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Regulation of plasmid-mediated iron transport and virulence in *Vibrio anguillarum*

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Summary. Iron is essential for bacterial growth and metabolism. In vertebrates this metal is complexed by high-affinity iron-binding proteins, such as transferrin in serum. The fish pathogen *Vibrio anguillarum* possesses a very efficient iron-uptake system which is encoded in the virulence plasmid pJM1. This allows the bacterium to utilize the otherwise unavailable iron in the fish host, resulting in the septicemic disease vibriosis. This system includes the siderophore anguibactin and transport components. We have cloned this iron-utpake system and have defined several genetic units by transposition mutagenesis. Nucleotide sequence analysis identified four open reading frames in the transport region, one of these corresponding to the gene for the outer membrane protein OM2 and another to a 40-kDa polypeptide. Complementation analysis indicated that products from all four reading frames are required for the transport of iron–anguibactin complexes. We have also identified positive and negative-acting regulatory elements that modulate in concert the expression of anguibactin biosynthetic genes and iron transport. The deletion or mutation of the positive-acting regulatory genes results in an iron-uptake-deficient phenotype and leads to an attenuation of virulence, underscoring the importance of this iron-uptake system as a virulence attribute of *V. anguillarum*.

Key words: Iron-utapke system – Vibriosis – Siderophore – *Vibrio anguillarum*

Introduction

An important attribute of a bacterial pathogen is its ability to grow in the host vertebrate's fluids and tissues. One essential element for bacterial growth is iron (Crosa 1980; Weinberg 1978), which is tightly bound by high-affinity binding proteins, such as transferrin and lactoferrin, present in tissues and fluids. Thus, the available concentration of iron for bacterial utilization is too low (Weinberg 1984). Pathogenic bacteria developed different systems to scavenge the host's iron (Crosa 1989). *Vibrio anguillarum* has a plasmid-mediated iron-uptake system consisting of the diffusible siderophore anguibactin (Jalal et al. 1989) and an iron-transport system that internalizes the anguibactin-bound iron. This siderophore competes for iron with the host's high-affinity binding proteins; the ferric–anguibactin complex is then recognized and internalized by a receptor system which includes at least two proteins: OM2, which is exposed on the surface of the cell (Actis et al. 1985), and a 40-kDa polypeptide, P40 (Crosa 1989). The components of the *V. anguillarum* iron-sequestering system, as well as regulatory elements, are encoded by the plasmid pJM1 (Crosa et al. 1980). Regulatory elements that modulate the synthesis of anguibactin as well as OM2 are the AngR protein and Taf (Tolmasky and Crosa 1984; Tolmasky et al. 1988a).

In this paper we describe features of the regulation of biosynthesis of components of the iron-uptake system.

The *trans*-acting factor Taf

Molecular cloning of the pJM1 iron-uptake region (Fig. 1) demonstrated that *V. anguillarum* harboring such a clone (pJHC-T2612) could synthesize anguibactin as well as OM2 and P40 (Table 1; Tolmasky and Crosa 1984). However, the levels of production of anguibactin were so low that these strains could not grow in the presence of iron chelators such as ethylenediamine-*N,N'*-bis(2-hydroxyphenylacetic acid) (EDDA) and were avirulent (Tables 1 and 2; Tolmasky and Crosa 1984). It was determined that these clones lacked the genetic determinants encoding a regulator that was designated Taf. Transfer of a clone carrying these genetic determinants (Fig. 1) to the *V. anguillarum* strains already harboring the iron-uptake clones increased the

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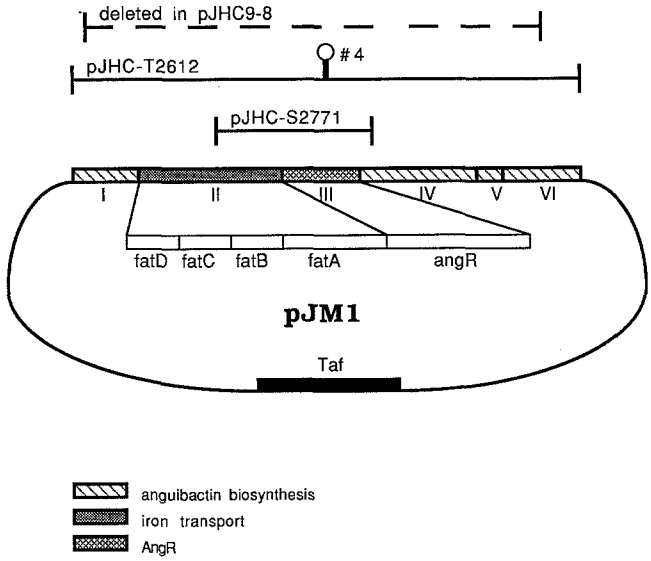

Fig. 1. Diagram of pJM1 and derivatives. The diagram shows pJM1 DNA with the roman numerals indicating different genetic units, as defined before, and the region were the genetic determinants for Taf are found (Tolmasky et al. 1988b). *fatA* and *fatB* are the genes encoding OM2 and P40 respectively. All four fat genes as well as *angR* have been sequenced (Actis et al. 1988, Farell et al. 1990). The lines on top of pJM1 represent DNA fragments cloned and the broken line shows the extension of the deletion in pJHC9-8. The circle on a stick labeled #4 represents the Tn3-HoHo1 insertion #4, which is located in the *angR* gene of pJHC-T2612

Table 1. Properties of *V. anguillarum* strains

Plasmids	AngR	Taf	Growth in EDDA	Anguibactin	OM2	Receptor
None	–	–	–	–	–	–
pJHC-T2612	+	–	–	L	+	+
pJHC-T2612, pJHC9-8	+	+	+	+	+	+
pJHC-T2612#4, pJHC9-8	–	+	–	–	+	+
pJHC-T2612#4, pJHC9-8, pJHC-S2771	+	+	+	+	+	+

Growth in EDDA was determined by incubation of the cells in M9 minimal medium with the addition of 10 μM EDDA. Anguibactin activity was determined by using bioassays as previously described (Walter et al. 1983); L indicates anguibactin could be detected but only at very low levels. OM2 was detected by immunoblots as described by Actis et al. (1985). Receptor activity was detected by crossfeeding bioassays using the strain to be tested as lawn and supernatant form *V. anguillarum* 775 as source of anguibactin (Tolmasky and Crosa 1984)

levels of anguibactin production and conferred on the bacteria the ability to grow in minimal medium with EDDA as well as restoring virulence (Tables 1 and 2; Tolmasky et al. 1988a). *lacZ* gene fusions generated by insertion mutagenesis with Tn3-HoHo1 (Stachel et al. 1985) in anguibactin biosynthetic genes were used to test the action of Taf. β-Galactosidase activity, as well as mRNA levels, were determined in the presence and

Table 2. Comparison of *V. anguillarum* strains

Plasmid	EDDA MIC	Relative anguibactin activity	Virulence
pJM1	40	1	<10
pJHC1	160	5.5	<10
pJHC-T2612	2	n.d.	3×10^6
pJHC-T2612, pJHC9-8	40	1	10^4
pJHC-T5.12, pJHC9-8	160	5.7	10^4
pJHC9-8	2	0	$>10^8$

The minimum inhibitory concentrations (MICs) of EDDA were determined on plates containing minimal medium plus 0.7% agarose with increasing concentrations of EDDA. Anguibactin activity was determined by crossfeeding bioassays. The values shown in the table were determined by measuring the growth halos and normalizing to the value obtained for *V. anguillarum* (pJM1). Each virulence value represents the number of cells that killed 50% of the fish. Assays were performed as described previously (Tolmasky and Crosa 1984). Plasmid pJHC-T5.12 is a recombinant clone equivalent to pJHC-T2612 but obtained from pJHC1. In strains carrying pJHC-T2612 or pJHC-T5.12, pJHC9-8 was included to provide Taf. n.d. = not determined: the halo, although existed, was too small to be accurately measured

absence of Taf. The results indicated that both were increased, suggesting that Taf acts at the transcriptional level (Tolmasky et al. 1988a).

The regulator AngR

Analysis of some of the mutants mentioned above, e.g. pJHC-T2612#4 (Fig. 1), led to the finding of another regulator that appears to work as an activator of anguibactin biosynthetic genes (Table 1). AngR, the product of the *angR* gene, regulates several genetic loci on the pJM1 plasmid in *trans* and maps to a region immediately downstream from the *fatA* gene which encodes the protein OM2 (Fig. 1). This gene has been recently cloned and sequenced (Farrell et al. 1990). Fig. 2 shows a diagram of a special feature that was found by analyzing *angR:* the presence of a helix-turn-helix motif similar to those found in DNA-binding proteins. Interestingly, one of the helices shows very strong similarity with the DNA-binding domain of the Cro protein from the *Salmonella* phage P22 (Sauer et al. 1982), 8 out of

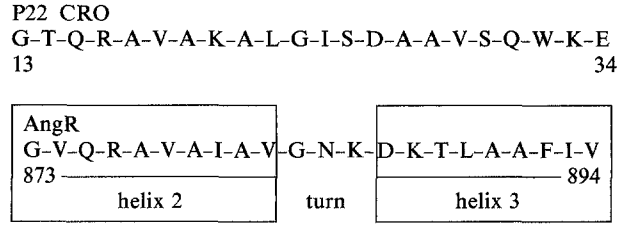

Fig. 2. Amino acid sequence of the helix-turn-helix regions of AngR and P22 Cro. Both DNA binding domains are aligned (Poteete et al. 1986; Farrell et al. 1990)

11 amino acids being identical. The conformation and the similarity of AngR to a known DNA-binding domain suggest that AngR may interact with DNA. Given the strong similarity between the helix 2 domain of these two proteins, it is probable that both serve the same function, which is to align the helix 3 in the major groove of the DNA. It is also likely that helix 3 of AngR is the recognition portion. The similarity of these two proteins was coincident with a similarity of P22 Cro operator sites with a region located at the −35 to −10 region of *angR* (Farrell et al. 1990).

In a recent study of the plasmid content and iron-uptake characteristics of several *V. anguillarum* strains obtained from various geographical regions, we determined that some pJM1-like plasmids, such as pJHC1, encoded iron-uptake systems that allowed bacteria to grow at higher EDDA concentrations as compared to *V. anguillarum* 775 which carries pJM1 (Table 2). Comparison of the components of the iron-uptake systems encoded by both pJM1 and pJHC1 showed that this latter plasmid encoded biosynthesis of higher levels of anguibactin (Table 2; Tolmasky et al. 1988b). However, this difference was not translated into higher virulence of *V. anguillarum* strains carrying pJHC1 or its cloned derivatives as compared to those carrying pJM1 or clones from the iron-uptake region of pJM1 (Table 2). Molecular cloning and insertion mutant complementation analysis demonstrated that the factor responsible for these higher levels of anguibactin production was the *angR* gene (Salinas et al. 1989). All comparisons between both *angR* regions, carried out by restriction endonuclease mapping and hybridization resulted in apparent identical DNA fragments. It is thus likely that the difference between these two *angR* genes is subtle, possibly one or only a few nucleotides have changed. We are presently sequencing the pJHC1 *angR* gene. Identification of the difference(s) between both AngR proteins at the nucleotide level and site-directed mutagenesis studies will be essential to correlate protein domains associated with regulatory functions and those responsible for DNA binding.

Conclusions

The pJM1 plasmid-mediated iron-transport system shows a complex regulation in which positive regulatory factors may play an important role in the expression of genes involved in the biosynthesis of anguibactin as well as in those associated with the actual transport of iron into the cell cytosol. Furthermore, expression of this system is repressed by high iron concentration. We conclude from present evidence that this repression occurs at both the transcriptional as well as the translational level, involving both a Fur-like product and antisense RNA. Our present efforts are thus directed to elucidating the role of these positive and negative regulatory components in the global regulation of this fascinating plasmid-mediated iron-uptake system.

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