Sensitivity of Non-flagellate *Salmonella* Mutants to the Flagellotropic Bacteriophage χ

By S. YAMAGUCHI AND H. FUJITA*

*Department of Biology, School of Education, Waseda University,
Nishiwaseda, Shinjuku-ku, Tokyo 160, Japan*

T. KUROIWA† AND T. IINO

*Department of Genetics, Faculty of Science, University of Tokyo,
Hongo, Bunkyo-ku, Tokyo 113, Japan*

(Received 25 May 1976; revised 9 September 1976)

SUMMARY

Non-flagellate *Salmonella* mutants representing complementation groups *H1*, *H2*, *flaAI*, *flaAII*, *flaAIII*, *flaB*, *flaC*, *flaD*, *flaE*, *flaF*, *flaK*, *flaL*, *flaM*, *flaN*, *flaP*, *flaQ* and *flaR* were examined for their sensitivity to the flagellotropic bacteriophage χ . *H1*, *H2*, *flaL* and *flaR* mutants were shown to be sensitive to χ by spot tests, although they were less sensitive than a parental flagellate strain; *flaAIII* mutants were also shown to be sensitive, although even less so, by their ability to propagate the phage at the spotted area. In liquid medium neither adsorption nor propagation of χ was observed on any of these sensitive non-flagellate mutants. Electron micrographs showed that χ particles were attached to the superhooks of *flaR* mutants, but the receptor sites of other sensitive non-flagellate mutants could not be recognized.

INTRODUCTION

Bacteriophage χ attacks motile strains of *Salmonella*, *Escherichia* and *Serratia* (Sertic & Boulgakov, 1936; Meynell, 1961; Iino & Mitani, 1967). Since the phage did not attack non-flagellate mutants or paralysed (flagellate but non-motile) *Salmonella*, Meynell (1961) concluded that an active flagellum was required for its infection. Examination in an electron microscope showed that χ became attached to a flagellum by its tail fibre, and then travelled to the base of the flagellum where it injected its DNA (Schade, Adler & Ris, 1967). Since then, morphological and genetical studies on flagella have progressed considerably. A flagellum consists of three parts: filament, hook, and basal body (DePamphilis & Adler, 1971). The filament is the distal helical part and is a polymer of flagellin; the basal body is the proximal structure composed of several substructures; and the hook connects the filament to the basal body. Genetical studies revealed 17 genes responsible for flagellar formation in *Salmonella*: two *H* genes (*H1* and *H2*, the structural genes for phase-1 and phase-2 flagellins, respectively), and 15 *fla* genes (*flaAI*, *AII*, *AIII*, *B*, *C*, *D*, *E*, *F*, *K*, *L*, *M*, *N*, *P*, *Q* and *R*: Yamaguchi *et al.*, 1972a; Patterson-Delafield *et al.*, 1973). Electron microscope studies showed that *H1*, *H2* and *flaL* mutants carried apparently normal basal bodies and hooks, and *flaR* mutants carried basal bodies and abnormal hooks – superhooks

* Present address: Tokyo Metropolitan Research Laboratory of Public Health, Hyakunin-cho, Shinjuku-ku, Tokyo 160, Japan.

† Present address: Department of Biology, Faculty of Science, Okayama University, Okayama 700, Japan.

Table 1. *Representative non-flagellate Salmonella strains used*

Strain no.	Genotype	Parent strain*
SJW412	<i>H1</i>	SJ241
SJW414	<i>H1</i>	SJ241
SJW417	<i>H1</i>	SJ241
SJW814	<i>H2</i>	SJW806
SJW310	<i>flaA1</i>	SJ241
SJW297	<i>flaAII</i>	SJ241
SJW251	<i>flaAIII</i>	SJ241
SJW315	<i>flaAIII</i>	SJ241
SJW341	<i>flaAIII</i>	SJ241
SJW272	<i>flaB</i>	SJ241
SJW141	<i>flaC</i>	SJ925
SJW227	<i>flaD</i>	SJ241
SJW152	<i>flaE</i>	SJ925
SJW164	<i>flaF</i>	SJ925
SJW159	<i>flaK</i>	SJ925
SJW411	<i>flaL</i>	SJ241
SJW413	<i>flaL</i>	SJ241
SJW415	<i>flaL</i>	SJ241
SJW467	<i>flaM</i>	SJ925
SJW291	<i>flaN</i>	SJ925
SJW337	<i>flaP</i>	SJ241
SJW235	<i>flaQ</i>	SJ241
SJW822	<i>flaR</i>	SJW806
SJW880	<i>flaR</i>	SJW806

* SJ241 and SJ925 are phase-1 stable derivatives of *Salmonella abortusequi* (Yamaguchi *et al.*, 1972*a*). SJW806 is a phase-2 stable derivative of *Salmonella typhimurium*.

(Yamaguchi, Iino & Kuroiwa, 1972*b*; Patterson-Delafield *et al.*, 1973; Suzuki & Iino, unpublished).

If the final attachment site for χ is at the base (the hook and/or the basal body) of a flagellum, as observed by Schade *et al.* (1967), non-flagellate mutants which retain the bases should be sensitive to the phage. We have therefore surveyed the χ sensitivity of the non-flagellate mutants representing all the known *H* and *fla* complementation groups.

METHODS

Bacteria and bacteriophage. The flagellate strain SJ241 is a phase-1 stable strain of *Salmonella abortusequi*. The representative non-flagellate *Salmonella* strains used are listed in Table 1. Bacteriophage χ was usually propagated and titrated on *Salmonella abortusequi* SJ241.

Media. The composition of nutrient broth, nutrient agar and nutrient gelatin agar (semi-solid medium) has been described (Yamaguchi *et al.*, 1972*a*).

Phage techniques. Sensitivity of bacterial strains to χ was examined by two methods.

- (i) Spot test. A portion (0.1 ml) of overnight broth culture (1×10^9 viable units ml^{-1}) of the test strain was spread on a nutrient agar plate and dried. Then, 0.02 ml drops of serial 10-fold dilutions of χ suspension containing 3×10^{10} plaque-forming units (p.f.u.) ml^{-1} were spotted on the lawn. After overnight incubation, the degree of clearing was recorded.
- (ii) Test for propagation of χ on the spotted area. A portion (0.05 ml) of overnight broth culture (1×10^9 viable units ml^{-1}) of the test strain was spread on a nutrient agar plate (3 cm diam.) and dried. Then, 0.01 ml of χ suspension containing 1.0×10^8 p.f.u. was spotted on the lawn. After incubation for 8 h, the bacteria and phage were suspended in

1.0 ml saline. The bacteria were killed by addition of chloroform and the number of p.f.u. in the suspension was counted using the soft agar layer method, with SJ241 as the indicator strain.

Other phage techniques were according to Adams (1959).

Electron microscopy of phage-bacterium complexes. A portion (0.1 ml) of bacterial suspension containing 2×10^8 viable units ml^{-1} was spread on a nutrient agar plate and dried. Then, 0.1 ml of χ suspension containing 1×10^{11} p.f.u. ml^{-1} was spread on it. After incubation for 10 min at 37 °C, the bacteria and phage were gently suspended in 0.5 ml 0.5% formalin. After washing with distilled water, samples were negatively stained with uranyl acetate at pH 4 and examined in a JEM7A electron microscope. More than 500 bacteria were examined in each survey.

RESULTS

Sensitivity of non-flagellate mutants to χ phage

The sensitivity to χ of non-flagellate *Salmonella* mutants carrying a mutation in the *H1*, *H2*, *flaAI*, *flaAII*, *flaAIII*, *flaB*, *flaC*, *flaD*, *flaE*, *flaF*, *flaK*, *flaL*, *flaM*, *flaN*, *flaP*, *flaQ* or *flaR* gene was examined. To avoid the confusion which could be caused by spontaneous reversion of the mutants to the flagellate form or by leakiness of the mutants, stable, or almost stable, and non-leaky mutants were used – except mutants of *flaAIII*, *B*, *P* and *R* in which non-leaky mutants have never been obtained. Using the spotting method, four groups of mutants, *H1*, *H2*, *flaL* and *flaR*, were sensitive to the phage, i.e. χ suspensions containing more than 3×10^5 p.f.u. ml^{-1} produced ill-defined patches on the lawns of these mutant bacteria. Discrete plaques were sometimes formed at higher dilutions, but they were too small and ill-defined to be counted. However, on the other *fla* mutants examined, even undiluted χ suspension (3×10^{10} p.f.u. ml^{-1}) had no effect.

Sensitivity of *H1*, *H2*, *flaL* and *flaR* mutants to χ was more clearly shown by examining propagation of the phage spotted on their lawns. After incubation for 8 h, the number of χ (p.f.u.) was 10^4 to 10^6 times that in the suspension originally spotted on the lawns (Fig. 1). In addition to these four groups of mutants, all the *flaAIII* mutants tested were also sensitive to χ , i.e. on the *flaAIII* mutants, the number of χ increased to 10^2 to 10^3 times that in the original suspension. All the *flaAIII* mutants are leaky; about 0.1% of bacteria carry a few flagella. However, as their degree of leakiness is not greater than that of *flaB* and *flaP* mutants, their sensitivity to χ cannot be due to this.

To examine the kinetics of χ infection of the sensitive non-flagellate bacteria, the rate constant for adsorption of the phage was measured. A broth culture containing 1×10^8 bacteria ml^{-1} was mixed with the same volume of χ suspension containing 1×10^9 p.f.u. ml^{-1} . After incubation for various periods at 37 °C, the mixture was centrifuged at 3000 rev. min^{-1} and unadsorbed phage in the supernatant were counted using the soft agar layer method. The rate constant for χ adsorption to the wild-type SJ241 cells was 8.5×10^{-9} ml min^{-1} , but adsorption to any of the sensitive non-flagellate mutants was not detected ($< 0.05 \times 10^{-9}$ ml min^{-1}). Furthermore, no significant increase of χ in the mixture was detected after incubation for 8 h.

Electron microscopy of χ -bacterium complexes

To identify the receptor site for χ , sensitive non-flagellate bacteria were mixed with the phage and examined in an electron microscope. When they were mixed in broth, no phage-bacterium complexes were observed even when the mixture contained 1×10^9 bacteria ml^{-1}

Fig. 1. Propagation of χ on non-flagellate *Salmonella* strains on nutrient agar plates. A portion (0.05 ml) of overnight broth culture (1×10^8 viable units ml^{-1}) of a non-flagellate *Salmonella* strain was spread on a nutrient agar plate (3 cm diam.) and 0.01 ml of χ suspension containing 1.0×10^8 p.f.u. was spotted on the lawn. After incubation for 8 h, the bacteria and phage were suspended in 1.0 ml saline. The bacteria were killed with chloroform and the number of p.f.u. in the suspension was counted using the soft agar layer method, with *Salmonella abortusequi* SJ241 as the indicator strain. The vertical dashed line indicates the number of p.f.u. (1×10^8) in the χ suspension originally spotted on the plate.

and 1×10^{11} p.f.u. ml^{-1} . A few complexes were found, however, when they were mixed on nutrient agar plates.

About 2% or less of *H1* and *flaL* bacteria had adsorbed one or two χ particles on to their surface but, because phage-bacterium complexes were rare, it was impossible to recognize the type of structure on the bacterial surface to which the phage particles were attached. The most distinct result was obtained with *flaR* mutants which carry four to eight superhooks per cell. About 10% of the *flaR* bacteria had adsorbed χ particles. High magnification electron micrographs showed χ particles attached by their tail fibres to the superhooks (Fig. 2). No *flaAIII* bacteria with adsorbed χ particles were seen.

Fig. 2. Attachment of bacteriophage χ to superhooks of *flaR* *Salmonella* mutants. (a) *Salmonella* strain sjw822 *flaR*. (b) *Salmonella* strain sjw880 *flaR*. Bar markers represent 100 nm.

DISCUSSION

All non-flagellate *Salmonella* mutants lack the flagellar filament, but they may be divided, on the basis of their hooks, into three classes: (i) those which carry apparently normal hooks – *H1*, *H2* and *flaL* mutants; (ii) those which carry superhooks – *flaR* mutants; and (iii) those which lack hooks – all other groups of *fla* mutants. The first two classes of mutants carry apparently normal basal bodies (Patterson-Delafield *et al.*, 1973; Suzuki & Iino, unpublished), but whether or not the third class of mutants carry the basal body or a part of it is unknown. Since Meynell (1961) reported that bacteriophage χ did not attack non-motile *Salmonella* strains, it has been believed that non-flagellate mutants are resistant to χ . In this study, however, all the non-flagellate groups of classes (i) and (ii), and the *flaAIII* mutants in class (iii) were found to be sensitive to χ .

Schade *et al.* (1967) proposed the following steps by which χ attacks motile bacteria: (1) χ attaches to the filament of the flagellum; (2) it travels to the base of the flagellum; and (3) it releases its DNA into the bacterium at the base of the flagellum. Our results support their proposal that the ultimate receptor site for χ is at the base of a flagellum, and electron micrographs show that in *flaR* mutants the superhook is the receptor for χ . The structure of the receptor for χ on the *H1*, *H2*, *flaL* and *flaAIII* bacteria could not be recog-

nized. However, since *H1*, *H2* and *flaL* mutants carry apparently normal hooks (Yamaguchi *et al.*, 1972*b*; Patterson-Delafield *et al.*, 1973; Suzuki & Iino, unpublished), it is most likely that the hook is the χ -receptor for these mutants. On *flaAIII* bacteria, neither filament nor hook was observed, but the bacteria were still sensitive to χ . Thus, they have a receptor for χ other than the hook and the filament. It is possible that the basal body is the final receptor for χ and that *flaAIII* bacteria carry the basal body or the part of it which acts as the χ -receptor.

The gene *flaR* determines the length of the hook (Patterson-Delafield *et al.*, 1973), but the structural gene for hook protein is as yet undetermined. Mutants in the latter gene would be expected to carry complete basal bodies, and if, as postulated, the basal body is the site of the χ -receptor, they should be sensitive to χ . At present, *flaAIII* mutants are the only known *fla* group that lack the hook but are sensitive to χ . However, before concluding that *flaAIII* is the structural gene for hook protein, it is necessary to confirm that these bacteria retain complete basal bodies. The remaining flagellar structure in non-flagellate strains, including *flaAIII* mutants, is now being examined (Suzuki, personal communication).

Because χ attacks motile *Salmonella*, it has been used as an agent for selecting non-motile mutants from motile bacteria. The groups of non-flagellate mutants which were sensitive to χ can be selected using χ as efficiently as other *fla* groups. This may be because of their low sensitivity to χ especially in liquid medium. Neither adsorption nor propagation of the phage could be demonstrated in liquid medium for any of the sensitive mutants, suggesting that the complexes of χ and these bacteria are so unstable that even Brownian movement disturbs their formation or facilitates their dissociation.

We are grateful to Professor T. Taira of Waseda University and T. Suzuki of University of Tokyo for their helpful discussion.

REFERENCES

- ADAMS, M. H. (1959). *Bacteriophages*. New York: Interscience Publishers.
- DEPAMPHILIS, M. L. & ADLER, J. (1971). Fine structure and isolation of the hook-basal body complex of flagella from *Escherichia coli* and *Bacillus subtilis*. *Journal of Bacteriology* **105**, 384-395.
- IINO, T. & MITANI, M. (1967). Infection of *Serratia marcescens* by bacteriophage χ . *Journal of Virology* **1**, 445-447.
- MEYNELL, E. W. (1961). A phage, $\phi\chi$, which attacks motile bacteria. *Journal of General Microbiology* **25**, 253-290.
- PATTERSON-DELAFIELD, J., MARTINEZ, R. J., STOCKER, B. A. D. & YAMAGUCHI, S. (1973). A new *fla* gene in *Salmonella typhimurium* - *flaR* - and its mutant phenotype. *Archiv für Mikrobiologie* **90**, 107-120.
- SCHADE, S. Z., ADLER, J. & RIS, H. (1967). How bacteriophage χ attacks motile bacteria. *Journal of Virology* **1**, 599-609.
- SERTIC, B. & BOULGAKOV, N. A. (1936). Bactériophages spécifiques pour des variétés bactériennes flagellées. *Comptes rendus des séances de la Société de biologie et de ses filiales, Paris* **123**, 887-888.
- YAMAGUCHI, S., IINO, T., HORIGUCHI, T. & OHTA, K. (1972*a*). Genetic analysis of *fla* and *mot* cistrons closely linked to *H1* in *Salmonella abortusequi* and its derivatives. *Journal of General Microbiology* **70**, 59-75.
- YAMAGUCHI, S., IINO, T. & KUROIWA, T. (1972*b*). Possession of flagellar hooks by some non-flagellate mutants of *Salmonella abortusequi*. *Journal of General Microbiology* **70**, 299-303.