SHORT COMMUNICATION

Isolation of a Non-adhesive Mutant of *Vibrio cholerae* and Chromosomal Localization of the Gene Controlling Mannose-sensitive Adherence

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A mutant (CD11) of *Vibrio cholerae* was isolated after exposure of a wild-type pathogenic strain (KB207) to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Although the mutant was motile and chemotactic, it adhered poorly to rabbit intestinal mucosa. Pretreatment of vibrios with 10 mg D-mannose ml⁻¹ and of intestinal mucosa with 100 mmol sodium metaperiodate inhibited adherence of KB207 but not of CD11. The gene (*ams*) controlling mannose-sensitive adherence was mapped on the bacterial chromosome closely linked to the *pur* locus.

INTRODUCTION

The adherence of pathogenic *Escherichia coli* to porcine and calf intestinal mucosa is mediated through the surface antigens K88 and K99, respectively, both of which are plasmid products (Stirm *et al.*, 1967; Smith & Linggood, 1971; Burrows *et al.*, 1976). Although the adherence of *Vibrio cholerae* to intestinal mucosa has been reported (LaBrec *et al.*, 1965; Freter & Jones, 1976; Nelson *et al.*, 1976; Bhattacharjee & Srivastava, 1978), nothing is known about the morphology, genetics or biochemistry of the surface components responsible for adhesion. The rational approach would be the isolation of distinct non-adhesive mutants and their comparison with wild-type parent strains.

This paper reports the isolation of such a mutant of *V. cholerae* which adhered poorly to intestinal mucosa as compared with the parent strain. Contrary to the findings with *E. coli*, the gene controlling adhesion of vibrios to intestinal mucosa was found to be situated on the bacterial chromosome.

METHODS

Vibrio strains. *Vibrio cholerae* strain KB207 (*str-r*) was the wild-type pathogenic strain from which the poorly adhesive mutant CD11 (*str-r, pur, ams*) was isolated. The gene controlling mannose-sensitive adherence of *V. cholerae* has been termed *ams*. Strain KB11 (*P*⁺, *ilv, arg, his, str-r*) was the donor in bacterial conjugation.

Media and growth condition. Bacteria were grown in either nutrient broth (Difco) or brain heart infusion (BHI, Difco). Nutrient broth was solidified with 1% (w/v) agar no. 3 (Oxoid) for nutrient agar plates and slants. Overnight cultures on slants were harvested in phosphate-buffered saline (PBS) pH 7.2, diluted 50-fold in BHI and incubated for 3 h at 37 °C. Such cultures had a viable count of about 1 × 10⁹ to 2 × 10⁹ vibrios ml⁻¹.

Isolation of mutant CD11. A fresh 3 h BHI culture of strain KB207 was treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich-Europe, Beerse, Belgium) at 30 µg ml⁻¹ for 20 min at 37 °C (Adelberg *et al.*,

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Table 1. *Properties of strains KB11, KB207 and CD11*

Strain	Adherence index*				Reciprocal of haemagglutination titre	
	<i>In vivo</i>	<i>In vitro</i>			Without D-mannose	With D-mannose (100 µg ml ⁻¹)
		Without D-mannose	With D-mannose (10 mg ml ⁻¹)	With NaIO ₄ (100 mmol)		
KB11	51	3.5	ND	ND	128	ND
KB207	60	3.0	0.009	0.016	128	16
CD11	2	0.01	0.008	0.009	64	64

ND, Not done.

* Adherence index is the number of adherent vibrios expressed as a percentage of the total number of vibrios to which the disc of intact intestinal mucosa (*in vitro*) or the entire intestinal mucosa of the ileal loop (*in vivo*) was exposed. Indices given are the average of several experiments.

1965). Treated bacteria were centrifuged and washed three times with PBS and the washed pellet was then incubated overnight at 37 °C in 7 ml nutrient broth. This culture was plated on nutrient agar for single colonies. About 500 colonies distributed over 10 plates were examined for motility and chemotactic response (Armstrong *et al.*, 1967). The non-motile and non-chemotactic mutants were rejected because such mutants are poorly adhesive (Freter & Jones, 1976; Allweiss *et al.*, 1977). The adherence of the remaining colonies was examined, and out of 240 colonies tested two were found to be less adhesive than KB207 of which CD11 was one. In addition CD11 was found to require hypoxanthine.

Adherence. The adherence *in vitro* of vibrios to discs of freshly isolated rabbit intestine and the effect of 10 mg D-mannose ml⁻¹ was measured as described earlier (Bhattacharjee & Srivastava, 1978). The adherence *in vivo* in the ileal loops of rabbit was measured as described by Srivastava *et al.* (1979). The adherence *in vitro* of untreated vibrios to discs of intestinal mucosa previously incubated with sodium metaperiodate (100 mmol) for 15 min was also investigated. The adherence index is the number of adherent vibrios expressed as a percentage of the number of vibrios to which the intestinal mucosa was exposed.

Haemagglutination. The agglutination of human red blood cells (group A) by vibrios and the effect of D-mannose was studied as described earlier (Bhattacharjee & Srivastava, 1978).

Bacterial mating. The donor (KB11) and the recipient (CD11) were grown separately in BHI for 3 h at 37 °C. Then 1 ml donor (1.5 × 10⁹ colony-forming units ml⁻¹) and 9 ml recipient (1.2 × 10⁹ colony-forming units ml⁻¹) were mixed and centrifuged. The pellet was suspended in 1 ml prewarmed (37 °C) nutrient broth and incubated at 37 °C. After 90 min, the mating mixture was made up to 10 ml and 0.2 ml was plated on to selective minimal agar plates and incubated at 37 °C for 48 to 72 h. The minimal medium and supplementations were as described by Bhaskaran (1964). All colonies isolated were purified twice on the same selective medium. The non-selective nutritional markers and adherence to discs of intestinal mucosa of all the recombinants were then tested.

RESULTS

Properties of mutant CD11

Compared with the parent strain KB207, the mutant CD11 showed little affinity, either *in vivo* or *in vitro*, for rabbit intestinal mucosa. Pretreatment of KB207 with D-mannose (10 mg ml⁻¹) decreased its level of adherence to that of the non-adhesive mutant CD11. Pretreatment of the intestinal mucosa *in vitro* with sodium metaperiodate (100 mmol) caused a similar decrease in the adherence of KB207. Neither treatment had any effect on the adherence of CD11 (Table 1).

Treatment of strains KB207 and CD11 with D-mannose (100 µg ml⁻¹) decreased the ability of the parent strain to agglutinate human red blood cells (group A) but had no effect on this property in the mutant (Table 1).

Whilst the parent strain KB207, being pathogenic, caused fluid to accumulate *in vivo* within the ileal loops, mutant CD11 was less effective. On average, only 3/10 loops challenged with the mutant accumulated fluid.

Table 2. Segregation by adherence of recombinants of strains KB11 and CD11

Recombinants		No. (%) of recombinants having the indicated adherence properties	
Type	No.	Low adherence index*	High adherence index*
<i>pur</i> ⁺	253	63 (24.8)	190 (75.2)
<i>pur</i> ⁺ <i>ilv</i>	210	83 (39.5)	127 (60.5)

* For definition, see Table 1.

Genetic mapping of *ams*

The frequency of recombination with *pur* was between 10^{-6} and 10^{-7} per donor cell. The frequency of *pur*⁺ revertants of CD11 was below 10^{-9} ; when 10 minimal agar plates were each seeded with 2×10^9 colony-forming units, no *pur*⁺ revertants of strain CD11 were found. The bacterial cross was designed according to the linkage map of *V. cholerae* described by Bhaskaran (1964) which gives the gene order as : *str-pur-ilv-arg-leu-his*.

In the cross between strains KB11 and CD11, selection was made for *pur*⁺ recombinants of the recipient, but with the medium adjusted to permit growth of either *ilv*, *arg* or *his* recombinants as non-selected markers. Only *ilv* showed linkage with *pur*⁺. About 75% of the *pur*⁺ recombinants adhered to discs of intestinal mucosa with an efficiency comparable to that of strain KB207, the remainder behaving like strain CD11. Among the *pur*⁺*ilv* recombinants, about 60% had a similar adherence index to strain KB207 (Table 2).

Although we did not examine all the recombinants for haemagglutinating ability and sensitivity to mannose, random examination of recombinants with low and high adherence indices revealed that restoration of adhesiveness in 83% of the recombinants was associated with mannose-sensitive haemagglutinating ability, whereas poorly adhesive recombinants exhibited, like CD11, mannose-resistant haemagglutination.

As well as selecting recombinants in the cross between strains KB11 and CD11, the mating mixture was plated on minimal medium supplemented with hypoxanthine. This allowed the re-selection of strain CD11. One hundred colonies were picked at random and tested for hypoxanthine requirement and the presence of the P plasmid. All were hypoxanthine-requiring and 84 colonies were P⁺ and 16 P⁻. This suggested that 84% of the CD11 cells had mated and received the P plasmid. All of these 100 colonies, whether P⁺ or P⁻, were poorly adhesive like strain CD11.

DISCUSSION

The adherence of *V. cholerae* to the mannose or mannose-like receptors on intestinal mucosa (Bhattacharjee & Srivastava, 1978) is confirmed by the finding that the treatment of intestinal mucosa *in vitro* with sodium metaperiodate, a reagent which cleaves the C-C bond between adjacent hydroxyl groups of sugars, abolished the adherence of strain KB207 but had no effect on the non-adhesive mutant CD11. Similar results with sodium metaperiodate were obtained for non-pathogenic *E. coli* (Ofek *et al.*, 1977).

Our results demonstrated that the *ams* gene could be transferred from an *ams*⁺ donor to mutant CD11. The linkage of *ams*⁺ to *pur*⁺ suggests that the gene controlling adherence of *V. cholerae* is chromosomal and is not situated on a plasmid. This is supported by the results of the experiment in which, instead of selecting recombinants of the KB11 $\times$ CD11 cross, CD11 was re-selected. The P plasmid was transferred to 84% of the recipients. If *ams*⁺ was situated on a plasmid, among the P⁺ CD11 colonies tested a reasonable number should have been adhesive like strain KB11. The presence of the P plasmid confirms that conjugal mating did occur allowing the transfer of plasmid(s) from the donor to the recipient. The high frequency of linkage of *ams*⁺ to *pur*⁺, the latter being on the chromosome,

rules out the possibility that *ams*⁺ might be situated on a plasmid which is poorly mobilized by the P plasmid.

Genes controlling adherence and toxin synthesis in enteropathogenic *E. coli* are known to be plasmid encoded (Stirm *et al.*, 1967; Smith & Halls, 1968). However, the gene (*tox*) controlling toxinogenesis in *V. cholerae* is located on the chromosome (Vasil *et al.*, 1975).

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REFERENCES

- ADELBERG, E. A., MANDEL, M. & CHEN, G. C. C. (1965). Optimal conditions for mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in *Escherichia coli* K12. *Biochemical and Biophysical Research Communications* **18**, 788–792.
- ALLWEISS, B., DOSTAL, J., CAREY, K. E., EDWARDS, T. F. & FRETER, R. (1977). The role of chemotaxis in the ecology of bacterial pathogens of mucosal surfaces. *Nature, London* **266**, 448–450.
- ARMSTRONG, J. B., ADLER, J. & DAHL, M. M. (1967). Non-chemotactic mutants of *Escherichia coli*. *Journal of Bacteriology* **93**, 390–398.
- BHASKARAN, K. (1964). Segregation of genetic factors during recombination in *Vibrio cholerae* strain 162. *Bulletin of the World Health Organisation* **30**, 845–853.
- BHATTACHARJEE, J. W. & SRIVASTAVA, B. S. (1978). Mannose-sensitive haemagglutinins in adherence of *Vibrio cholerae* eltor to intestine. *Journal of General Microbiology* **107**, 407–410.
- BURROWS, M. R., SELLWOOD, R. & GIBBONS, R. A. (1976). Haemagglutinating and adhesive properties associated with the K99 antigen of bovine strains of *Escherichia coli*. *Journal of General Microbiology* **96**, 269–275.
- FRETER, R. & JONES, G. W. (1976). Adhesive properties of *Vibrio cholerae*. Nature of the interaction with intact mucosal surfaces. *Infection and Immunity* **14**, 246–256.
- LABREC, E. H., SPRING, J., SCHNEIDER, H. & FORMAL, S. B. (1965). Localisation of vibrios in experimental cholera: a fluorescent antibody study in guinea pigs. In *Proceedings of the Cholera Research Symposium*, pp. 272–276. Washington, D.C.: U.S. Government Printing Office.
- NELSON, E. T., CLEMENTS, J. D. & FINKELSTEIN, R. A. (1976). *Vibrio cholerae* adherence and colonization in experimental cholera: electron microscope studies. *Infection and Immunity* **14**, 527–547.
- OFEK, I., MIRELMAN, D. & SHARON, N. (1977). Adherence of *Escherichia coli* to human mucosal cells mediated by mannose receptors. *Nature, London* **265**, 623–625.
- SMITH, H. W. & HALLS, S. (1968). The transmissible nature of the genetic factor in *Escherichia coli* that controls enterotoxin production. *Journal of General Microbiology* **52**, 319–334.
- SMITH, H. W. & LINGGOOD, M. A. (1971). Observations on the pathogenic properties of the K88, Hyl and Ent plasmids of *Escherichia coli* with particular reference to porcine diarrhoea. *Journal of Medical Microbiology* **4**, 467–485.
- SRIVASTAVA, R., SINHA, V. B. & SRIVASTAVA, B. S. (1979). Re-evaluation of antibacterial and anti-toxin immunities in experimental cholera. *Indian Journal of Medical Research* **70**, 368–372.
- STIRM, S., ØRSKOV, F., ØRSKOV, I. & MANSA, B. (1967). Episome carried surface antigen K88 of *Escherichia coli*. II. Isolation and chemical analysis. *Journal of Bacteriology* **93**, 731–739.
- VASIL, M. L., HOLMES, R. K. & FINKELSTEIN, R. A. (1975). Conjugal transfer of a gene determining production of enterotoxin in *Vibrio cholerae*. *Science* **187**, 849–850.