RpoN of the fish pathogen Vibrio (Listonella) anguillarum is essential for flagellum production and virulence by the water-borne but not intraperitoneal route of inoculation

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To investigate the involvement of RpoN in flagellum production and pathogenicity of Vibrio (Listonella) anguillarum, the rpoN gene was cloned and sequenced. The deduced product of the rpoN gene displayed strong homology to the alternative σ54 factor (RpoN) of numerous species of bacteria. In addition, partial sequencing of rpoN-linked ORFs revealed a marked resemblance to similarly located ORFs in other bacterial species. A polar insertion or an in-frame deletion in the coding region of rpoN abolished expression of the flagellin subunits and resulted in loss of motility. Introduction of the rpoN gene of V. anguillarum or Pseudomonas putida into the rpoN mutants restored flagellation and motility. The rpoN mutants were proficient in the expression of other proposed virulence determinants of V. anguillarum, such as ability to grow under low available iron conditions, and expression of the LPS O-antigen and of haemolytic and proteolytic extracellular products. The infectivity of the rpoN mutants with respect to the wild-type strain was unaffected following intraperitoneal injection of fish but was reduced significantly when fish were immersed in bacteria-containing water. Thus, RpoN does not appear to regulate any factors required for virulence subsequent to penetration of the fish epithelium, but is important in the infection of fish by water-borne V. anguillarum.

Keywords: Vibrio (Listonella) anguillarum, fish pathogen, vibriosis, σ54 (RpoN), flagellum expression

INTRODUCTION

Vibriosis in fish is generally described as a haemorrhagic septicaemia caused by members of the genus Vibrio and is regarded as one of the economically most serious diseases in marine fish culture, affecting a variety of fish species (Egidius, 1987). While several marine Vibrio species have been associated with the disease, the major importance in terms of distribution, frequency of outbreaks and the financial losses ensuing has been attributed to vibriosis manifested by Vibrio (Listonella) anguillarum (Austin & Austin, 1993). A number of factors have been implicated in the virulence of V. anguillarum, including the iron-sequestering system (Crosa, 1980; Crosa et al., 1980), LPS (Norqvist & Wolf-Watz, 1993) and extracellular products which have haemolytic and proteolytic properties (Munn, 1980; Toranzo et al., 1983; Inamura et al., 1985). Previous work done in our laboratory revealed that the polarly located flagellum and the chemotactic motility it mediates are required for virulence of V. anguillarum when fish are immersed in water containing the pathogen but not when fish are inoculated by intraperitoneal injection (O’Toole et al., 1996). In the course of those studies, an rpoN transposon mutant was isolated and found to be deficient for flagellum expression and hence motility. In addition, this mutant, unlike the other non-motile transposon mutants isolated, failed to grow under conditions of low iron availability in vitro and was avirulent by the intraperitoneal mode of inoculation (O’Toole et al., 1996). These findings indicated that RpoN is involved in the expression of the polar flagellum

The GenBank/EMBL/DDBJ accession number for the sequence reported in this paper is u86585.
of *V. anguillarum* and also suggested a possible role for RpoN in ability to grow at low iron concentrations.

RpoN is known to regulate a wide range of cellular functions in response to environmental and metabolic variations (Merrick, 1993). A number of these functions are directly related to virulence, such as the production of pili by *Neisseria gonorrhoeae* (Meyer et al., 1984; Boyle-Vavra et al., 1993) and *Pseudomonas aeruginosa* (Ishimoto & Lory, 1989) and expression of the Hpr and Wts virulence proteins by the plant pathogens *Pseudomonas syringae* pv. *phaseolicola* (Fellay et al., 1991) and *Erwinia stewarti* (Frederick et al., 1993), respectively. Moreover, expression of the flagellum of *P. aeruginosa*, which is an important virulence factor in the burned mouse model (Drake & Montie, 1988), is dependent upon RpoN (Totten et al., 1990). rpoN mutants of *Pseudomonas putida* and *Caulobacter crescentus* also fail to synthesise flagella (Inouye et al., 1990; Brun & Shapiro, 1992).

To assess the role of RpoN in the virulence of *V. anguillarum*, the rpoN gene was cloned from a genomic library and sequenced. Additional mutations, consisting of a plasmid insertion and an in-frame deletion, were constructed in the rpoN gene and the mutants were analysed for various phenotypes, including flagellum expression and motility, iron-limited growth and virulence in a natural model of vibriosis, i.e. rainbow trout. Complementation studies were performed by re-introducing the rpoN gene on a plasmid into the mutants. The ability of the rpoN gene from *P. putida* to complement mutations in the rpoN gene of *V. anguillarum* was also tested.

**METHODS**

**Bacterial strains, phages and plasmids.** *Vibrio anguillarum* NB10 (serotype O1) was isolated at the Umeå Marine Research Centre, Norrbys, Sweden, by our laboratory during a natural outbreak of vibriosis (Norgqvist et al., 1990). *V. anguillarum* aflagellate strain ABE8 is an rpoN transposon mutant derived from strain NB10 (O’Toole et al., 1996). *V. anguillarum* strain OTR7 is an aflagellate flhA transposon mutant of strain NB10 (O’Toole et al., 1996). *V. anguillarum* strain 775E39 is a plasmid-cured derivative of serotype O1 strain 775 (both strains were gifts from Trevor Trust, British Columbia, Canada) and was used as an iron-uptake-deficient control in the assays for growth under low iron conditions. The highly competent *Escherichia coli* SY327 strain (Δ(lac pro) argE [Am]) rif mlaA recA56) (Miller & Mekalanos, 1988) was used for transformation after ligation of fragments into the pDM4 and pSUP202 vectors. All plasmids to be conjugated into *V. anguillarum* were transformed into *E. coli* S17-1 (thi pro hsdR pSUP202) derivatives. pDM4 (Milton et al., 1996) is a chloramphenicol-resistant derivative of pGP704 (Miller & Mekalanos, 1988) which contains the sacBR genes of *Bacillus subtilis*. pRpoN-Va is a derivative of the mobilizable plasmid pSUP202 (Simon et al., 1983) which contains the rpoN gene of *V. anguillarum* and its flanking ORFs. The 6.5 kb EcoRI fragment containing the rpoN gene was excised from pBS7-1, gel-purified using the GeneClean kit (Biolol) and then ligated to pSUP202 which had been digested with EcoRI and dephosphorylated using calf-intestinal phosphatase. pVLT31 (de Lorenzo et al., 1993) is a tetracycline-resistant derivative of the mobilizable broad-host-range plasmid pMMB207 (Morales et al., 1991) which has a tac promoter directed towards the multiple cloning site. pFHB (Cases et al., 1996) is a derivative of pVLT31 containing the rpoN gene of *P. putida*, but no neighbouring genes, such that rpoN gene expression is directed by the tac promoter.

**Media and growth conditions.** *E. coli* was routinely grown at 37 °C in L broth (1% tryptone, 0.5% yeast extract, 1% NaCl) and on L agar (L broth plus 1.5%, w/v, agar). *V. anguillarum* was grown at room temperature in Trypticase soy broth (TSB; BBL) or on TSA (TSB plus 1.5%, w/v, agar). The soft agar used for assaying motility of *V. anguillarum* consisted of TSB plus 0.3% (w/v) agar. The Vibrio-selective medium used was TCBS agar (Difco).

**Antibiotics and enzymes.** Antibiotic concentrations used for all *E. coli* strains were as follows: ampicillin, 100 μg/ml; chloramphenicol, 25 μg/ml; and tetracycline, 10 μg/ml. For *V. anguillarum* the concentrations used were: chloramphenicol, 10 μg/ml; and tetracycline, 5 μg/ml. Restriction enzymes were purchased from a variety of sources, and KGB buffer (Sambrook et al., 1989) was used for all digests. T4 DNA ligase and calf-intestinal phosphatase were purchased from Promega. Incubation conditions for all DNA-modifying enzymes were in accordance with those recommended by the manufacturers.

**Bacterial matings.** *E. coli* S17-1 was used as the donor strain for the transfer of all plasmid derivatives to *V. anguillarum* by bacterial conjugation. Strains were grown to the mid-exponential growth phase. At a ratio of 10:1 based on OD_{600} values (measured with a Hitachi 150-20 spectrophotometer), the recipient and donor strains were mixed and centrifuged. The bacterial pellet was resuspended in one-tenth the original volume of TSB and 25 μl amounts were spotted onto TSA. The matings were allowed to progress at 30 °C overnight, after which antibiotic-resistant transconjugants were selected on TCBS agar containing 10 μg chloramphenicol ml⁻¹ in the case of pDM4 derivatives or 5 μg tetracycline ml⁻¹ for pSUP202 and pVLT31 derivatives.

**Screening of the genomic library.** Screening of the genomic library was performed as described previously by Milton et al. (1996). The probe, a 250 bp internal fragment of the rpoN gene, was generated by PCR using the primers 5'-GCAAGACTTTGCAATGCTTAA-3' (bases 936-957; Fig. 1) and 5'-TAATGTAAAGCAGGCA-3' (bases 1185-1168; Fig. 1) and labelled with [α-32P]dCTP by random oligonucleotide priming as described previously by Sambrook et al. (1989).

**Southern blot analysis.** The pBluescript clones pBS1-1, pBS7-1 and pBS8-1 were digested with the EcoRI and XbaI restriction endonucleases and electrophoresed through a 1.0% (w/v) agarose gel in Tris/borate/EDTA buffer (TBE;
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Sambrook et al., 1989). Southern blotting was then performed as described previously (O'Toole et al., 1996). PCR products generated from sequence either upstream or downstream of the single XbaI restriction site on the rpoN gene (120 bp downstream of its proposed start codon; Fig. 1), which was identified from a partial sequence of rpoN obtained previously (O'Toole et al., 1996), were labelled with [α-32P]dCTP by random oligonucleotide priming and used as probes. The sizes of the fragments upstream and downstream of the single XbaI restriction site, which contained rpoN sequence, were calculated for each of the pBluescript clones. From this analysis, a pBluescript clone(s) which contained the entire rpoN gene could be identified. The probe which was specific for sequence upstream of the XbaI restriction site of rpoN was generated by PCR using the primers 5'-GGTACACCACATGACGTTAGCAGGCTC-3' (bases 421-441; Fig. 1) and 5'-CCAAATGTTGGAGAGTTTGCAAACA-3' (bases 637-617; Fig. 1). The probe which was specific for sequence downstream of the XbaI restriction site of rpoN was the same as that used to screen the genomic library.

DNA techniques and sequencing. Oligonucleotide primers were synthesized with an Applied Biosystems model 394 automated DNA/RNA synthesizer. Double-stranded DNA sequencing was performed by the dideoxy chain-termination method (Sanger et al., 1977) with T7 DNA polymerase (Boehringer Mannheim). The T3 and T7 primers were used for sequencing of small DNA inserts within the pBluescript linker region. Using the pBS7-1 plasmid, both strands of the rpoN gene were sequenced by primer walking in both directions from the partial sequence of rpoN obtained previously (O'Toole et al., 1996). Unless otherwise stated, all conditions for the various DNA techniques used were as described by Sambrook et al. (1989).

PCR conditions. The PCR conditions were as described previously (O'Toole et al., 1996). Template was either genomic DNA or bacterial cells. For analysis, one-tenth of the PCR product obtained previously (O'Toole et al., 1996), was labelled with [α-32P]dCTP by random oligonucleotide priming and used as probes. The sizes of the fragments downstream of the XbaI restriction site of rpoN allele were calculated for each of the pBluescript clones. From this analysis, a pBluescript clone(s) which contained the entire rpoN gene could be identified. The probe which was specific for sequence downstream of the XbaI restriction site of rpoN allele was the same as that used to screen the genomic library.

Construction of mutations in the rpoN gene. Two types of mutations were made in the coding region of rpoN, i.e. a polar insertion and an in-frame deletion. To construct an insertion mutant of rpoN (mutant OTR81), an 825 bp internal fragment of rpoN, rpoNeh, was generated by PCR and was subsequently cloned into the XbaI–Sac1 restriction endonuclease sites of the suicide vector pDM4, producing the plasmid pDM-rpoNeh1. The recombinant plasmid pDM-rpoNeh1 was mobilized into V. anguillarum wild-type strain NB10 by conjugal mating and transconjugants were selected on TCBS containing 10 μg chloramphenicol ml−1. Integration of the plasmid into the rpoN gene was confirmed by PCR analysis using a primer complementary to the plasmid just outside the linker region of pDM4 and another primer complementary to the rpoN gene just outside of the rpoNeh region, and by subsequent restriction endonuclease analysis of the PCR products obtained. The two primers used in the PCR to generate the rpoNeh fragment were rpoNe (5'-CTAGTCTACAGGCTAGGTATGAGTGG-3') and rpoNh (5'-CTAGGAGCTCTTCATCTTTTCAAACATT-3') whereby an XbaI and a SacI enzyme restriction site, preceded by the sequence CTAG, are present at the 5'-end of primers rpoNe and rpoNh, respectively. The priming sites on the rpoN gene sequence of both oligonucleotides are indicated in Fig. 1.

To construct an in-frame deletion in rpoN, use was made of two BglII restriction sites, separated by 348 bp, in the rpoN nucleotide sequence (located at nucleotides 960 and 1308; Fig. 1). pDM-rpoNeh1 was treated with BglII endonuclease to remove the 348 bp internal fragment and was subsequently ligated to itself, producing an in-frame deletion within the rpoNeh fragment. Loss of the 348 bp fragment from the resulting recombinant plasmid, pDM-rpoNeh2, was confirmed by PCR and restriction analysis. A 348 bp deletion was then generated in the rpoN gene of V. anguillarum by allelic exchange as previously described by Milton et al. (1996). A PCR product from the mutated rpoN allele displayed by a recombinant bacterium (mutant OTR83) was cloned into pBluescript and sequenced to verify the presence of a 348 bp in-frame deletion in the rpoN gene of this mutant.

Haemolytic and proteolytic activity assays. Assays to assess the haemolytic and proteolytic activities of bacterial cultures were performed as described previously (O'Toole et al., 1996).

Western blot analysis. A rabbit polyclonal antiserum against the purified flagellin subunits of V. anguillarum strain NB10 was used to detect the flagellin proteins via immunoblotting. A second rabbit polyclonal antiserum, raised against formalin-killed whole cells of a serotype O1 strain of V. anguillarum, 775.17B (Norqvist & Wolf-Watz, 1993), was used to detect LPS O-antigen expression. The OD600 values of overnight cultures of the V. anguillarum strains were measured. To obtain equivalent amounts of cells, the concentration of each bacterial strain was adjusted to an OD600 value of 1.0 by appropriate dilution with TSB. Aliquots of 50 μl cells were centrifuged and the bacterial pellets were resuspended in 50 μl SDS-PAGE loading buffer (Sambrook et al., 1989) and boiled for 10 min; 7.5 μl of each bacterial lysate was separated on an SDS-11% (w/v) polyacrylamide gel as described previously by Laemmli (1970). The immunoblotting techniques used were as previously reported (O'Toole et al., 1996).

Motility assays. Bacterial strains were grown overnight and subsequently adjusted to an OD600 value of 1.0. Aliquots of 3 μl were spotted onto the surface of 0.3% TSA plates and migration through the agar was monitored over a period of 24 h. The swimming motility of the bacterial strains in liquid media was assessed using phase-contrast light microscopy. The Ptac promoter of pH30, which harbours the rpoN gene of P. putida, was induced by addition of 0.5 mM IPTG to the growth media.

Fish infections. Rainbow trout (Oncorhynchus mykiss) weighing approximately 5–15 g were inoculated with V. anguillarum either by intraperitoneal injection or by immersion in water containing bacteria as described previously by our laboratory (Norqvist et al., 1989). For the determination of 50% lethal dose (LD50) values, five fish were infected at each of four different 10-fold dilutions of bacteria and were monitored over a period of 7 d. Mean LD50 calculations were made by the method of Reed & Muench (1938) on the basis of results from two infection experiments.

Electron microscopy. The negative staining and electron microscopic techniques used were as previously outlined by McGee et al. (1996). The grids were examined with a Zeiss EM 109 transmission electron microscope operated at an accelerating voltage of 50 kV.

Assay for growth under iron-limiting conditions. Assays for growth at low iron availability were performed using the iron-chelating agent EDDA (Sigma) as described previously (O'Toole et al., 1996). Antibiotics were present where appropriate.

Computer analysis. Database searches were conducted using
the Genetics Computer Group Sequence Analysis software (Devereux et al., 1984) of the Genetics Computer Group (University of Wisconsin).

RESULTS

Cloning and sequencing of the rpoN gene of V. anguillarum

To facilitate the construction of additional rpoN mutants and allow subsequent complementation studies, the rpoN-containing locus of V. anguillarum strain NB10 was cloned and sequenced. A PCR product generated from a partial rpoN sequence determined in a previous study (O’Toole et al., 1996) was radiolabelled and used as a probe to screen a genomic bacteriophage library of V. anguillarum. Approximately 3000 bacteriophage plaques were screened, of which three plaques hybridized to the probe. Three plaques, named 1-1, 7-1 and 8-1, were picked and re-probed for the purpose of single plaque isolation. From the three selected bacteriophages, their respective pBluescript plasmids, pBS1-1, pBS7-1 and pBS8-1, were excised and maintained in E. coli XL-1 Blue. Restriction digestion using EcoRI endonuclease to determine the size of the insert revealed that pBS1-1 contained an approximately 5.5 kb fragment while pBS7-1 and pBS8-1 both contained an insert of approximately 6.5 kb. Southern blot analysis (see Methods) indicated that pBS7-1 contained the entire rpoN gene (data not shown) and thus this clone was chosen for sequencing of rpoN. Sequencing was performed by walking along the DNA in both directions with oligonucleotide primers. The complete nucleotide and deduced amino acid sequence of rpoN is shown in Fig. 1.

The rpoN coding region extends for 1458 bp (nucleotides 544–2001 inclusive; Fig. 1) and encodes a potential 486 amino acid polypeptide which exhibits strong homology to the RpoN proteins from several bacteria. The highest degree of similarity was to the rpoN gene product of Salmonella typhimurium (65.1% identity and 82.2% similarity over the entire polypeptide). A highly conserved stretch of 10 amino acids (ARRTI-KYRE) known as the RpoN box, which is diagnostic of the σ^54 factor (Merrick, 1993) and is proposed to play a role in promoter recognition (Taylor et al., 1996), is encoded at the 3'-end of the rpoN gene of V. anguillarum (nucleotides 1930–1959 inclusive; Fig. 1). Two additional ORFs were found adjacent to the rpoN gene, one upstream and one downstream, with both transcribing in the same direction as rpoN (Fig. 1). The stop codon of the upstream ORF, ORF 1, is separated by 51 bp from the proposed start codon of the rpoN gene (Fig. 1). The deduced amino acid sequence of ORF 1 shows strong similarity to ORFs located just upstream of the rpoN gene of other bacterial species such as E. coli (75.4% identity and 88.3% similarity over a 163 amino acid region) which encode ATP-binding cassette transport-like proteins. A third ORF, ORF III, begins 26 bp downstream of the stop codon of rpoN (Fig. 1) and likewise exhibits homology to similarly located ORFs in other bacteria such as Klebsiella pneumoniae (58.7% identity and 79.4% similarity over a 63 amino acid region). This rpoN-linked ORF in K. pneumoniae, ORF95, encodes a so-called RpoN-modulation protein whereby mutations in ORF95 affect expression from certain RpoN-dependent promoters (Merrick & Coppard, 1989).

Construction of mutations in the rpoN gene

Two types of mutations were made in the rpoN gene of V. anguillarum strain NB10, i.e. insertion of plasmid DNA and an in-frame deletion. An insertion mutant, OTR81, was made by the integration of a mobilizable suicide plasmid, pDM4, into the rpoN gene. As stated above, two ORFs were identified closely aligning the rpoN gene of V. anguillarum and to eliminate the possible polar effects that a mutation in rpoN could have on neighbouring genes, an in-frame deletion of 348 bp (residues 961–1308 inclusive; Fig. 1) was constructed in the coding region of rpoN by allelic exchange, generating mutant OTR83.

Analysis of rpoN mutants for motility

Bacterial strains were grown overnight at room temperature and the cultures were examined for motility using phase-contrast light microscopy. Mutants OTR81 and OTR83 were non-motile, whereas wild-type strain NB10 exhibited rapid swimming motility. The strains were also tested for motility on soft agar and after incubation for 24 h, mutants OTR81 and OTR83 failed to migrate through the soft agar, in contrast to the wild-type strain NB10 (Fig. 2).

Western blot analysis for flagellin expression in the rpoN mutants

Previous results from our laboratory have shown that the flagellar filament of V. anguillarum is composed of four flagellin subunits, FlaA–D, with the sizes 40, 41, 42 and 45 kDa, respectively (Milton et al., 1996). Whole-cell lysates of the rpoN mutants were separated by SDS-PAGE and analysed by Western blotting using antibodies specific for the flagellin subunits. No flagellin expression was detected in mutants OTR81 or OTR83 (Fig. 3).

Electron microscopy of rpoN mutants

Analysis of the rpoN mutants OTR81 and OTR83 by negative staining and electron microscopy showed that both lacked a flagellar filament (Fig. 4).

Transcomplementation of mutations in rpoN

To determine whether flagellum expression and motility could be restored in both the rpoN polar insertion and deletion mutants, the mobilizable vector pRpoN-Va containing the rpoN gene of V. anguillarum was introduced into mutants OTR81 and OTR83 by conjugation. Western blot analysis demonstrated that flagellin subunit expression was regained in their re-
spective transconjugant strains, OTR81/pRpoN-Va and OTR83/pRpoN-Va (Fig. 3), which correlated with the formation of a full-length flagellar filament as seen by electron microscopy (Fig. 4). Motility assays showed that the transcomplemented strains, like NB10, migrated through soft agar (Fig. 2) and swam rapidly in liquid broth using light microscopy, a small percentage of bacteria displayed rapid swimming motility. This percentage increased greatly following the addition of 0.5 mM IPTG to the growth media. Furthermore, OTR81/pFH30 and OTR83/pFH30 migrated through soft agar at levels which increased under thermore, OTR81/pFH30 and OTR83/pFH30 mi-

Given the high sequence conservation of ε54 amongst bacteria, the question of whether the rpoN gene from another bacterial species could compensate for a disruption in the rpoN gene of V. anguillarum was also investigated. pFH30, a derivative of the mobilizable broad-host-range vector pVLT31, contains the rpoN gene of P. putida, but no neighbouring genes, inserted in the multiple cloning site of pVLT31 downstream of a tac promoter (Cases et al., 1996). Both plasmids pVLT31 and pFH30 were introduced into mutants OTR81 and OTR83 by conjugation. When transconjugants OTR81/pFH30 and OTR83/pFH30 were examined for motility in liquid broth using light microscopy, a small percentage of the bacteria displayed rapid swimming motility. This percentage increased greatly following the addition of 0.5 mM IPTG to the growth media. Furthermore, OTR81/pFH30 and OTR83/pFH30 migrated through soft agar at levels which increased under IPTG induction (Fig. 2). This was in agreement with the elevated flagellin expression observed in OTR81/pFH30 and OTR83/pFH30 grown in the presence of 0.5 mM IPTG (Fig. 3). However, transconjugants OTR81/pVLT31 and OTR83/pVLT31 failed to exhibit any motility (Fig. 2) or flagellin expression (Fig. 3).

To ensure that the plasmid insertion in the rpoN gene was maintained, all transcomplemented strains of OTR81 were grown in the presence of chloramphenicol. Furthermore, to verify that the rpoN in-frame deletion was still intact in strains OTR83/pVLT31 and OTR83/pFH30, three pairs of convergent PCR primers complementary to the rpoN gene of V. anguillarum. 

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**Fig. 1.** Nucleotide sequence of the rpoN gene of V. anguillarum and its deduced amino acid sequence. The rpoN gene extends from nucleotides 544 to 2001 and encodes a potential 486 amino acid polypeptide. The highly conserved stretch of 10 amino acids (ARRTIAKYRE) known as the RpoN box is underlined. Partial sequences of the two oligonucleotides rpoNe and rpoNh used to construct mutations in rpoN are indicated.
were used. All primer sets, which did not yield products from the rpoN gene of P. putida and were therefore considered to be specific to the V. anguillarum rpoN gene under the PCR conditions applied, confirmed the existence of the rpoN deleted allele (but not the wild-type allele) in OTR83/pVLT31 and OTR83/pFH30 by PCR analysis (data not shown). In addition, a convergent PCR primer pair complementary to the rpoN gene of P. putida which did not generate a product from the rpoN gene of V. anguillarum showed that the rpoN gene of P. putida was present in OTR81/pFH30 and OTR83/pFH30 but not in OTR81/pVLT31 and OTR83/pVLT31 (data not shown).

Analysis of rpoN mutants for the expression of virulence-associated phenotypes

To examine whether the disruption of the rpoN gene had any effect on other virulence-related functions, expression of the LPS O-antigen and the haemolytic and proteolytic activities were tested for rpoN mutants OTR81 and OTR83. Neither of the mutants differed from the wild-type strain with respect to these properties (data not shown). Similarly, both rpoN mutants, like wild-type strain NB10, grew successfully in TSB containing up to 1 mM EDDA and were thus proficient for iron-limited growth. This was in contrast to previous findings with the aflagellate rpoN transposon mutant ABE8 (O'Toole et al., 1996), which was unable to grow under low iron conditions (see Discussion).

Virulence studies on the rpoN mutants

To examine whether RpoN was required for expression of any other factors essential for virulence following penetration of the fish epithelial barrier, fish were infected with rpoN mutants OTR81 and OTR83 by intraperitoneal injection. In contrast to ABE8, which had an LD50 value of 16 × 106 bacteria (O'Toole et al., 1996), mutants OTR81 and OTR83 exhibited no de-
Fig. 4. Flagellum production in *V. anguillarum* strains viewed by electron microscopy. (a) Flagellated wild-type strain NB10. (b) Aflagellate *rpoN* deletion mutant OTR83. *rpoN* insertion mutant OTR81 was similarly aflagellate (not shown). (c) Transcomplemented strain OTR83/pRpoN-Va. Strain OTR83/pRpoN-Va also produced a flagellum (not shown). Bars, 1 μm.

**Table 1.** Virulence analyses of the wild-type, *rpoN* mutant and transcomplemented strains of *V. anguillarum*

pRpoN-Va contains the *rpoN* gene (and its neighbouring ORFs) of *V. anguillarum*. LD<sub>50</sub> values were calculated based on either the number of bacteria injected intraperitoneally (IP; mean value obtained from two separate experiments) or the number of bacteria (ml infected water)<sup>-1</sup> (mean value derived from two separate immersion infections). ND, Not determined.

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* Polar insertion of plasmid DNA in the *rpoN* gene.
† In-frame deletion in the coding region of *rpoN*.
‡ Transposon insertion in the *V. anguillarum* NB10 homologue of the *flhA* gene (O’Toole et al., 1996) which encodes a component of the flagellum-specific export apparatus essential for flagellation. Aflagellate mutant OTR7 was infected in parallel with the *rpoN* mutants for comparison.
§ A serotype O1 strain of *V. anguillarum* of lower virulence with respect to NB10.

crease in virulence with respect to the wild-type strain (LD<sub>50</sub> value of less than 10 bacteria) in intraperitoneal inoculation experiments (Table 1). Since a moderate shift in the virulence of the *rpoN* mutants would be difficult to detect where the LD<sub>50</sub> value is in the region of less than 10 bacteria, an in-frame deletion, identical to
the mutation in OTR83, was made in the rpoN gene of a less virulent serotype O1 V. anguillarum strain, 775. The resulting rpoN mutant, 775O83, had a similar LD50 value to its parent strain, 775 (Table 1).

In immersion infections of fish, the LD50 value of rpoN mutant OTR83 was 1.5 x 10^6 bacteria (ml infected water)^-1 while in parallel experiments, the wild-type strain NB10 had an LD50 value of 1.8 x 10^6 bacteria ml^-1 (Table 1). The aflagellate flaA transposon mutant OTR7, isolated previously (O'Toole et al., 1996), exhibited an LD50 value of 1.1 x 10^6 bacteria ml^-1 in concurrent immersion infections. Similarly, mutant OTR81 was attenuated for virulence in immersion infections (Table 1). Whether virulence could be restored in the rpoN mutants after introduction of the rpoN gene of V. anguillarum on a plasmid was also tested. The transcenplemented strains OTR81/pRpoN-Va and OTR83/pRpoN-Va partially regained virulence with respect to OTR81 and OTR83, i.e. had LD50 values of 3.0 x 10^6 bacteria ml^-1 and 5.5 x 10^6 bacteria ml^-1, respectively (Table 1).

DISCUSSION

In this study, a genomic bacteriophage library of V. anguillarum NB10 was screened and the rpoN gene was cloned and sequenced. The complete rpoN ORF encodes a potential 486 amino acid polypeptide which from computer analysis shows extensive homology to the RpoN protein of several species of bacteria. The rpoN gene is flanked by a number of ORFs which have the same direction of transcription as rpoN. The deduced amino acid sequence of the partially sequenced ORFs revealed that they are highly homologous to similarly located ORFs in the rpoN loci of other bacterial species.

In earlier work, an rpoN transposon mutant of V. anguillarum, ABE8, was found to be deficient for flagellum production and iron-limited growth (O'Toole et al., 1996). Introduction of the rpoN gene of V. anguillarum on a plasmid into mutant ABE8 restored flagellum expression and motility but failed to revive ability to grow under low available iron conditions (data not shown). ABE8 was subsequently found to lack the approximately 67 kb plasmid which is present in strain NB10 (data not shown) and most other pathogenic strains of V. anguillarum serotype O1 (Wiik et al., 1989; Larsen & Olsen, 1991) and encodes genes essential for iron sequestration (Actis et al., 1988) and, hence, virulence (Crosa et al., 1980; Singer et al., 1991). However, these findings alone did not rule out a regulatory role for RpoN in the ability to grow under low iron conditions since the plasmid-encoded effector proteins required for iron uptake are absent in mutant ABE8. Thus, to determine the importance of RpoN in the flagellation and virulence of V. anguillarum and to resolve whether it is required for iron-limited growth, additional rpoN mutants were constructed and complementation studies were performed.

Both the plasmid-insertion and the deletion rpoN mutants generated in this study grew as successfully as the wild-type strain at high concentrations of an iron-chelating agent. This demonstrates that neither rpoN nor any possible co-transcribed downstream genes are required for growth of V. anguillarum under low iron conditions. However, like ABE8, the rpoN mutants lacked expression of all of the flagellin subunits (Fig. 3) and exhibited a non-motile aflagellate phenotype (Figs 2 and 4). Complementation of the rpoN mutants with the wild-type rpoN gene of V. anguillarum restored flagellum expression (Figs 3 and 4) and motility (Fig. 2). Thus, RpoN is crucial for flagellum biosynthesis in V. anguillarum.

While the α5-dependent flagellum production observed in V. anguillarum would not appear to be a general feature of motile bacteria, it is possible that some species use RpoN and its activator proteins as a means to regulate flagellum expression in response to environmental and metabolic changes. Environmental modulation of the α5-dependent promoter of the flaB flagellin gene of Campylobacter coli has been reported (Alm et al., 1993).

Other recent data have indicated the involvement of RpoN in expression of structural components of the polar flagellum of V. anguillarum. Potential RpoN-dependent promoters have been found upstream of all but one of the flagellin genes of V. anguillarum, i.e. flaD (McGee et al., 1996). Work with other Vibrio species has also implied an important role for RpoN in flagellation and motility. RpoN-dependent promoters have been suggested for the flaC and flaE flagellin genes of Vibrio parahaemolyticus (McCarter, 1995) and the motY motilify genes of both Vibrio alginolyticus and V. parahaemolyticus (Okunishi et al., 1996). Moreover, a gene in V. parahaemolyticus, designated flaK, whose deduced product resembles members of a family of RpoN activators such as NtrC, is essential for production of at least one of the flagellins, FlaC (Starnbach et al., 1996) although transcription of the flaC gene itself is independent of RpoN (Starnbach & Lory, 1992). However, despite the appearance of a consensus promoter for α5 (RpoF) but not α5 (RpoN) upstream of the flaD flagellin gene of V. anguillarum (McGee et al., 1996), FlaD flagellin expression was not detected in the rpoN mutants (Fig. 3). In P. aeruginosa, expression of the flagellin gene, which is under the direct control of a α8-dependent promoter (Totten & Lory, 1990; Starnbach & Lory, 1992), also requires RpoN (Totten et al., 1990) although transcription of the rpoN gene itself is independent of RpoN (Starnbach & Lory, 1992). This indicates that in certain bacteria RpoN may control a regulator of flagellum biosynthesis. Alternatively, transcription of one or more of the early assembled flagellum components may depend on RpoN and their lack of expression in an rpoN mutant might result in down-regulation of genes, e.g. flagellin genes, which are transcribed later in the flagellar gene cascade. This latter

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hypothesis is supported by the identification of an RpoN-specific consensus sequence upstream of the flgB gene homologue of V. anguillarum (R. O'Toole & H. Wolf-Watz, unpublished). The FlgB rod protein is probably expressed at an earlier stage during flagellum assembly than flagellin subunits FlaC and FlaD, since an flgB transposon mutant fails to express these flagellins (O'Toole et al., 1996). Hierarchical control of flagellar gene expression which coincides with the order of assembly of components into a functional flagellum has been well-documented in E. coli and S. typhimurium (Komeda, 1982; Kutsukake et al., 1990; Kubori et al., 1992).

As mentioned above, the deduced product of the rpoN gene of V. anguillarum exhibits considerable sequence conservation with respect to the RpoN proteins from a variety of other bacteria. Introduction of solely the rpoN gene of P. putida into both the V. anguillarum rpoN polar insertion and deletion mutants re-established flagellin expression (Fig. 3) and motility (Fig. 2), indicating that conservation of RpoN function between V. anguillarum and P. putida, and possibly other bacterial species, exists.

Fish infection studies were performed to assess the role of RpoN in the pathogenicity of V. anguillarum. Mutations in the rpoN gene of V. anguillarum strain NB10 and of the less virulent strain 775 had no effect on virulence when the mutants were injected intraperitoneally into fish (Table 1). RpoN, therefore, does not appear to function as a regulator of any virulence or metabolic functions which are important in infection subsequent to penetration of the fish epithelium by the pathogen. In agreement with this were the findings that RpoN is not required by V. anguillarum for expression of LPS O-antigen and growth proficiency under low iron conditions, both of which are essential for the virulence of V. anguillarum when injected into the fish interior (Norqvist & Wolf-Watz, 1993; Crosa et al., 1980; Singer et al., 1991).

However, the virulence of the rpoN mutants was significantly reduced, with respect to the wild-type strain, when fish were exposed to water containing V. anguillarum. The rpoN deletion mutant exhibited an approximately 800-fold loss in virulence in immersion infections, which was partially complemented following re-introduction of the rpoN gene on a plasmid (Table 1). Previous infection studies with transposon mutants of V. anguillarum demonstrated that loss of the polar flagellum resulted in a decrease in pathogenicity via immersion but not intraperitoneal inoculation (O'Toole et al., 1996). For direct comparison, fish infections with the rpoN mutants were performed in parallel with an aflagellate flaB transposon mutant, OTR7. Mutant OTR7, which like the rpoN mutants lacks expression of all of the flagellin subunits (O'Toole et al., 1996), exhibits virulence typical of aflagellate mutant derivatives of V. anguillarum NB10. An approximately 600-fold decrease in virulence was observed for OTR7 in immersion infections (Table 1). Thus, much of the decrease in virulence of water-borne V. anguillarum due to loss of RpoN can be attributed to the elimination of the flagellum, which is required to mediate chemotactically directed motility during infection (O'Toole et al., 1996). Other RpoN-dependent factors which assist V. anguillarum in immersion infections of fish may, however, exist, but the large reduction in virulence due to loss of the flagellum may obscure their contribution to virulence.

In summary, the sequence data presented in this work show that the rpoN-containing locus of V. anguillarum closely resembles its counterparts in other species of bacteria in terms of conservation of sequence and gene linkage. Expression of the flagellin subunits is dependent upon RpoN, and rpoN mutants of V. anguillarum are aflagellate and non-motile. RpoN therefore functions as a regulator of an important virulence factor of V. anguillarum, the polar flagellum, which is involved in the infection of fish by water-borne bacteria. Interestingly, RpoN does not mediate expression of any factors required for pathogenicity once V. anguillarum has penetrated the fish epithelial barrier.

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