Mol Gen Genet (1989) 218:367–370

MGG

Mapping of chromosomal loci associated with lipopolysaccharide synthesis and serotype specificity in *Vibrio cholerae* 01 by transposon mutagenesis using Tn*5* and Tn*2680*

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Summary. *Vibrio cholerae* strains of the 01 serotype have been classified into three subclasses, Ogawa, Inaba and Hikojima, which are associated with the O-antigen of the lipopolysaccharide (LPS). The DNA encoding the biosynthesis of the O-antigen, the *rfb* locus, has been cloned and analysed (Manning et al. 1986; Ward et al. 1987). Transposon mutagenesis of the Inaba and Ogawa strains of *V. cholerae*, using Tn*5* or Tn*2680* allowed the isolation of a series of independent mutants in each of these serotypes. Some of the insertions were mapped to the *rfb* region by Southern hybridization using the cloned *rfb* DNA as a probe, confirming this location to be responsible for both O-antigen production and serotype specificity. The other insertions allowed a second region to be identified which is involved in *V. cholerae* LPS biosynthesis.

Key words: LPS – Bacteriophage resistance; O-antigen – P-factor – Conjugation

Introduction

Vibrio cholerae 01 strains can be classified into three serotypes, Ogawa, Inaba and Hikojima, associated with the O-antigen of the lipopolysaccharide (LPS). Three antigens, A, B and C and the relative amounts of each on the various serotypes have been defined. Ogawa contains A, B and a small amount of C; Inaba contains A and C, and Hikojima possesses A, B and C. However, little information is available on the Hikojima strains as they appear to be unstable. The precise nature of the A, B and C antigens is unknown (Sakazaki and Tamura 1971).

Studies on the chemical composition of *V. cholerae* LPS (Redmond 1978, 1979; Kenne et al. 1982; Hisatsune and Kondo 1980; Sen et al. 1980) have revealed the types of sugars present and some of the linkages involved. The LPS is thought to consist of a perosamine backbone substituted at various positions by minor sugars including a number of amino sugars. Perosamine and quinovosamine are associated exclusively with the O-antigen as is 4-amino-4-deoxy-L-arabinose which has been found only in Ogawa LPS and may be associated with the B antigen which is characteristic of this serotype (Redmond 1978). Monoclonal antibodies directed against core antigens and also the A, B and C antigens have been described (Gustaffson and Holme 1985; Ward HM, Thomas CJ and Manning PA manuscript in preparation) and these may allow the structure and composition of particular determinants to be identified.

Conjugation studies in *V. cholerae* (Bhaskaran 1959; Parker et al. 1979) have allowed the construction of a linkage map of the *V. cholerae* chromosome. The locus encoding serotype specificity, referred to as *oag*, has been mapped between *ilv* and *arg* and is closely linked to the *mot* locus (*strA – ilv-1 – spcA – mot – oag – arg-1*). It is predicted that *oag* actually represents the *rfb* gene cluster which encodes the biosynthesis of O-antigen of the LPS (Manning et al. 1986; Ward et al. 1987) and accordingly should be designated *rfb* as is the convention.

The genes encoding the biosynthesis of the O-antigen of Ogawa and Inaba have been cloned (Manning et al. 1986), and a detailed analysis of the cloned DNA has been described (Ward et al. 1987). These studies have enabled a physical map of contiguous chromosomal DNA to be derived and a minimal region consisting of a 19.5 kb *Sst*I fragment, has been defined as containing all the necessary information for *V. cholerae* O-antigen expression in *Escherichia coli* K12. Thus, the plasmid pEVX7 containing this *Sst*I fragment can be used to probe transposon insertion mutants in the O-antigen region and so detect any DNA rearrangements.

This paper describes the isolation and characterization of a series of transposon insertion mutants and their use to define and map the *rfb* locus of *V. cholerae*.

Results

Neutralization of bacteriophage VcII using purified LPS

Phage neutralization studies performed according to Guidolin and Manning (1985) using phenol/water extracted LPS (Jann and Westphal 1975) show that LPS from strains 569B (Classical, Inaba), 1621 (El Tor, Inaba), 029 (Classical, Ogawa) and 017 (Eltor, Ogawa) were capable of inactivating phage VcII, but no effect was observed with LPS from the O-antigen-less strain Kasauli R (Classical, rough) or the hybrid strain 569B-165. This latter strain differs only from strain 569B in that it has the O-antigen of a non-01 type but all other surface antigens are identical. About 5 μg/ml of LPS was sufficient for 50% neutralization. These data indicate that VcII, like phage CP-T1 (Guidolin and Manning 1985), uses the O-antigen of LPS as its receptor.

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It should be noted that although VcII will only plaque on classical strains, it can be neutralized by LPS from any *V. cholerae* strain regardless of biotype. Thus, it can be concluded that the biotype specificity of the phage is not at the level of the receptor, but due to immunity as a result of lysogeny or to the presence of biotype specific restriction-modification systems (Imbesi and Manning 1982).

Characterization of the LPS of transposon insertion mutants

By simultaneous selection for phage and antibiotic resistance it was possible to isolate Tn*2680* and Tn*5* insertion mutants in strains 569B, 1621 and 017. Bacterial agglutination tests indicated that most of the putative transposon insertion mutants either did not express or expressed greatly reduced amounts of O-antigen on their surface (Table 1). The LPS of all the transposon insertion mutants were examined by SDS-polyacrylamide gel electrophoresis (Lugtenberg et al. 1975) followed by silver staining (Tsai and Frasch 1982) of whole cell lysates. Most mutants exhibited the characteristic pattern of the type rough strain Kasauli R with no material corresponding to LPS substituted with O-antigen being detected. Instead a broad region was detected at the bottom of the gel, representing LPS molecules consisting of lipid A substituted with the core oligosaccharide. In rough mutants where there is no O-antigen these molecules are more numerous than in the smooth parent strain since all molecules, not just a small proportion, are unsubstituted. Therefore the region is more diffuse than that of the smooth parent strain.

Table 1. Bacterial agglutination

Strain	Mutation	Antiserum titre[a]		
		α-Ogawa	α-Inaba	α-Kasauli R
1621	–	28		2^5
V638	*rfa*::Tn*5*	<2		2^{10}
V640[b]	*rfb*::Tn*5*-1	–		–
V641	*rfb*::Tn*5*-2	<2		2^{10}
V462	*rfb*::Tn*5*-3	<2		2^9
V643	*rfb*::Tn*5*-4	<2		2^{11}
V644	*rfb*::Tn*5*-5	<2		2^{11}
569B	–		2^{10}	2^6
V661	*rfb*::Tn*5*-6		2^2	2^{11}
V663	*rfb*::Tn*5*-7		<2	2^{11}
V665	*rfb*::Tn*5*-8		<2	2^{11}
V667	*rfb*::Tn*5*-9		<2	2^{11}
V669	*rfb*::Tn*5*-10		<2	2^{11}
V671	*rfb*::Tn*5*-11		2^7	2^{11}
Kasauli R	*rfb*?	<2	<2	2^{11}

[a] Bacterial cells were used at a density of 4×10^9 cells/ml and the titre is the reciprocal of the lowest dilution which produced agglutination

[b] Strain V640 auto-agglutinated

Southern hybridization analysis

It was possible to map precisely most of the transposon insertions by Southern hybridization analysis (Southern 1975; Maniatis et al. 1982). Chromosomal DNA from the transposon mutants was digested with various restriction enzymes and after transfer to nitrocellulose, was probed with [^{32}P]labelled pEVX7. The use of *Cla*I, *Eco*RI, *Sst*I and *Bam*HI digests allowed the precise positions of the various Tn*5* insertions to be located on the restriction map of the *rfb* region (Fig. 1). It can be seen that the majority of insertions are localized in the right-hand end (in the 11.7 kb *Cla*I fragment) of the cloned DNA or just beyond this region. Interestingly, RNA polymerase binding studies and other data (unpublished) have suggested that there are several transcriptional units and that the insertions indicated here would all map within two of these.

The transposon insertion in strain V644 did not appear to map within the DNA region included in pEVX7, yet it did alter the size of the 11 kb *Bam*HI fragment. Probing digests of V644 with pPM1003, which includes additional contiguous chromosomal DNA beyond the limits of the *Sst*I fragment contained in pEVX7 showed that the mutation in this strain maps in the 5 kb *Sst*I fragment to the right of the other insertions (Fig. 1), that is, in DNA immediately contiguous with but outside the minimal 19.5 kb *Sst*I fragment. The most likely explanation for this result is that this region encodes a function which can be complemented by *E. coli* K12 so that although the minimal *Sst*I fragment of pEVX7 is sufficient to give O-antigen expression in such a heterologous host, insertion of a transposon

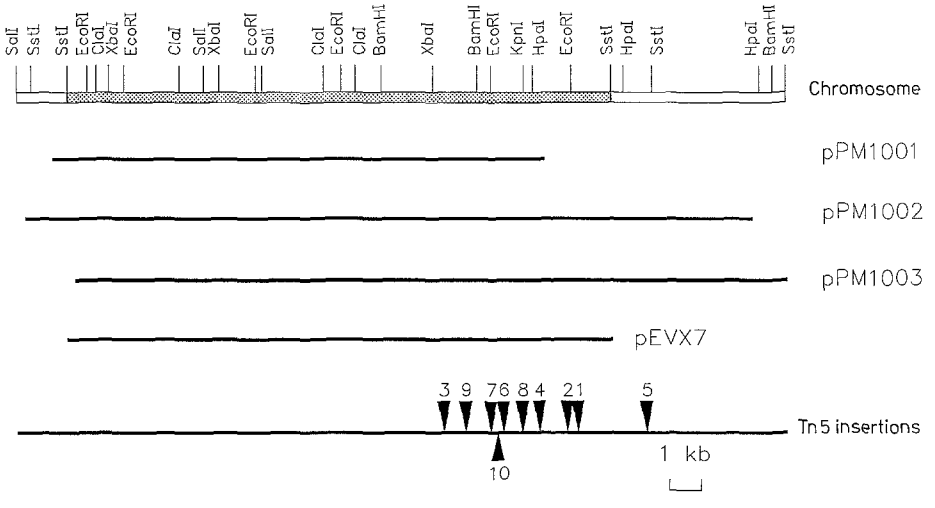

Fig. 1. Restriction map of the *rfb* region of *Vibrio cholerae* 01, showing sites of Tn*5* insertions (*arrowheads*) in 1621 (*rfb*::Tn*5*-1–5) and 569B (*rfb*::Tn*5*-6–10). The contiguous chromosomal DNA contained in the plasmids, pPM1001, pPM1002, pPM1003 and pEVX7 according to Ward et al. (1987) is also shown. The shaded region corresponds to the *Sst*I fragment capable of mediating O-antigen expression in *E. coli* K-12

Table 2. Linkage analysis

Selected marker	Unselected frequency	No. distance	Linkage markers	Relative[a]
A				
ilv-1	*arg*-1	34	0.028	3.57
	his-1	21	0.017	4.07
Total 1200	*str*	199	0.165	1.8
	Km^r (*rfb*)	542	0.45	0.79
arg-1	*ilv*-1	139	0.125	2.08
	his-1	49	0.044	3.12
Total 1106	*spc*	726	0.656	0.42
	str	113	0.102	2.28
	Km^r (*rfb*)	652	0.589	0.52
B				
ilv-1	*arg*-1	30	0.16	1.83
	his-1	1	0.005	5.2
Total 179	Km^r	148	0.82	0.19
arg-1	*ilv*-1	3	0.04	3.2
	his-1	1	0.013	4.3
Total 75	Km^r	14	0.18	1.71

[a] Relative distance = ln (1/linkage frequency). Linkage frequency is defined according to Parker et al. (1979) as the ratio of unselected donor markers to selected donor markers. In panel A are shown the results of matings of V689 (V667[P::Tn*3*]) as donor with V692 as recipient. In panel B, V671[P::Tn*3*] was used as donor

Table 3. Multi-factor analysis

Selected marker	Recombinant class	Frequency	
		A[a]	B[b]
ilv	*ilv*	0.37	0
	ilv, arg	0.003	0.034
	ilv Km^r	0.42	0.59
	ilv, arg, Km^r	0.026	0.23
	ilv, his	0.008	0
	ilv, arg, his	0.009	0.14
	ilv, str	0.14	–
	ilv, str, arg	0.023	–
	ilv, his, Km^r	–[c]	0
arg	*arg*	0.39	0.77
	arg, ilv	0.008	0.027
	arg, Km^r	0.48	0.13
	arg, ilv, Km^r	0.124	0.053
	arg, his	0.016	0.013
	arg, his, ilv	0.028	0
	arg, str	0.004	–
	arg, str, ilv	0.099	–

[a] Column A corresponds to multi-factor cross analysis from panel A in Table 1, using the Tn*5* mutant in V667
[b] Column B corresponds to multi-factor cross analysis from panel B in Table 1, using the Tn*5*-11 mutant in V671
[c] Data not available

in this region in the *V. cholerae* chromosome will inactivate this function and so affect O-antigen biosynthesis. This mutant indicates that the *rfb* region of *V. cholerae* must extend beyond the 19.5 kb *Sst*I fragment to include at least part of the 5 kb *Sst*I fragment on the right.

When the protein composition of whole cell membranes of the Tn*5* insertion mutants was analysed by SDS-polyacrylamide gel electrophoresis all mutants, regardless of serotype or biotype, exhibited a similar pattern to the type rough strain, Kasauli R. They all overproduced a 23 kDa protein, which was a minor protein in the parent strain. The significance of this alteration is unknown, but similar effects have been previously observed in LPS mutants (Koplow and Goldfine 1974; Ames et al. 1974; Lugtenberg et al. 1976; Guidolin and Manning 1985).

Characterization of putative rfa *mutants*

Some of the mutants were not transposon insertions in the *rfb* region as judged by Southern hybridization with either pEVX7 or pPM1003. The Tn*2680* insertions exhibited a strong tendency to auto-agglutinate and colonies had a rough appearance on solid media. This was not observed with any of the Tn*5* mutants. It was proposed that these mutants had insertions in a second locus on the chromosome, the *rfa* region, associated with biosynthesis of the LPS core. DNA alterations could not be detected with either of our probes.

Analysis of the core (+ lipid A) regions of the LPS of these mutants, using whole cells failed to detect any difference when compared with the parent strains (data not shown).

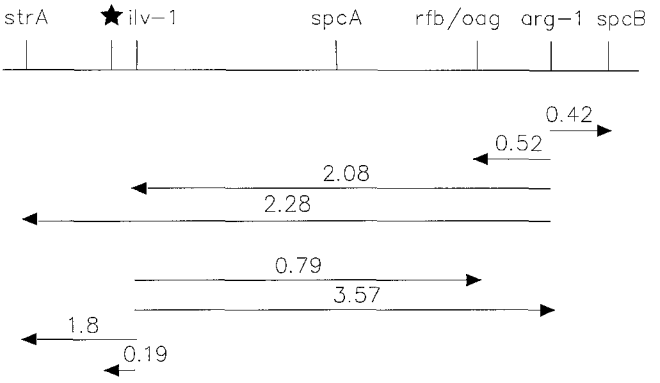

Fig. 2. Genetic map of the *rfb* region in *Vibrio cholerae* 01 showing the linkage of *rfb* to *ilv* and *arg*. Distances (R.D.) and gene order are based on the linkage data shown in Tables 2 and 3. *Star* indicates suggested map position of the Tn*5*-11 mutation in strain V671

Mapping rfb *using* P^+ *donor strains*

Recombinants were initially selected as *arg*$^+$ or *ilv*$^+$ and then screened for the presence of various unselected markers. The donor was V689 (*rfb*::Tn*5*-9) into which the P factor (P::Tn*3*) had been introduced and Table 2 (panel A) shows the data from a number of matings with strain V692 (*ilv*-1, *arg*-1, *his*-1, *spc, rif*) and the linkage of *rfb* to *arg* and *ilv*-1. In Table 3, these data are presented in the form of multi-factor cross analysis, which allows the order of the genes to be determined. From these results, it is possible to place the *rfb* locus between *ilv*-1 and *arg*-1 and to construct a map of various markers on the chromosome in this region as shown in Fig. 2.

*Mapping of the non-*rfb *mutants*

The strain V671, in which Tn*5* maps outside *rfb*, was converted to a donor by introducing P::Tn*3* and used to map the Tn*5* insertion. Linkage analysis is shown in Table 2 (panel B) and the multi-factor cross data for both *ilv*$^+$ and *arg*$^+$ recombinants are shown in Table 3 (column B). It can thus be concluded that, in this strain, Tn*5* maps closer to *ilv*-1 than *arg*-1 and from the multi-factor cross analysis, Tn*5* would appear not to be inserted between *ilv* and *arg*. The suggested map position of this mutation (Tn*5*-11) is indicated in Fig. 2.

Discussion

There are several reasons why the transposon insertion mutations of *V. cholerae* appear to be clustered. Firstly, any mutation which still allows sufficient O-antigen to be made such that it could function as a phage receptor would not come through the selection process. It is also possible that insertions in the left-hand region (Fig. 1) are lethal because they block O-antigen synthesis entirely by preventing the carrier lipid (undecaprenol-phosphate), which is also used in peptidoglycan and other syntheses, from being re-utilized. Because of the genetic defect a complete O-antigen molecule is not made and the partial O-antigen molecules are unable to be removed and so destabilize the structural rigidity of the entire cell wall.

A number of transposon insertions do not map within the *rfb* region. This indicates that the site of transposon insertion may be the *rfa* gene cluster responsible for the biosynthesis of the core oligosaccharide. As this region has not yet been cloned from *V. cholerae*, it was not possible to analyse further these putative *rfa* mutants by Southern hybridizations.

The *rfb* locus was mapped relative to the *ilv*-1 and *arg*-1 genes. The gene order obtained agrees with that reported by Bhaskaran (1960) and Parker et al. (1979). It can be concluded that the *rfb* locus corresponds to the *oag* locus described by previous workers. Thus, a single gene cluster has been identified, mapping between *ilv*-1 and *arg*-1, which encodes O-antigen biosynthesis and serotype specificity of the *V. cholerae* LPS (Manning et al. 1986; Ward et al. 1987; Ward et al. manuscript in preparation). Thus, since it is possible to get reciprocal serotype conversion between Inaba and Ogawa, (Gangarosa et al. 1967; Sack and Miller 1969; Sakazaki and Tamura 1971) the changes which occur in this process must map at this locus.

Future experiments using the transposon insertion mutants described in this paper will be aimed at defining the effect of the mutations, both genetically and chemically.

Acknowledgements. The authors wish to thank the National Health and Medical Research Council of Australia, the Australian Research Committee and the Clive and Vera Ramaciotti Foundations for support. H.M.W. was the recipient of a Commonwealth Postgraduate Research Award.

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Communicated by W. Goebel

Received November 8, 1988