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Antigenic Changes in *Vibrio cholerae* biotype *eltor* Serotype Ogawa After Bacteriophage Infection

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Vibrio cholerae biotype *eltor* serotype Ogawa cultures infected with temperate bacteriophage from lysogens of biotype *eltor* serotype Hikojima yielded types with altered agglutination reactions.

Antigenic changes in *Vibrio cholerae* biotype *cholerae* occurred subsequent to infection with bacteriophage from either of two lysogens of *V. cholerae* biotype *eltor* serotype Hikojima (5). A phage-treated strain of biotype *cholerae* serotype Ogawa (O-factors AB) gained the ability to agglutinate in Inaba type-specific antiserum and hence was able to synthesize antigenic factor C.

We now report modifications in serological reactions of *V. cholerae* biotype *eltor* serotype Ogawa cultures after infection with temperate phage from biotype *eltor* serotype Hikojima strains.

The biotypes and serotypes of the *V. cholerae* strains used are listed in Table 1; the history of these isolates is given in a previous report (5). The methods of culturing the organisms, induction of the lysogens for phage production, and titration of phage lysates were essentially those described earlier (5); however, T_1N_1 broth and agar (Trypticase, 1%; NaCl, 1% [agar 1.5%], pH 7.4) were used in place of nutrient broth and agar.

The lysogens (strains 1633, 2001, 92TP) were induced with either ultraviolet light or heat. After incubation of the induced cultures for 7 to 8 h at 37°C, chloroform was added to kill the surviving cells. For some experiments, phage from the two donor lysogens (1633, 2001) was propagated on strain 1621 by inoculating 0.1 ml of phage lysate from an induced culture into 10 ml of 7-h broth cultures. These phage lysates were labeled 1633 (1621) and 2001 (1621).

A cell-free phage suspension was mixed with cells from a log-phase culture established from a single O-form colony of the recipient strain. The ratio of cells to phage was about 2:1. Phage-infected cultures, along with appropriate controls (recipient cells in broth; phage in broth), were incubated for 4 h at 30°C and then for 7 h at 37°C. The phage "sterility" control was plated on agar, incubated for 48 h, and examined for bacterial growth. Single colonies selected at random from plates streaked with the phage-treated cultures, or with the control culture of recipient cells, were picked and transferred to broth. After incubation for 8 h, agar plates were streaked to isolate colonies for serological identification. After this initial serotyping, single colonies were selected from these plates and the procedure was repeated. A total of five subcultures was thus carried out, testing for serological characteristics at each transfer.

Slide agglutinations were done with monospecific Ogawa, Inaba, and antirough serums obtained from H. L. Smith of the Vibrio Reference Laboratory. When any isolates agglutinated in saline, the tests were repeated using sodium chloride at a lower concentration of 0.4% in all menstrua (8).

Selected isolates from the phage-treated cultures which exhibited alterations in serological patterns and retained their seroconverted characteristics after five passages were tested for immunity to infection with phage from the donor lysogens and for lysogeny using procedures described previously (5). Three inducing agents—ultraviolet light, heat, and mitomycin C—were used in attempts to induce phage production in

TABLE 1. *Characteristics of phage donor and recipient strains of V. cholerae*

Strain	Biotype	Serotype	Antigenic factors
Donor lysogens			
1633	*eltor*	Hikojima	ABC
2001	*eltor*	Hikojima	ABC
92TP[a]	*cholerae*	Hikojima-(Rough)	ABC(R)
Recipient strains			
407	*eltor*	Ogawa	AB
429	*eltor*	Ogawa	AB
1423	*eltor*	Ogawa	AB
1621	*eltor*	Ogawa	AB
1714	*eltor*	Ogawa	AB

[a] 92TP is a serologically converted isolant derived from strain 029 (5) after treatment with phage from strain 2001 which agglutinates in antirough serum and is serotype Hikojima.

the seroconverted strains. Induction of cultures with mitomycin C followed the procedure of Parker et al. (6).

V. cholerae strains of biotype *eltor* serotype Ogawa yielded forms with altered agglutination reactions subsequent to infection with phage from lysogens of serotype Hikojima (Table 2). The most pronounced serological modification was the gain in ability to agglutinate in Inaba or antirough serum, or both. Cells of isolates clumping in type-specific Inaba serum are synthesizing somatic antigen C, whereas those that gave a positive agglutination reaction in Inaba and antirough serums (Inaba-Rough) possess somatic O-factors ACR. Strains exhibiting trivalent agglutinative properties in Ogawa, Inaba, and antirough serums were designated Hikojima-Rough (O-factors ABCR). Some isolates of the phage-treated cultures were saline agglutinable and were untypable. These may be rough variants which synthesize little or no polysaccharide smooth antigens (12). No changes in agglutination reaction were detected in cultures of strains 407 and 1423 after infection with phage preparation 2001 (1621), but perhaps not enough colonies were screened to detect seroconverted cells.

Isolates exhibiting changes in serological characteristics from eight of the phage-treated cultures (Table 2) were subcultured in broth. During this recloning process, some cells from the phage-treated cultures which initially exhibited

TABLE 2. *Serological types of Vibrio cholerae biotype eltor serotype Ogawa isolated subsequent to phage infection*

Treated cells code no.	Phage source[a] code no.	No. of single colonies tested	Serotype of isolates	Antigenic factors
407	2001 (1621)	5	5 Ogawa	AB
429	1633	8[b]	2 Ogawa	AB
			2 Ogawa-(Rough)	AB(R)
			2 Hikojima-(Rough)	ABC(R)
			2 Salt-agglutinable	
429	1633 (1621)	4[b]	1 Ogawa	AB
			1 Ogawa-(Rough)	AB(R)
			2 Hikojima-(Rough)	ABC(R)
429	2001 (1621)	5	4 Ogawa	AB
			1 Hikojima	ABC
429	92TP	4	3 Ogawa-(Rough)	AB(R)
			1 Hikojima-(Rough)	ABC(R)
1423	1633	5[b]	3 Ogawa	AB
			2 Hikojima	ABC
1423	2001 (1621)	5	5 Ogawa	AB
1423	92TP	4[b]	2 Ogawa-(Rough)	AB(R)
			2 Hikojima-(Rough)	ABC(R)
1621	2001 (1621)	10[b]	5 Ogawa	AB
			5 Hikojima	ABC
1621	92TP	3	1 Ogawa-(Rough)	AB(R)
			2 Salt-agglutinable	
1714	1633	6[b]	5 Ogawa-(Rough)	AB(R)
			1 Hikojima-(Rough)	ABC(R)
1714	1633 (1621)	4	1 Ogawa	AB
			2 Hikojima-(Rough)	ABC(R)
			1 Salt-agglutinable	
1714	2001 (1621)	6[b]	1 Ogawa	AB
			1 Ogawa-(Rough)	AB(R)
			3 Hikojima-(Rough)	ABC(R)
			1 Salt-agglutinable	
1714	92TP	11[b]	3 Ogawa	AB
			2 Ogawa-(Rough)	AB(R)
			1 Hikojima	ABC
			1 Hikojima-(Rough)	ABC(R)
			4 Salt-agglutinable	
Controls: untreated recipient cells		3	3 Ogawa	AB

[a] 92TP is biotype *cholerae* serotype Hikojima. 1633 and 2001 are biotype *eltor* serotype Hikojima.

[b] Isolates exhibiting changes in serological characteristics which were carried through five serial cultures.

TABLE 3. *Antigenic pattern of selected isolates from phage-treated cultures of Vibrio cholerae biotype eltor during serial subculture*

Treated cells code no.	Phage source code no.	Subculture									
		1		2		3		4		5	
		No.[a]	Antigenic factors[b]	No.	Antigenic factors	No.	Antigenic factors	No.	Antigenic factors	No.	Antigenic factors
429	1633	(8)	2 AB 2 ABR 2 ABCR 2 (SA)	(7)	2 ABR 2 ABCR 3 (SA)	(2) (6) (2)	2 ABR 2 ABR 4 ABCR 2 ABR	(4) (3) (1) (6) (4)	1 AB 1 ABR 2 ABCR 6 ABCR 1 ABR 2 ABCR 1 ACR	(2) (1) (2) (2)	1 AB 2 ABR 1 ABCR 2 ABCR 1 ACR 1 ABCR 1 ACR 1 ACR 1 AC
429	1633 (1621)	(4)	1 AB 1 ABR 2 ABCR	(6)	1 AB 3 ABCR 2 ACR	(3) (3)	3 ABCR 3 ABCR	(3) (3)	3 ABCR 3 ABCR	(1) (1)	1 ABCR 1 ABCR
1714	92TP	(11)	3 AB 2 ABR 1 ABC 1 ABCR 4 (SA)	(2) (5)	2 ABR 2 AB 1 ABC 1 ABCR 1 (SA)	(1) (3) (3)	1 AB 2 AB 1 ABC 2 ABCR 1 (SA)	(1) (3) (2) (5) (2)	1 AB 3 AB 1 ABR 1 ABC 5 ABCR 2 ABR	(1) (1) (1) (1) (2)	1 AB 1 AB 1 AB 1 ABC 1 ABCR 1 ABR 1 ABC
429 1714	(Controls)	(3)	3 AB	(3)	3 AB	(3)	3 AB	(3)	3 AB	(3)	3 AB

[a] Number indicates number of single colonies subcultured.

[b] Abbreviations: AB, Ogawa; ABR, Ogawa-Rough; ABC, Hikojima; ABCR, Hikojima-Rough; AC, Inaba; ACR, Inaba-Rough; SA, salt-agglutinable.

changes in agglutination reactions were not stable and yielded descendants of different types (Table 3). Representative pedigrees illustrated in Table 3 showed the following. (i) Gain in ability to synthesize agglutinogenic factor C was not necessarily a permanent characteristic. Some Ogawa-Rough and Ogawa types appeared on subculture of the Hikojima-Rough isolates. (ii) O-factor B appeared masked in some isolates with the emergence of serotype Inaba and Inaba-Rough forms. (iii) Some saline-agglutinable forms became typable on transfer. (iv) The rough characteristic was not necessarily stable, appearing and disappearing during the subculturing procedures.

The serotypes of the control cultures remained unchanged throughout the five transfers. However, the limited number of discrete colonies (three) screened from each subculture may not detect rough variant forms which have been reported to appear in some *V. cholerae* cultures at a frequency of about 1 in 10^4 divisions or less (3).

The seroconverted isolates which lost their ability to agglutinate in Inaba and antirough sera became sensitive to phage released by the donor lysogens, i.e., those having only antigenic factors AB after five subcultures (Table 3). Cultures from phage-treated strains which retained their serological change after five serial subcultures (e.g., those expressing antigenic factor C, or R, or both) were resistant to infection with phage from the donor lysogens. However, since attempts to induce phage production in these "immune" types with ultraviolet light, heat, or mitomycin C were unsuccessful, it remains unknown whether those stable isolates exhibiting "phage-induced" serological changes are lysogens. Thus, the seroconverted strains of biotype *eltor* isolated subsequent to phage infection differ from those isolated from a biotype *cholerae* serotype Ogawa strain. The latter seroconvertants undergo spontaneous induction and readily respond to inducing agents (5).

The variation in somatic antigens in cells isolated from phage-treated cultures of biotype *eltor* serotype Ogawa may be due to a number of factors. Two possibilities are the following.

(i) The phage may be acting as a selection agent for phage-resistant forms in serotype Ogawa cultures which have characteristics ranging between smoothness and extreme roughness with expression of antigenic factor C in some of the surviving cells. Rough variants of *V. cholerae* have been isolated with the aid of A-type choleraphages (10, 13). Presumably, some A-type phage may lyse cells of only serotype Ogawa strains which possess a type-specific polysaccharide-containing antigen (the phage receptor), and the surviving variant cells, lacking phage receptors, have rough characteristics. A similar mechanism of selection of spontaneous mutants in a parent culture is thought to be operative when *V. cholerae* is cultivated in the presence of immune serum or various chemicals (2, 3, 8). Some of the rough and saline-agglutinable forms detected in our phage-treated cultures may be the rough-type bar mutants that are phage resistant and normally present in cultures. However, the roughness, as well as salt agglutinability, characteristic was not stable in some isolates. The expression of the R antigen in these latter types may be due to superficial modifications of the serological phenotype and not to genotypic changes.

Concomitant with the loss of ability of seroconvertants to agglutinate in antirough and Inaba serums was a return to sensitivity to phage from the donor lysogens. The possibility exists that the temperate phage used in our experiments are specific for the serotype Ogawa strains used. The variant Inaba types and the intermediate Hikojima forms, reported to occur spontaneously in some Ogawa cultures (7, 11), could survive the phage infection and become the dominant types in the secondary cultures. Thus, the presumed seroconvertants may not be lysogens carrying the infecting phage, which could explain our inability to induce phage in those isolates exhibiting agglutination reactions different from the original cultures. Yet, the finding that the temperate phage from our donor lysogens also will lyse some of our serotype Inaba strains (unpublished observation) tends to discount that only serotype Ogawa strains are sensitive to the phage from the Hikojima lysogens used in the experiments reported here.

(ii) If the biotype *eltor* serotype Ogawa strains, which exhibit serotype changes subsequent to phage infection, prove in further testing to be lysogens for the original infecting phage, as was found for the seroconverted biotype *cholerae* serotype Ogawa strains (5), then the phage genome from lysogens 1633, 2001, and 92TP may affect or effect the synthesis of lipopolysaccharide antigenic factors B and C which helps confer antigenic specificity on the three main serotypes of *V. cholerae*. A similar mechanism has been reported in *Salmonella* where conversion phages bring about alterations in the chemical structure of O antigen polysaccharides (1, 4, 9).

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