DNA was performed by the method of Davis et al. (5), and DNA sequencing was performed by the method of Sanger et al. (13). For this last analysis, we used primers homologous to the vector (pBR322) near the *Hin*dIII site, and only one strand in each region was sequenced.

Analysis of *V. salmonicida* virulence and serotyping. The virulence tests were performed as described previously (14), and the serotyping was performed by the method of Espelid et al. (8).

RESULTS

Physical characterization of the plasmid present in strain TEO 83.001. *V. salmonicida* TEO 83.001 contains only one plasmid (here designated pVS1), and we have used this strain as a model in our experiments. A restriction endonuclease map of pVS1 was constructed (Fig. 1), and the map shows that the molecular size of the plasmid is about 11.5 kb, a value which was significantly lower than the size (10 MDa = 15.4 kb) reported previously (18). During our experiments on analysis of restriction endonuclease sites in pVS1, the patterns in some of the digests (enzymes with many sites) seemed to indicate that pVS1 contained repeated DNA sequences. This hypothesis was analyzed further by hybridizing different parts of pVS1 DNA against each other (Fig. 2). Lane 1 shows an agarose gel of the separated *Hin*dIII fragments from pVS1. The DNAs in three such lanes were transferred to individual membranes, and each membrane was used for hybridization against each of the same three fragments labelled with ^{32}P. As can be seen in lane 2, the 4.4-kb *Hin*dIII fragment hybridized strongly against both the 3.3- and the 3.8-kb *Hin*dIII fragments. Interestingly, however, the 3.8- and 3.3-kb DNA fragments did not hybridize against each other (lanes 3 and 4, respectively).

One way to explain these results would be to assume that the two *Hin*dIII sites generating the 4.4-kb fragment are within a repeated DNA sequence and that the repeat is direct (explaining the lack of hybridization between the 3.3- and 3.8-kb fragments). This model would predict that it might be possible to form heteroduplexes between the 4.4-kb *Hin*dIII fragment and each of the other two *Hin*dIII fragments from pVS1. We tested this hypothesis for one of these combinations (the 4.4- and 3.8-kb fragments), and the analysis showed that such structures were formed, indicating that there exists a homologous sequence of approximately 1 kb at the ends of the two fragments (Fig. 3). We also sequenced between 200 and 300 bp at the ends of each of the three

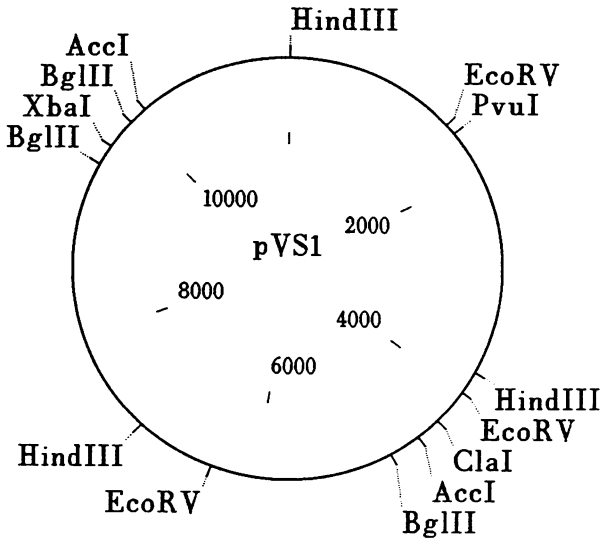

FIG. 1. Restriction endonuclease map of plasmid pVS1. pVS1 was digested with the relevant single enzymes and combinations of them. In addition, we also digested each of the three *Hin*dIII fragments separately by using recombinant plasmids where the three fragments were cloned separately into the *Hin*dIII site of plasmid pBR322.

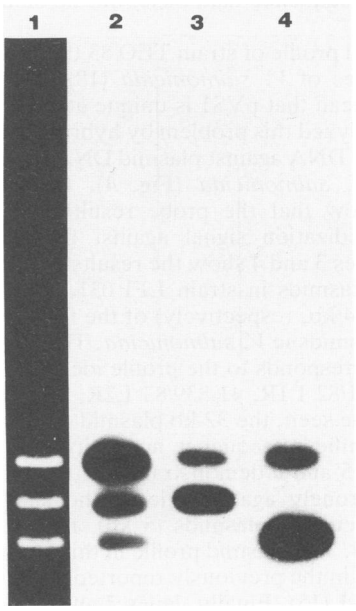

FIG. 2. Analysis of repeated DNA sequences in pVS1. Lanes: 1, *Hin*dIII-digested pVS1 separated by agarose gel electrophoresis; 2 to 4, hybridization of ^{32}P-labelled 4.4-kb (lane 2), 3.8-kb (lane 3), and 3.3-kb (lane 4) pVS1 *Hin*dIII fragments against the DNA in lane 1 (or equivalent lanes). The probes were prepared by labelling the recombinant plasmids containing the relevant fragments (see legend to Fig. 1).

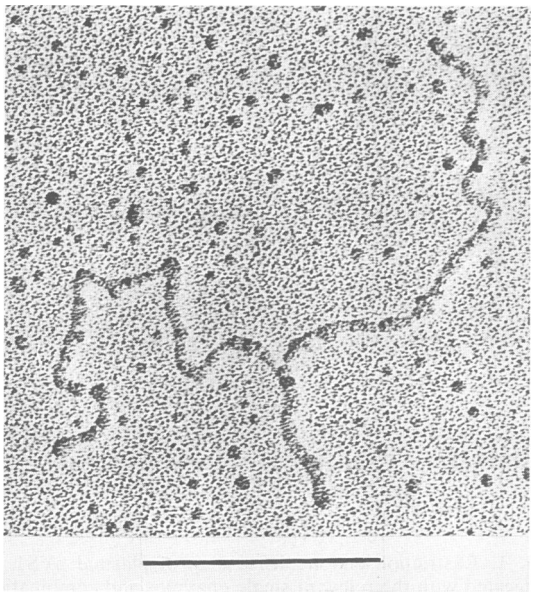

FIG. 3. Electron micrograph of heteroduplex formed between the 4.4- and 3.8-kb *Hin*dIII fragments of pVS1. Bar, 0.5 μm.

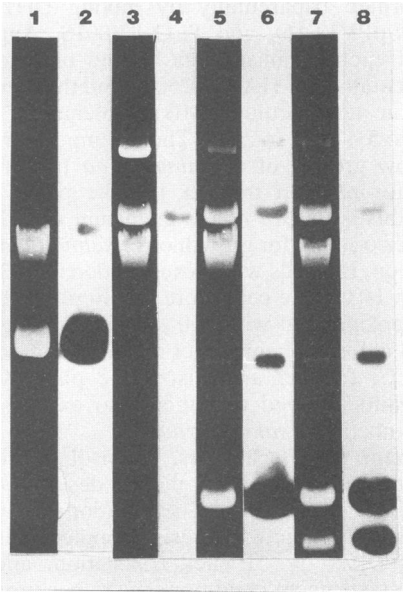

FIG. 4. Hybridization of pVS1 DNA against agarose gel electrophoresis-separated plasmids from different natural isolates of *V. salmonicida*. Lanes: 1, 3, 5, and 7, plasmids from TEO 83.001, LFI 031, LFI 024, and LFI 029, respectively; 2, 4, 6, and 8, hybridization of ^{32}P-labelled pVS1 DNA against DNA in lanes 1, 3, 5, and 7, respectively. The major band common to all lanes represents chromosomal DNA fragments.

cloned *Hin*dIII fragments, and the results of these analyses showed that the sequence at one end of the 4.4-kb fragment was identical to the sequence at one end of the 3.8-kb fragment. Correspondingly, the sequence at the opposite end of the 4.4-kb fragment was identical to one of the ends of the 3.3-kb fragment. Finally, the sequences at the other end of the 3.8- and 3.3-kb fragments did not show any similarity to the other sequenced regions. All sequences determined were compared with the published sequences in the GENE/EMBL DNA sequence data base, but no similar sequences were found.

The plasmid profile of strain TEO 83.001 is unusual among natural isolates of *V. salmonicida* (18), but this does not necessarily mean that pVS1 is unique at the DNA sequence level. We analyzed this problem by hybridizing radioactively labelled pVS1 DNA against plasmid DNA from other natural isolates of *V. salmonicida* (Fig. 4). Lanes 1 and 2, as expected, show that the probe resulted in a strong and specific hybridization signal against the same DNA sequences. Lanes 3 and 4 show the results of the hybridization against the plasmids in strain LFI 031, a strain containing two (32 and 94 kb, respectively) of the four most frequently occurring plasmids in *V. salmonicida*. The plasmid profile in this strain corresponds to the profile identified previously in strains A/2181/82 L1R, 41.839/83 L2R, and 870903629 L1R (15). As can be seen, the 32-kb plasmid appears to contain a weak but significant sequence similarity with sequences in pVS1. Lanes 5 and 6 demonstrate that plasmid pVS1 DNA hybridizes strongly against one of the smaller and most frequently occurring plasmids (5 kb), here represented by strain LFI 024. The plasmid profile in this strain corresponds to the profiles in the previously reported strains NCMB 2262 and HI 11366-1 (15). Finally, lanes 7 and 8 show that pVS1 hybridizes equally strongly against the slightly smaller plasmid (4 kb) present in strain LFI 029 (whose profile corresponds to strain HI 9724, reported previously by Sørum et al. [15]).

The fact that the hybridization experiments were performed under stringent conditions, and the lack of hybridization against the chromosomal DNA fraction, confirmed the specificity of the analyses described above. We therefore conclude that although the plasmid profile of strain TEO 83.001 is unique, DNA sequences in pVS1 are frequently present in independent natural isolates of *V. salmonicida*.

Development of a gene transfer system for *V. salmonicida* and plasmid curing. To analyze the function of the *V. salmonicida* plasmids, we initially tried to use plasmid curing agents (ethidium bromide and mitomycin) and the chemical mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine to remove pVS1 from TEO 83.001. Even though we also combined these methods with colony hybridization (with pVS1 as the probe), we were unable to identify a plasmid-free derivative of TEO 83.001, indicating that pVS1 is very stable and therefore not easily cured by standard plasmid curing techniques.

As an alternative method for plasmid curing, we developed a method which involved the use of plasmid incompatibility to construct a plasmid-free derivative of TEO 83.001. Such methods first require development of a gene transfer system for the organism, and we have tested broad-host-range plasmids from incompatibility groups P1 and Q for their ability to transfer to and replicate in a spontaneous streptomycin-resistant derivative (TEO 83.001S) of *V. salmonicida*. *E. coli* was used as the donor in these experiments, and we found that the IncQ-based replicon pPV14 could be transferred efficiently (frequencies of 10^{-4} to 10^{-5} per recipient cell) to *V. salmonicida* provided that the mating conditions were carefully controlled. The tested IncP plasmid (RP4) could not be transferred to *V. salmonicida* or was transferred very inefficiently.

The establishment of a gene transfer system for *V. salmonicida* opened the possibility of using more efficient approaches for obtaining a plasmid-free strain. The method we

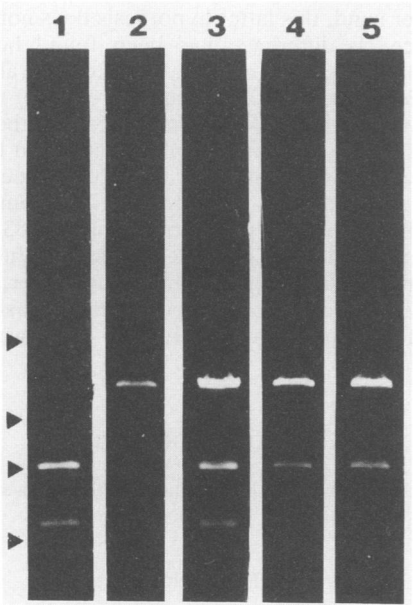

FIG. 5. Curing of pVS1. All lanes represent agarose gel electrophoresis separation of *Bgl*II-digested plasmids from *V. salmonicida* or *E. coli*. The 0.5-kb fragment is not visible in the figure. Lanes: 1, TEO 83.001; 2, DH1(pPV14); 3, TEO 83.001S(pPV14); 4, TEO 83.001S(pPV14.16); 5, DH1(pPV14.16). The triangles mark the positions of bacteriophage λ *Hin*dIII-digested size markers (23.1, 9.4, 6.6, and 4.4 kb, respectively).

used first involved cloning *Bgl*II-generated DNA fragments (6.5, 4.6, and 0.5 kb, respectively) from pVS1 into a unique *Bgl*II site of pPV14. The idea of this approach was that one or more of these fragments would, it is hoped, exert an incompatibility effect which could be used to induce loss of the original plasmid. One of the recombinant plasmids (designated pPV14.16), containing the 6.5-kb DNA fragment from pVS1, proved to have such a property. Figure 5 shows the results of an analysis of *Bgl*II-digested plasmids from the different *V. salmonicida* strains generated in the gene transfer experiments. Lane 1 represents pVS1, isolated from TEO 83.001, while lane 2 represents pPV14, isolated from *E. coli*. Lane 3 represents the plasmids isolated from TEO 83.001S(pPV14), and this analysis shows that both pVS1 and pPV14 are present in this *V. salmonicida* cell line. Lane 4 shows the *Bgl*II-digested plasmids from a clone isolated after growth of strain 83.001S(pPV14.16) in the presence of chloramphenicol for approximately 30 generations. As can be seen, the 4.4-kb (and 0.5-kb [not shown]) *Bgl*II fragment(s) is no longer present, while the 6.5-kb DNA fragment cloned in pPV14.16 is still present. Lane 5 shows the corresponding digest of pPV14.16 isolated from *E. coli*, and the results are thus consistent with the hypothesis that pVS1 has been lost during growth of TEO 83.001S(pPV14.16) under selective conditions. When the clone lacking pVS1 was grown further in the absence of antibiotics, the chloramphenicol and tetracycline resistances proved to be unstably maintained and were lost together. Analysis of the plasmid content of such Cm^S and Tc^S cells showed, as expected, that they no longer contained extrachromosomal DNA. In addition, hybridization analysis with ^{32}P-labelled pVS1 as the probe against total DNA from one of these clones confirmed that pVS1 and pPV14.16 were both lost from the cells (data not shown).

Pathogenicity and serological properties of the plasmid-cured strain. Strains TEO 83.001, TEO 83.001S, and TEO 83.001S.dVS1 (the plasmid-cured derivative of strain TEO 83.001S) were compared with respect to their ability to induce disease in Atlantic salmon (*Salmo salar*). The experiment was performed by injecting groups of fish intraperitoneally with different dosages of the three bacterial strains and then inspecting the fish individually with respect to disease development. Figure 6 illustrates the lethality as a function of bacterial dosage and time after inoculation. As can be seen, the dosages and time responses were similar for the three strains. We also isolated *V. salmonicida* from other fish (infected with TEO 83.001S.dVS1) that had developed the disease, and of the five tested isolates, all were streptomycin resistant and plasmid free.

We have also compared the serotypes of TEO 83.001 with those of its plasmid-free derivative. No significant differences were found when four different VS-P1 monoclonal antibodies (produced by clones 2B5, 7F3, 2G5, and 7D4) or four different monoclonal antibodies (produced from clones 7G1, 5E10, 4H4, and 1G11) reactive against unidentified determinants (8) were used. On the basis of the infection and serotyping experiments, it thus seems unlikely that there is any significant correlation between the virulence and the plasmids of *V. salmonicida*.

DISCUSSION

The physical analysis of the plasmid (pVS1) in strain TEO 83.001 showed that it contains sequences strongly homologous to the extrachromosomal DNA in other natural isolates of *V. salmonicida*. Two small (4- and 5-kb) plasmids present in a large fraction of natural isolates of *V. salmonicida* share strong sequence similarity with pVS1. Since we could show that pVS1 contains a directly repeated DNA element, it seems possible that this is an IS-like sequence that perhaps has caused a spontaneous fusion between the two smaller plasmids, generating pVS1. Such a hypothesis would explain the strong hybridization signals obtained when labelled pVS1 was hybridized against the two smaller plasmids, and if the cointegrate formation was mediated by an IS element, one would also expect the element to be directly repeated in the cointegrate (10). The distances between the internal *Hin*dIII sites are consistent with this model, particularly if one assumes that the element originated from the smallest plasmid.

Virulence tests and serotyping experiments gave no evidence in support of the hypothesis that plasmids are required for the virulence of strain TEO 83.001. We also tested the sensitivity of growth for this strain and its plasmid-cured derivative in the presence of the iron-binding protein transferrin. Both strains grew slowly in the presence of 1 μg of transferrin per ml, while none of the strains grew in the presence of 10 μg/ml (data not shown). These experiments thus indicated that plasmid pVS1 probably does not encode the equivalent of the iron-sequestering system present in *V. anguillarum* (3). The previously reported speculations on a possible correlation between virulence and plasmids in *V. salmonicida* (18) are therefore not supported by the experiments described here.

The virulence tests reported in these experiments were performed by intraperitoneal injections of the bacteria. It is therefore difficult to exclude the possibility that the plasmids may be of some importance when the bacteria are invading the fish from the external environment. Similarly, our experiments do not exclude the possibility that plasmids present in other strains of *V. salmonicida* are required for virulence.

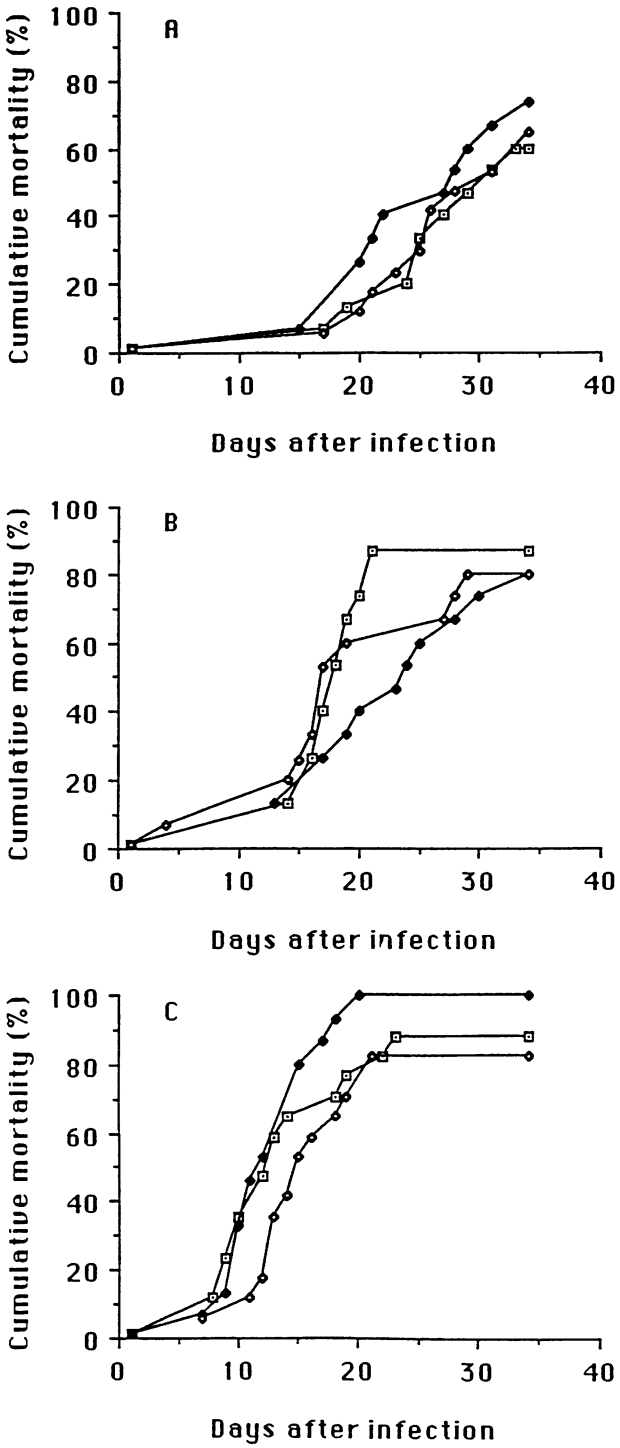

FIG. 6. Lethality of Atlantic salmon after intraperitoneal injection with TEO 83.001, TEO 83.001S, and TEO 83.001S.dVS1. Injection with 10^6 (A), 10^7 (B), and 10^8 (C) bacteria, respectively, is shown. The numbers of individual fish in each infection experiment varied between 15 and 20. Symbols: ⊡, TEO 83.001; ◆, TEO 83.001S; ◇, TEO 83.001S.dVS1.

On the other hand, this latter hypothesis does not seem very likely, since no difference has been found between the virulence of TEO 83.001 and other tested natural isolates of *V. salmonicida* (unpublished results).

One way to obtain more information about the biological function of the *V. salmonicida* plasmids would be to carry out a more extensive analysis of the DNA sequences in the plasmids. Alternatively, a more detailed comparison of potential phenotypic differences between TEO 83.001 and its plasmid-cured derivative might represent a useful approach. The outcome of such experiments is, however, uncertain, and it may therefore prove to be a difficult task to obtain definite information about the role of the plasmids in *V. salmonicida*.

ACKNOWLEDGMENT

This work was supported by a grant from the Norwegian Council of Fisheries Research.

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