NATURAL OCCURRENCE OF FOUR REVERSIBLE FLAGELLAR PHASES IN CULTURES OF *SALMONELLA MIKAWASHIMA*

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ABSTRACT

EDWARDS, P. R. (Communicable Disease Center, U.S. Public Health Service, Atlanta, Ga.), R. SAKAZAKI, AND I. KATO. Natural occurrence of four reversible flagellar phases in cultures of *Salmonella mikawashima*. J. Bacteriol. **84**:99–103. 1962—Study of cultures of *Salmonella mikawashima* isolated from reptiles revealed that single colonies of some strains contained three distinct H phases and that colonies of other cultures contained four such phases. In addition to the usual *y* and *e,n,z*$_{15}$ phases of *S. mikawashima*, two additional antigens which occurred naturally and which were identical with naturally occurring H antigens of the Arizona group, designated z_{47} and z_{50}, were recognized. Some cultures were represented by the formula *6,7:y:-e,n,z*$_{15}$*:z*$_{47}$, some by *6,7:y:e,n,z*$_{15}$*:z*$_{50}$, and some by *6,7:y:e,n,z*$_{15}$*:z*$_{47}$*:z*$_{50}$. The four H antigens were serologically unrelated, and each could be caused to revert to the others. Spontaneous reversions also were observed. The organisms underwent loss variation, and stable diphasic forms were encountered in which the *e,n,z*$_{15}$ phase occurred in conjunction with *y*, z_{47}, or z_{50}, indicating that the three antigens may be alternative forms of phase 1.

The phenomenon of phase variation, which affects flagellar antigens, and the presence of three flagellar phases in cultures of *Enterobacteriaceae* were reviewed briefly by Fife et al. (1961). Attention was called to the fact that the presence of three readily reversible, naturally occurring antigens in a single bacterium had been observed only in three serotypes of the Arizona group. In the *Salmonella* group, readily reversible flagellar variation involving naturally occurring antigens has been observed only in serotypes which possessed two alternative H (flagellar) antigens as described by Andrewes (1922). Cultures such as *S. salinatis* (*4,12:-d,e,h:d,e,n,z*$_{15}$) possessing complex H phases, which were irreversibly separable into simpler components through loss variation, have been described (Edwards and Bruner, 1942; Edwards, 1950). Further, organisms which occurred in one phase and which changed irreversibly to diphasic forms with two other phases, as the *S. worthington* culture (*1,13,23:z*$_{43}$ → *1,13,23:z:l,w*) of Taylor et al. (1960) and the *S. goerlitz* culture (*3,15:z*$_{27}$ → *3,15;e,h:1,2*) of LeMinor and Edwards (1960), have been reported. However, unpublished data now at hand indicate that these irreversible variations actually involve only two complex phases and that variation in such cultures is completely comparable to that observed in *S. salinatis*. Finally, many "induced" or artificial phases have been obtained by continued growth of salmonellae in antisera, but these are not readily reversible and can hardly be considered natural or "normal" H antigens. Thus, to the present time, spontaneous, reversible phase variation in salmonellae has been described only in organisms which display two alternative H antigen complexes. The purpose of the present paper is to describe cultures of *S. mikawashima*, some of which have three and some four naturally occurring reversible H antigens.

MATERIALS AND METHODS

During 1959 and 1960, 40 cultures of *S. mikawashima* were isolated from the feces of snakes (*Agkistrodon halys blomhaffi* and other species). These cultures later were found to be divisible into the following serological varieties: (i) *6,7:y:e,n,z*$_{15}$ (normal *S. mikawashima*), 17 cultures; (ii) *6,7:y:e,n,z*$_{15}$*:z*$_{47}$, 11 cultures; (iii) *6,7:y:e,n,z*$_{15}$*:z*$_{50}$, 9 cultures; and (iv) *6,7:y:-e,n,z*$_{15}$*:z*$_{47}$*:z*$_{50}$, 3 cultures. The incidence and serological properties of the z_{47} and z_{50} antigens, neither of which previously had been encountered in *Salmonella* cultures, are described below. The antigenic formulas are recorded above without

prejudice as to the genetic function of the various antigens (i.e., which components constitute phase 1, 2, 3, or 4). Some evidence regarding this point will be presented.

Although all of the cultures were examined in some detail, the majority of the work was done with four cultures. The methods used in the biochemical and serological studies were those described by Edwards and Ewing (1955). Changes in H phases were accomplished in the usual way by the addition of one or more suitably absorbed H antisera to semisolid medium. Each time the phases were changed, the cultures were plated and well-isolated single colonies were selected for examination. Each of the changes in flagellar antigens listed below was accomplished not once, but was repeated many times with multiple colony isolations. In those instances in which change in H antigens did not occur when colony isolates were cultivated in semisolid medium containing two or more antisera, the organisms were transferred serially in similar tubes over periods of 3 months to 1 year to assure the fact that the cultures were stabilized and that other antigens would not appear.

RESULTS

All the cultures examined possessed the usual biochemical properties of *S. mikawashima*, and all were members of *Salmonella* O antigen group C_1. In absorption tests, all completely removed agglutinins from C_1 (*6,7*) serum. The results obtained in the examination of the H antigens of the four cultures with which most of the work was done are outlined below.

Culture 2122-59. When first examined, this culture was agglutinated strongly by *y* serum but was unaffected by other *Salmonella* H antisera. A single *y* colony was placed in semisolid medium that contained *y* serum with the expectation that *e,n,z*$_{15}$ phase would appear. However, from the spreading growth was isolated a form which was not agglutinated by sera for the recognized H antigens of salmonellae. This form was agglutinated strongly and to the titer of antiserum for Arizona H antigen 39. To this form the symbol z_{47} was assigned. More than 400 colonies of the original culture were examined and only *y* and z_{47} colonies were found. When either of these forms was placed in semisolid medium containing both *y* and z_{47} sera, *e,n,z*$_{15}$ phases were isolated without difficulty, and these *e,n,z*$_{15}$ forms could be caused to revert to *y* or z_{47} at will by growth in proper combinations of sera. Further, spontaneous change from *e,n,z*$_{15}$ to both *y* and z_{47} was observed as well as spontaneous change from *y* to z_{47} and vice versa. These spontaneous changes occurred in the absence of antisera.

When the culture was placed in semisolid medium to which had been added *y*; *e,n,z*$_{15}$; and z_{47} sera, a fourth antigenic form emerged. At first it was thought that this was an "induced" or "artificial" antigen, but further experience with this and other cultures indicated that this was not the case. This fourth H antigen was assigned the symbol z_{50}. (We are indebted to F. Kauffmann for the examination of the culture and for confirmation of the symbols z_{47} and z_{50}. Dr. Kauffmann noted rapid hydrolysis of urea by the culture. This was not apparent in our tests.) It was not related to the then recognized antigens of the *Salmonella* or Arizona groups. Later, an identical antigen was found to occur naturally in four cultures of a new Arizona serotype and was assigned the Arizona symbol H42.

A single colony in the z_{47} phase on plating gave rise to both *y* and z_{47} colonies. From the z_{47} colonies, forms were obtained which had the formuld *6,7 : y : e,n,z*$_{15}$ *: z*$_{47}$. These three phases were reversible at will, and single colonies, isolated at each change of phase, continued to be reversible to each of the other phases without difficulty. Antigen z_{50} was not recovered from these z_{47} forms which were isolated from the original z_{47} colony. On the contrary, *y* forms, which segregated naturally from the original z_{47} colony, possessed the formula *6,7 : y : e,n,z*$_{15}$ *: z*$_{50}$. These three phases also were fully reversible at will in all directions, and each phase could be readily changed to either of the others. However, z_{47} could not be recovered. Thus, it seemed that a single colony, from which four distinct H antigens could be recovered, gave rise to two different forms, each of which yielded only three of the antigens, z_{47} appearing in one and z_{50} in the other (Fig. 1).

Culture 2090-60. Culture 2090-60 possessed the formula *6,7 : y : e,n,z*$_{15}$ *: z*$_{47}$. It is mentioned only because it was predominantly in the z_{47} phase when isolated. Of 350 colonies examined, 348 were agglutinated by z_{47} serum, and only two by *y* serum. On passage through semisolid medium containing both *y* and z_{47} sera, *e,n,z*$_{15}$ phases were obtained from both *y* and z_{47} colonies. The three phases were fully reversible.

Culture 2105-60. This culture possessed the

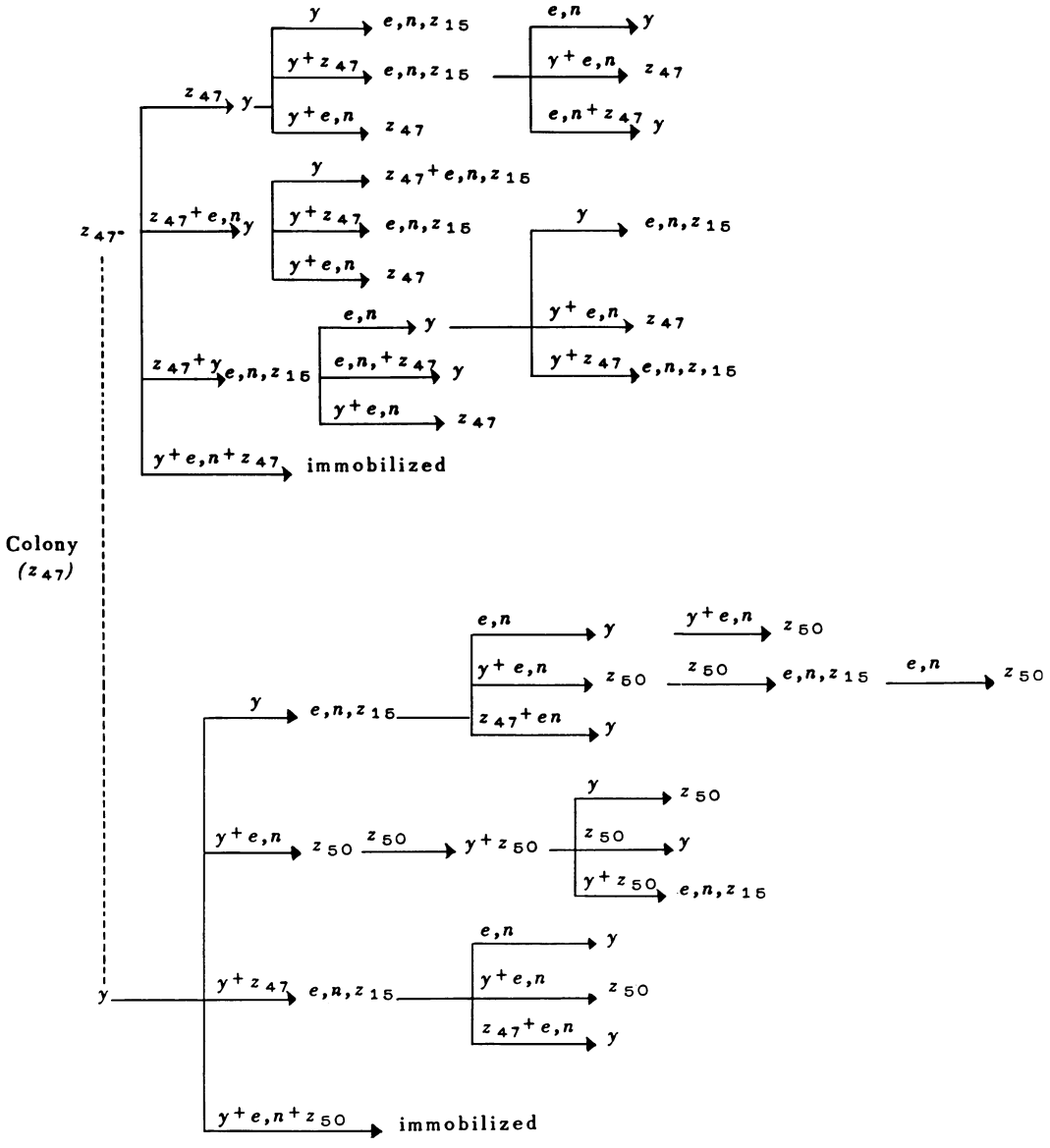

FIG. 1. *Variation in a colony of culture 2122-59. Dotted lines indicate spontaneous variations. Solid lines indicate phase changes accomplished by use of sera. Symbols on arrows indicate H sera added to medium to accomplish changes in phase.*

formula $6,7:y:e,n,z_{15}:z_{50}$. In this culture, the z_{50} phase was found in the original culture without resort to migration through antiserum. Of 286 colonies examined, 195 were agglutinated by y serum, 86 by e,n,z_{15} serum, and 5 by z_{50} serum. Each of the three phases was fully reversible to the other two by cultivation in appropriate antisera.

Culture 2316-60. Culture 2316-60 possessed antigens $6,7:y:e,n,z_{15}:z_{47}:z_{50}$, and all four phases were found in the original culture without serum treatment. Of 300 colonies examined, 93 were y, 76 were e,n,z_{15}, 126 were z_{47}, and 5 were z_{50}. Single colony isolations of each of the four phases could be changed to the other three without difficulty by cultivation in the proper sera. In this culture, spontaneous change from z_{50} to z_{47} was observed.

TABLE 1. *Relationships of flagellar antigens of Salmonella mikawashima, culture 2122-59*

Antigens	Sera			
	S. mikawashima phase 1 (*y*)	*S. mikawashima* phase 2 *e,n,z*$_{15}$	Arizona 1158-58 phase 1 (H39)	Culture 2122-59 z_{50}
S. mikawashima, phase 1	12,800	<100	<100	<100
2122-59, *y* phase	12,800	<100	<100	<100
S. mikawashima, phase 2	<100	6,400	<100	200
2122-59, *e,n,z*$_{15}$ phase	<100	6,400	<100	200
Arizona 1158-58, phase 1	<100	<100	12,800	<100
2122-59, z_{47} phase	<100	<100	12,800	<100
Arizona 3064-61, H42 phase	<100	<100	<100	6,400
Culture 2122-59, z_{50} phase	<100	<100	<100	6,400

In Fig. 1 an example is given of a single colony which gave rise to four distinct H antigens and which naturally segregated two variants, each of which possessed three H antigens. Among the hundreds of colonies examined during the work on the above described cultures, stable diphasic segregants were repeatedly observed. Diphasic forms having the formulas *y*:*e,n,z*$_{15}$; z_{47}:*e,n,z*$_{15}$; and z_{50}:*e,n,z*$_{15}$ were isolated from cultures which originally contained all the antigens. The results of attempts to demonstrate the antigens were by no means uniform in all colonies. Some colonies gave rise to four H antigens, some only to three, and others followed the usual diphasic behavior of salmonellae. Results of colony selection were unpredictable. The cultures seemed to undergo loss variation from the quatrephasic to the diphasic state. Spontaneous changes between the *y*, z_{47}, and z_{50} forms were observed and normal diphasic variation between those antigens and the *e,n,z*$_{15}$ form occurred.

Culture 2122-59. The serological reactions of the four H antigens of culture 2122-59 are given in Table 1. No relationships were apparent among them. The agglutination of the *e,n,z*$_{15}$ phase by z_{50} serum was due to the presence of a small amount of *e,n,z*$_{15}$ agglutinin, which resulted from the injection of a *6,7*:z_{50}:*e,n,z*$_{15}$ form. It was removed by absorption with *S. mikawashima* phase 2 or *S. sandiego* phase 2. Absorption of sera, prepared from phase 1 (*y*) and phase 2 (*e,n,z*$_{15}$) of the type strain of *S. mikawashima* by the corresponding phases of culture 2122-59, resulted in a complete removal of agglutinins. Arizona H39 serum was exhausted of H agglutinins by the z_{47} phase of culture 2122-59, as was z_{50} serum prepared from culture 2122-59, when absorbed with the H42 phase of Arizona serotype 23:23:30:42.

DISCUSSION

From the results obtained, it was apparent that among cultures of *S. mikawashima*, isolated from reptiles, strains which yielded two, three, or four distinct H antigens could be found. The question immediately arose as to whether the antigens z_{47} and z_{50} (i.e., antigens not found in the usual diphasic *S. mikawashima* culture) were "induced" or "artificial" antigens like the *j* and z_5 antigens, which can be obtained, respectively, by prolonged cultivation in antisera of the *d* and *b* phases of a number of salmonellae. The observations recorded here indicate that z_{47} and z_{50} were not induced phases, since they occurred repeatedly in nature and reverted spontaneously to "normal" phases. These two properties are not characteristic of induced phases, which occur rarely in nature and which are reversible to normal phases only with difficulty, if, indeed, reversion can be accomplished.

Are z_{47} and z_{50} "R phases" in the larger sense of Kauffmann (1961), which includes not only induced antigens but also a number of antigens which are found rarely, usually do not exhibit reversible phase variation, and are sometimes found in conjunction with two well-known H antigens? As R phases are described by Kauffmann, z_{47} and z_{50} could well be included. However, he is careful to point out that no absolute criteria for the differentiation of normal and R phases now are available. In the case of the naturally occurring antigens, and particularly those which revert spontaneously to "normal" antigens, their designation as R phases at present is completely arbitrary.

As stated above, the complex cultures described here had a tendency to segregate simpler forms, and stable diphasic types were isolated from cultures which exhibited three or four phases. One seemingly pertinent observation was that the *e,n,z*$_{15}$ phase was present in all these stabilized diphasic forms and that it occurred in combination with one of the other three antigens (*y*, z_{47}, or

z_{50}). Since e,n,z_{15} occurs typically as phase 2 of many *Salmonella* types, it would seem that y, z_{47}, and z_{50} were alternative phase 1 components. The repeated occurrence of the four antigens in single colony cultures, the reversibility of each to the other three, and the occasional spontaneous reversions observed demonstrate that salmonellae do not necessarily exist only in the monophasic and diphasic states, but may be multiphasic as well.

LITERATURE CITED

ANDREWES, F. W. 1922. Studies in group agglutination. I. The Salmonella group and its antigenic structure. J. Pathol. Bacteriol. **25**:505–521.

EDWARDS, P. R. 1950. Unusual antigenic variation in an enteric bacterium. Proc. Soc. Exptl. Biol. Med. **74**:471–474.

EDWARDS, P. R., AND D. W. BRUNER. 1942. A description of an unusual Salmonella type with with special reference to the evolution of Salmonella species. J. Bacteriol. **44**:289–300.

EDWARDS, P. R., AND W. H. EWING. 1955. Identification of Enterobacteriaceae. Burgess Publishing Co., Minneapolis.

FIFE, M. A., P. R. EDWARDS, R. SAKAZAKI, M. NOZAWA, AND M. MURATA. 1961. The occurrence of three flagellar phases in Arizona serotypes. Japan. J. Med. Sci. Biol. **13**:173–178.

KAUFFMANN, F. 1961. Die Bakteriologie der Salmonella Species. Munksgaard, Copenhagen.

LEMINOR, L., AND P. R. EDWARDS. 1960. Presence de trois phases de l'antigene flagellaire chez des bacteries du groupe *Salmonella*. Ann. inst. Pasteur. **99**:469–474.

TAYLOR, J., M. M. LEE, P. R. EDWARDS, AND C. H. RAMSEY. 1960. A new flagellar variation associated with new antigens in the Salmonella group. J. Gen. Microbiol. **23**:583–588.