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NOTES

Cloning of a *Vibrio alginolyticus rpoN* Gene That Is Required for Polar Flagellar Formation

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A fragment of DNA was cloned which complemented a polar flagellum-defective (*pof*) mutation of *Vibrio alginolyticus*. The fragment contained two complete and two partial open reading frames (ORFs) (ORF2 and -3 and ORF1 and -4, respectively). The presumed product of ORF2 has an amino acid sequence with a high degree of similarity to that of RpoN, which is an alternative sigma factor (σ^{54}) for other microorganisms. The other ORFs are also homologous to the genes adjacent to other *rpoN* genes. Deletion analysis suggests that ORF2 complements the *pof* mutation. These results demonstrate that RpoN is involved in the expression of polar flagellar genes.

Many bacteria move by means of rotating flagella, located either peritrichously or at a pole on the cell body. Certain marine *Vibrio* species are unique in that they have two types of flagella: a single polar flagellum suited for swimming in a liquid environment and numerous lateral flagella suited for swarming over the surfaces of animate or inanimate objects (2, 4, 20, 24). In *Vibrio parahaemolyticus* and the closely related *Vibrio alginolyticus*, it has been shown that polar and lateral flagella are powered by different ion-motive forces: the coupling ion of the polar flagellar motor is sodium and that of the lateral flagellar motor is a proton (5, 15).

The lateral flagella are synthesized under viscous conditions (2, 6, 24, 31). An increase in viscosity is a cue for induction of lateral flagellar expression (6). Surprisingly, a decrease in the rotation of the polar flagellum is sensed and lateral flagellar expression is induced (14, 23). Thus, the polar flagellum functions not only as a locomotive organelle but also as a mechanosensor which couples viscous drag to cell differentiation. The polar flagella of *V. alginolyticus* rotate very fast, up to 1,700 rps, and the rotation is stable (22, 27). Furthermore, strictly speaking, the synthesis of polar flagella may not be constitutive. It should be more or less coupled with the cell division cycle, since most of the *Vibrio* cells have a single polar flagellum at one of the cell poles.

To investigate these unique features of the polar flagellum in *V. alginolyticus*, we wanted to clone the genes involved. From a lateral flagellum-defective mutant, we have isolated many polar flagellum-defective mutants which are defective in both polar and lateral flagellar formation (12, 15, 28). In this study, using one of those strains as a recipient for shotgun cloning by electroporation (16), we cloned a *pof* gene involved in polar flagellar formation. Nucleotide sequencing of the gene and the flanking region revealed that the *pof* gene encodes an alternative sigma factor, σ^{54} (RpoN).

Cloning of the *pof* gene. Most of the strains and plasmids used in this work have been listed previously (28). Plasmid pSU18 and chromosomal DNA from *V. alginolyticus* 138-2 were digested with *Eco*RI and *Bam*HI and ligated. These DNA libraries were transferred into YM14 cells by electroporation as described previously (16), and the cells were inoculated onto 0.3% agar VC plates (0.3% agar in VC medium [0.5% Polypeptone, 0.5% yeast extract, 0.4% K_2HPO_4, 3% NaCl, 0.2% glucose]) supplemented with chloramphenicol. The only colony which spread on the plate was isolated, and the plasmid DNA from the clone was recovered (Fig. 1A). It contained a 2.4-kb *Eco*RI fragment (Fig. 1B). The plasmid was named pVIK14 and was used for further analysis. When plasmid pVIK14 was introduced into YM14 cells, the resulting cells spread on 0.3% agar VC plates as fast as wild-type cells (data not shown), and the formation of polar flagella was confirmed by high-intensity dark-field microscopy (12). Therefore, we concluded that the *Eco*RI fragment contained the *pof* gene. When the plasmid pVIK14 was introduced into other *pof* mutants, YM44 cells also acquired motility.

The 2.4-kb fragment was hybridized to chromosomal DNA digested by *Eco*RI in high-stringency conditions (Fig. 2). Single bands of 2.4 and 5 kb were detected in *V. alginolyticus* and *V. parahaemolyticus*, respectively. In the two *pof* mutants, the 2.4-kb band was detected. No band was detected in *Salmonella typhimurium* or *Escherichia coli.*

Nucleotide sequence of the *pof* gene. We then subcloned the various regions of the 2.4-kb fragment into the vector pSU21, whose multiple cloning site is different from that of pSU18 (Fig. 1B). Only pMSA6 complemented the *pof* defect of YM14. Next, we determined the entire nucleotide sequence of the 2.4-kb *Eco*RI fragment by the dideoxy chain termination method with the Sequenase version 2.0 DNA sequencing kit (United States Biochemical Corp., Cleveland, Ohio). [α-^{32}P] dCTP (3,000 μCi/mmol; Amersham Japan, Tokyo) was used for radioactive labeling.

Two complete and two partial open reading frames (ORFs), all in the same orientation, were identified in the fragment and

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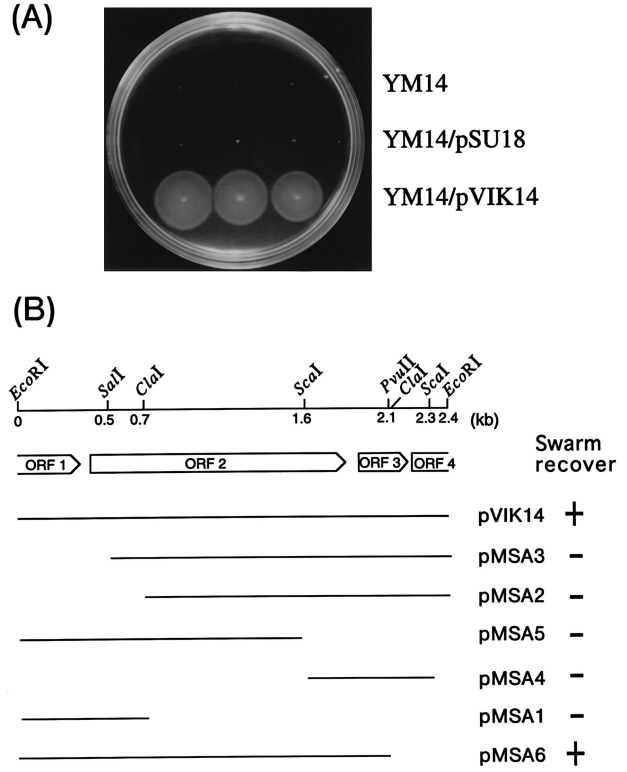

FIG. 1. Restriction map and complementation assay. (A) Swimming recovery of YM14 cells carrying the plasmid pVIK14. Single colonies of the chloramphenicol-resistant transformants with pSU18 (vector) or pVIK14 and of the host strain (YM14) were inoculated on a 0.3% agar VC plate and incubated at 30°C. (B) Restriction map of plasmid pVIK14 and its deletion derivatives. The plasmids carry the fragments indicated by solid lines. Their abilities to complement the defect of strain YM14 are indicated on the right side of the map. The coding regions, ORF1, -2, -3, and -4, were predicted from the DNA sequence.

named ORF1, ORF2, ORF3, and ORF4 in the order shown in Fig. 1B. The noncoding regions between the ORFs are 54, 27, and 5 bp. From the results of the complementation assay, we concluded that the longest ORF (ORF2; 489 amino acids) is the *pof* gene (Fig. 1B).

A search of the protein database revealed that the deduced amino acid sequence of the *pof* gene product is very similar to those of the *rpoN* gene products (σ^{54}) of a wide variety of bacteria (Fig. 3). The identity of ORF2 with the various RpoN sequences is about 60%. We found all the diagnostic features of RpoN (i.e., a glutamine-rich region, an acidic central region, a helix-turn-helix motif, and an RpoN box) in the deduced *pof* product. We therefore concluded that this gene encodes σ^{54} and named the gene *rpoN*. However, the mutants did not show glutamine auxotrophy and they grew on ammonium ions as the sole nitrogen source.

ORF1, ORF3, and ORF4 were also similar to the *rpoN*-linked genes (Fig. 4). The order of the homologous genes is the same as those in other species, such as *E. coli* (30). The ORF1 protein may be an ATP-binding protein and a member of the ABC transporter family (11), and the ORF1 homolog was suggested to be an essential gene in *Rhizobium meliloti* (1). The function of the ORF3 protein (95 amino acids), whose gene is just downstream of *rpoN*, is not known. The ORF4 protein is homologous to an enzyme, IIA, of the phosphoenolpyruvate-dependent phosphotransferase system (30).

Implications of regulation of polar flagellum gene expression. The extensive similarity of its gene product to σ^{54} in many

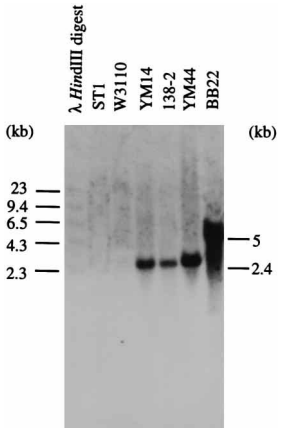

FIG. 2. Southern hybridization with the *rpoN* coding region. Total chromosomal DNAs, which were prepared for strains ST1 (*S. typhimurium*), W3110 (*E. coli*), and YM14, 138-2, YM44, and BB22 (*V. parahaemolyticus*) were digested with *Eco*RI and separated by agarose gel electrophoresis. Southern hybridization was performed by using a probe prepared from an *Eco*RI fragment of pVIK14 according to a manual from the manufacturer (ECL direct nucleic acid labeling and detection system; Amersham).

other bacteria and the existence of the three *rpoN*-flanking genes indicate that the *pof* gene encodes an alternative sigma factor, σ^{54} (RpoN). This is consistent with the previous finding that a gene named *motY*, which encodes a component of the sodium-driven polar flagellar motor, has a sequence very similar to the consensus of the σ^{54}-dependent promoters (28). In *V. parahaemolyticus*, a potential σ^{54} consensus promoter sequence in addition to the σ^{28} consensus promoter may direct the genes for hook-associated proteins or flagellin (25). Fur-

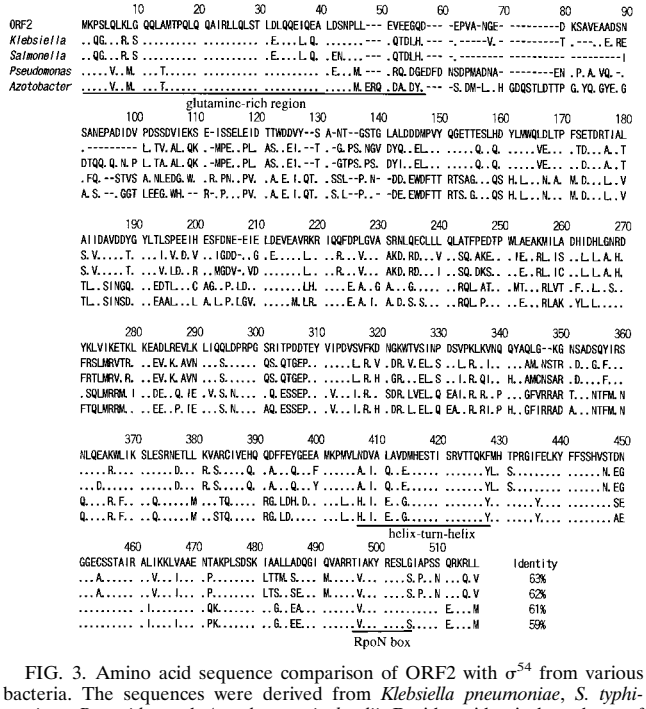

FIG. 3. Amino acid sequence comparison of ORF2 with σ^{54} from various bacteria. The sequences were derived from *Klebsiella pneumoniae*, *S. typhimurium*, *P. putida*, and *Azotobacter vinelandii*. Residues identical to those of ORF2 are indicated by periods, and gaps are indicated by hyphens. The percent identities of ORF2 with the various RpoN sequences are shown at the ends of the sequences.

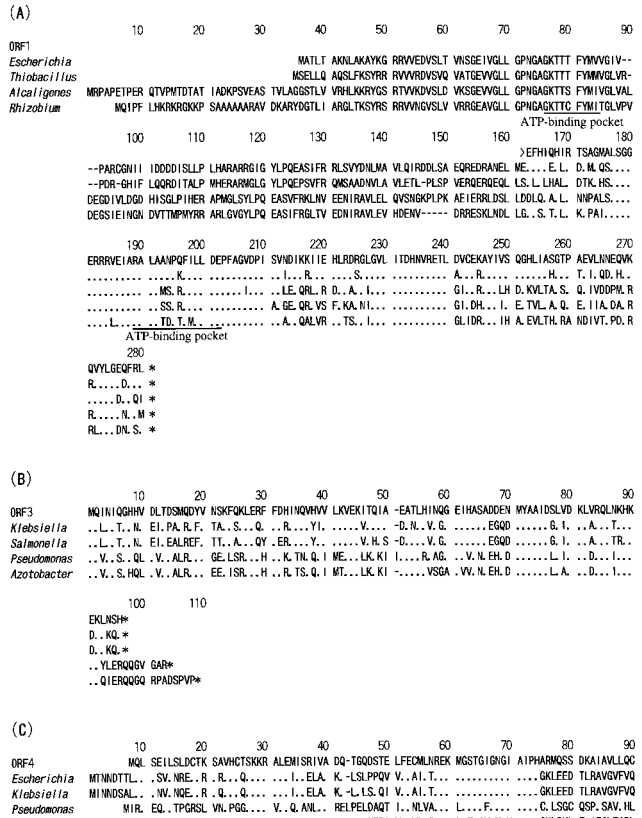

FIG. 4. Amino acid sequence comparison of the *rpoN*-flanking gene products of ORF1 (A), ORF3 (B), and ORF4 (C) of *V. alginolyticus* with those of various bacteria. The sequences were derived from *Klebsiella pneumoniae*, *S. typhimurium*, *P. aeruginosa*, *Azotobacter vinelandii*, *Thiobacillus ferrooxidans*, *Alcaligenes eutrophus*, *Rhizobium meliloti*, and *Bradyrhizobium japonicum*. Residues identical to those of ORF1, ORF3, or ORF4 are indicated by periods, and gaps are indicated by hyphens.

thermore, the predicted product of *flaK*, which is located in a cluster of polar flagellum genes of *V. parahaemolyticus*, is homologous to NtrC, a σ^{54}-interacting regulatory protein (33). Therefore, we suggest that some, if not all, polar flagellum-related genes, including those involved in flagellation, motility, and chemotaxis, are transcribed from σ^{54}-dependent promoters.

In *E. coli* and *S. typhimurium*, genes involved in flagellar formation and function form contiguous clusters and are organized into a regulon (for reviews, see references 7 and 21). Most of them are transcribed by RNA polymerase holoenzyme containing σ^{28}, encoded by *fliA*, which itself is a member of the regulon (18, 19). In *V. parahaemolyticus* also, expression of at least one lateral flagellum gene coding for the filament subunit protein (flagellin) depends on σ^{28}, encoded by *lafS* (26). In other bacteria, another alterative sigma factor, σ^{54}, is activated by environmental changes, such as lack of nutrients, to express the genes involved in responses to the environment (17). In several bacteria with polar flagella, such as *Pseudomonas aeruginosa* (34), *Pseudomonas putida* (13), *Caulobacter crescentus* (9), and *Vibrio anguillarum* (29), *rpoN* mutants do not produce flagella. Furthermore, it has been shown that some flagellum-related genes are transcribed from σ^{54}-dependent promoters (3, 35). As suggested for *P. aeruginosa* (32) and *C. crescentus* (9), it is possible that σ^{54} and σ^{28} in marine *Vibrio* spp. may regulate the expression of polar flagellum genes at different levels in the regulatory hierarchy. Interestingly, no σ^{54}-dependent promoter has been described in any gene for peritrichous flagella. Considering that polar flagella seem to be synthesized once in a cell cycle and that *rpoN* expression is cell cycle-dependent in *C. crescentus* (8–10), we speculate that the regulation of σ^{54} activity may be responsible for the determination of polar flagellation.

Nucleotide sequence accession number. The sequence data of the 2.4-kb fragment have been deposited with DDBJ under accession no. AB006709.

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