The Effect on Virulence of Converting the O Antigen of *Salmonella cholerae-suis* from 6₂7 to 6₁7 by Phage

By H. WILLIAMS SMITH AND ZÖE PARSELL

Houghton Poultry Research Station, Houghton, Huntingdon, PE17 2DA

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SUMMARY

Of 86 strains of *Salmonella cholerae-suis* isolated in the U.K., 83 possessed the O antigen 6₁7 and three the O antigen 6₂7. All the O 6₁7 strains were latently infected with the same phage which was active on the O 6₂7 strains; during lysogenization this phage converted O 6₂7 strains to O 6₁7. None of 900 strains of *Escherichia coli* was latently infected with this phage; nor were they susceptible to it.

Two O 6₂7 strains converted to O 6₁7 by lysogenization were significantly more virulent for chicks than were their O 6₂7 parent strains; lysogenization of one of these O 6₂7 strains by two different non-converting phages was not accompanied by an increase in virulence. One of the converted strains tested in mice was more virulent for these animals than was the O 6₂7 strain from which it was derived.

The O 6₁7 organisms were found at higher concentrations in the blood and liver of chickens into which they had been injected intravenously than were organisms of the O 6₂7 strain from which they were derived. No difference was detected between the survival rates of the two kinds of organisms in normal chicken serum.

INTRODUCTION

Phage conversion, whereby genes resident in phages can change the properties of the bacteria they lysogenize, is now a well-recognized phenomenon. For example, the bacterial polysaccharides, or O antigens, that serve as the basis for the serological classification of the *Salmonella* genus can be altered in this manner; the subject has been thoroughly studied in the E group (Hayes, 1968). Strains of *Salm. cholerae-suis*, members of the C1 group, may possess either of the O antigens 6₁7 or 6₂7, and Escobar & Edwards (1968), in the U.S.A., showed that all the 6₁7 strains they examined were lysogenized with a phage which could convert 6₂7 strains to 6₁7. We have confirmed this observation with strains of *Salm. cholerae-suis* isolated in the U.K. and now report our studies to determine whether the conversion from O 6₂7 to O 6₁7 was accompanied by a change in virulence.

METHODS

Bacteria. Most of the strains of *Salmonella cholerae-suis* had been isolated at veterinary investigation laboratories in England and Wales during the period 1970-1 and identified by Mr W. J. Sojka at the Central Veterinary Laboratory, Weybridge, Surrey; all except one of them had been isolated from pigs. The four used in infection experiments were all classed as variety H₂S+. The remaining strains were stock cultures maintained by us. All these strains and ones prepared in the laboratory were smooth. In describing them, the expressions 'lysogenic', 'carrying phages' and 'latently infected with phage' are used synonymously.

Media and cultural conditions. Nutrient broth contained (g/l): 'Lab Lemco' (Oxoid Ltd, London, S.E.1), 10; Oxoid peptone, L37, 10; glucose, 1; NaCl, 5; the pH was adjusted to 7.5 before autoclaving at 105 °C for 20 min. The nutrient agar was 'Difco' tryptose agar (Difco Laboratories, Detroit, Michigan, U.S.A.) containing 0.02 M-CaCl₂. Except where stated otherwise, broth cultures consisted of organisms grown in broth for 24 h in a shaking water bath at 37 °C.

The identification of lysogenic strains. Lysogenic strains were identified by a modification of the cross-culture method (Fisk, 1942). A selected group of strains was grown in nutrient broth and each was used in turn as a 'basal' strain by spreading 0.08 ml volumes of the culture over the whole surface of a nutrient agar plate. The plates were then dried and the remaining cultures, after heating to 58 °C for 30 min, were spotted on to them. This process of killing the bacteria in the cultures without destroying the phage that may be present is referred to below as heat treatment. The plates were incubated at 37 °C for 18 h and examined for phage action; this was revealed by zones of complete or semi-complete lysis, or by discrete plaques. Modifications of this method were used when necessary or convenient. Sometimes only a few known susceptible strains were used as the basal strain, or live cultures were used for spotting; the basal strain in this latter case was a nalidixic acid-resistant mutant and the nutrient agar employed contained 20 µg sodium nalidixate/ml. Occasionally, strains were incubated together in broth and, after heat treatment, the product tested for phage.

Purification and propagation of phages. A suitably diluted suspension of a heat-treated lysogenic strain was mixed with a broth culture of a non-lysogenic strain selected as the propagating strain and spread on nutrient agar and incubated at 37 °C. A discrete plaque was picked as soon as plaques were distinctly visible, and replated with the propagating strain. This process was repeated twice. Finally, a discrete plaque with some of the surrounding bacterial growth was picked into broth, and incubated without shaking at 37 °C until lysis occurred. If lysis occurred rapidly, more of the propagating strain was added and incubation continued until lysis occurred again. The lysed culture was then heat-treated and tested for bacteriological sterility. Each phage was designated by the number of the lysogenic strain from which it was derived followed by the number of the propagating strain.

The lysogenization of bacterial strains. One drop of a broth culture of the strain to be lysogenized was spread over a small area of the surface of a plate of nutrient agar. When this was dry, one drop of an undiluted phage preparation was spotted on to the surface of the culture which was then incubated at 37 °C. A zone of complete lysis was observed after 4 to 5 h. Incubation was continued until secondary growth appeared within this zone. A little of this secondary growth was picked off into broth and incubated for 24 h, the culture plated on agar, and a single colony picked and plated. Finally, a single colony from this plating was picked into broth and incubated. The final culture was tested on a plate of nutrient agar to confirm its complete resistance to the undiluted phage preparation. Its colonial morphology was also examined. Only cultures that yielded colonies normal in appearance were retained; any forming colonies with a 'nibbled' appearance were regarded as actively phage-infected and were discarded. Lysogenized strains were designated by the number of the susceptible strain followed by the number, in parentheses, of the phage with which it had been lysogenized.

Cross-resistance tests. These were performed by a modification of Bail's (1923) original method. Resistant strains were prepared by the action of several phages on one strain of *Salmonella cholerae-suis*. The phages were then tested for activity against these strains.

Identification of strains possessing O antigens 6₁ or 6₂. This was achieved in our laboratory by preparing antisera in rabbits against an O 6₂ strain of *Salmonella cholerae-suis*, A50, and against an O 6₁ strain, A50(A1/A50). The O 6₂ antiserum was then absorbed with the O 6₁ strain and the O 6₁ antiserum was absorbed with the O 6₂ strain. In slide tests, the pure O 6₂ antiserum so obtained only agglutinated O 6₂ strains and the pure O 6₁ antiserum agglutinated only O 6₁ strains.

Virulence tests. These were performed in 2-day-old Light Sussex chicks from a *Salmonella*-free flock and in adult white Swiss mice of the Tuck TO strain. The mice were injected subcutaneously with 0.1 ml volumes of a broth culture of one or other of the strains of *Salmonella cholerae-suis* under test. The chicks were infected in exactly the same manner except that in their case the broth cultures were diluted 1 in 20. The infective doses for mice and chicks were 7×10^7 and 3.5×10^6 viable organisms respectively. The mice and the chicks were fed *ad libitum* on commercial diets free from antibiotics. The numbers that died daily were recorded; a proportion was examined bacteriologically to confirm that deaths resulted from infection by the inoculated strain of *Salm. cholerae-suis*. Ten days after the commencement of the experiment, the survivors were killed and examined for the presence of macroscopic lesions: in mice these were the white foci in the liver that are commonly associated with *Salmonella* infections, and in the chicks were a proliferative pericarditis. The results were analysed in 2×2 contingency tables and examined for significance by the χ^2 test by Mr J. G. Rowell of the A.R.C. Statistics Group of Cambridge University.

The estimation of lysogenic and non-lysogenic forms of Salmonella cholerae-suis in the blood and liver of infected chickens. Chickens, 4 weeks old, were injected intravenously with 0.1 ml volumes of broth cultures of lysogenic or non-lysogenic forms of the same strain of *Salmonella cholerae-suis*. At intervals, viable numbers were estimated in blood removed from wing veins. For estimating viable numbers in the liver, 1 g organ samples were removed at the end of the experiment and ground with sterile sand and 9 ml volumes of phosphate buffer, pH 7.0, in a pestle and mortar. Some chickens were infected with 0.1 ml volumes of cultures of both the lysogenic and the non-lysogenic forms; colonies from blood and liver samples of these chickens were subcultured into broth and organisms classified according to whether or not they were lysogenic.

The survival of lysogenic and non-lysogenic forms of Salmonella cholerae-suis in chicken serum. Broth cultures of lysogenic and non-lysogenic forms of the same strain of *Salmonella cholerae-suis* were diluted 1 in 10 in phosphate buffer and equal volumes of each diluted culture mixed together and the mixture immediately diluted 1 in 1000 in buffer. One drop (0.02 ml) of the latter dilution was then added to 1.0 ml of chicken serum and incubated in a shaking water bath at 37 °C. At intervals, a 0.1 ml sample was removed and spread evenly over the surface of a plate of MacConkey's agar. The plate was incubated at 37 °C for 24 h, counted for colonies and an appropriate number of colonies sub-cultured into nutrient broth to determine whether or not they contained lysogenic organisms. In control tests a sample of the same serum was heated at 65 °C for 30 min before inoculation to confirm that the serum could support the multiplication of the *Salm. cholerae-suis* organisms.

Bacterial counts. Viable organisms in broth cultures and in the blood and liver suspensions of chickens were estimated by the method of Miles & Misra (1938), using MacConkey's agar.

Table 1. *Virulence for chicks of lysogenic and non-lysogenic strains of Salmonella cholerae-suis*

	Strains inoculated							
	A1	A50	A50(A1/ A50)*	A50(A99/ A50)*	A50(A102/ A50)*	A3	A58	A58(A3/ A58)*
Lysogenic status	+	—	+	+	+	+	—	+
O antigens possessed	6 ₁₇	6 ₂₇	6 ₁₇	6 ₂₇	6 ₂₇	6 ₁₇	6 ₂₇	6 ₁₇
No. of chicks that died	45	23	72	25	26	49	28	67
No. of surviving chicks with lesions	27	4	29	3	3	33	2	30
No. of chicks that died or had lesions	72	27	101	28	29	82	30	97

For each strain, doses of 3.5×10^6 viable organisms were inoculated subcutaneously into groups of 140 chicks.

* Strains that had been lysogenized in the laboratory are designated by their number before lysogenization followed by the number, in parenthesis, of the lysogenizing phage; the phages are designated by the lysogenic strain from which they were obtained followed by the number of the strain on which they were propagated. Strains A99 and A102 were strains of *Salm. thompson* and *montevideo* respectively.

RESULTS

The incidence of lysogenic strains of Salmonella cholerae-suis

Of the 86 strains of *Salmonella cholerae-suis* examined, 83 were lysogenic, all 83 being shown by cross-resistance tests to be latently infected with the same phage; this phage was lytic for the remaining three strains, A50, A52 and A58. Two of the lysogenic strains, A1 and A3; two non-lysogenic strains, A50 and A58; and strains A50(A1/A50) and A58(A3/A58), i.e. strains A50 and A58 after they had been lysogenized with the phage carried by strains A1 and A3 respectively, were examined serologically by Dr Bernard Rowe of the Salmonella Reference Laboratory, Colindale, London N.W.9. He reported strains A1, A3, A50(A1/A50) and A58(A3/A58) to possess the O antigens 6₁₇, and strains A50 and A58 to have the O antigens 6₂₇. Subsequent tests with antisera prepared in our laboratory revealed that the non-lysogenic strain A52, like A58 and A50, possessed the O antigen 6₂ and that all of a selection of the lysogenic strains tested possessed the O antigen 6₁.

None of 900 strains of *Escherichia coli* (266 isolated from human beings, 173 from cattle, 339 from pigs and 122 from poultry) were latently infected with phages active on the *Salmonella cholerae-suis* strains A50 and A1; nor were they susceptible to phage A1/A50.

The virulence for chicks and mice of strains of Salmonella cholerae-suis possessing O antigens 6₁₇ and 6₂₇

Chicks were infected with two of the non-lysogenic O 6₂₇ strains of *Salmonella cholerae-suis*, A50 and A58, two of the lysogenic O 6₁₇ strains, A1 and A3, and strains A50 and A58 after they had been converted to O 6₁₇ by lysogenizing them with the phages carried by A1 or A3 [designated strains A50(A1/A50) and A58(A3/A58)] (Table 1). Included in the Table are the results for strains A50(A99/A50) and A50(A102/A50). Phages A99/A50 and A102/A50, obtained from lysogenic strains of *Salm. thompson* and *Salm. montevideo*

Table 2. *Virulence for mice of the A50 and A50(A1/A50) strains of Salmonella cholerae-suis*

Strain inoculated	O antigen possessed	Inoculation time (days)							
		3	4	5	6	7	8	9	10
		No. of mouse deaths							
A50	6 ₂ 7	0	1	12	21	23	25	29	30
A50(A1/A50)	6 ₁ 7	2	6	29	40	46	48	49	50

For each strain, doses of 7×10^7 viable organisms were inoculated subcutaneously into groups of 60 mice. Strain A50(A1/A50) was derived by lysogenization of strain A50 with phage A1/A50.

Table 3. *Recovery of viable organisms from the blood and liver of chickens at intervals after inoculation of strains A50 and A50(A1/A50) of Salmonella cholerae-suis*

Strain inoculated	No.* of organisms in				
	Blood at (h)			Liver at (h)	
	3	6	24	6	24
A50	27 (4-150)	11 (1-33)	13 (7-38)	18000 (4000-22000)	3000 (2000-11000)
A50(A1/A50)	220 (18-365)	47 (12-111)	26 (17-55)	16000 (8000-43000)	9000 (3000-43000)

For each strain, doses of 7×10^7 viable organisms were inoculated intravenously into groups of ten chickens. Five chickens in each group were killed at 6 h and 24 h. Consequently, the results quoted for blood at 24 h and liver at 6 h and 24 h were those for 5 chicks only.

*The median value ($\times 10^2$ /ml of blood or ground tissue) is quoted followed, in parenthesis, by the range.

respectively, were different from each other and from the phage carried by the 83 *Salm. cholerae-suis* strains; they were not implicated in O 6₂ to O 6₁ conversion. Broth cultures of strain A50(A99/A50) contained large numbers of free phage particles; by contrast, broth cultures of strain A50(A102/A50) contained only small numbers.

The two 6₁7 strains, A1 and A3, were more virulent than the two 6₂7 strains, A50 and A58 ($0.001 < P < 0.01$, judged by the number of chicks that died, and $P < 0.001$, judged by the number that died or developed lesions); the converted 6₁7 strains, A50(A1/A50) and A58(A3/A58), were also more virulent ($P < 0.001$, judged by either of the two criteria). Strain A50(A1/A50) was even more virulent than strain A1 ($0.001 < P < 0.01$, judged by the number of chicks that died, and $P < 0.001$, judged by the number that died or developed lesions); strain A58(A3/A58) was only slightly more virulent than A3 ($0.01 < P < 0.05$, judged by the number of chicks that died, and $P > 0.05$, judged by the number that died or developed lesions). In contrast to strain A50(A1/A50), the virulence of A50(A99/A50) and A50(A102/A50) was no greater than that of A50 itself ($P > 0.05$ judged by either of the two criteria).

Mice were inoculated with strains A50 and A50(A1/A50) (Table 2). As in the chicks, the number of mice that died was significantly higher in the group inoculated with strain A50(A1/A50) than in that inoculated with strain A50 ($P < 0.001$). When the experiment was terminated after 10 days, all except one of the remaining mice in each group appeared well; only these two sick mice had severe lesions.

Table 4. *The ratio of Salmonella cholerae-suis A50(A1/A50) and A50 organisms in the blood and liver of chickens at intervals after inoculation of equal numbers of both organisms*

Chicken no.	No. of A50(A1/A50) colonies amongst 20 A50 colonies cultured from:				
	Blood at (h)			Liver at (h)	
	3	6	24	6	24
1	20	18	—	19	—
2	20	18	—	18	—
3	18	19	—	15	—
4	17	14	—	17	—
5	18	8	—	11	—
6	20	19	19	—	16
7	20	19	14	—	17
8	18	19	10	—	13
9	20	17	8	—	11
10	17	13	10	—	13
Average	18.8	16.4	12.2	16.0	14.0

Doses of 7×10^7 viable organisms of both strains were inoculated intravenously into ten chickens. Chickens nos. 1 to 5 were killed at 6 h and nos. 6 to 10 at 24 h.

Table 5. *Survival of mixtures of Salmonella cholerae-suis A50 and A50(A1/A50) organisms on incubation in normal chicken serum*

Time (h)	Viable nos.*/0.1 ml serum		Percentage of surviving organisms that were A50(A1/A50)	
	Average	Range	Average	Range
0	36	28-40	48	36-60
2	21	17-23	50	36-69
3	16	11-23	53	44-64

Equal viable nos. of the two organisms combined in 0.02 ml volumes of buffer were added to 1.0 ml volumes of each of 4 sera and incubated with shaking at 37 °C. Samples (0.1 ml) were removed at intervals for measurement of viable nos.; all the colonies cultured were examined by lysogenicity tests to determine whether they were A50 or A50(A1/A50).

* Average values and the range of results for the four sera are shown.

The concentration of Salmonella cholerae-suis A50 and A50(A1/A50) organisms in the blood and liver of infected chickens

Numbers of organism in the blood and liver of 4-week-old chickens were estimated at intervals after intravenous injection with *Salmonella cholerae-suis* A50 or A50(A1/A50) (Table 3). In a similar experiment, chickens received mixtures of equal numbers of the two kinds of organism and the ratios of the recovered organisms were studied (Table 4). In this latter experiment none of the discrete colonies that grew on culture plates inoculated with material from the chickens showed evidence of active phage infection, thus indicating that the A50 organisms were not being attacked *in vivo* by phage liberated by the lysogenic A50(A1/A50) organisms. In both experiments, the A50(A1/A50) organisms were present at higher concentrations in the blood at 3 and 6 h than were the A50 organisms; a similar trend was noted in the liver.

The survival of Salmonella cholerae-suis A50 and A50(A1/A50) organisms in chicken serum

Equal numbers of organisms of strains A50 and A50 (A1/A50) of *Salmonella cholerae-suis* were mixed and incubated in normal chicken sera (Table 5); each serum specimen tested had been collected sterilely from healthy 4-week-old chickens. The viable numbers of organisms in all four specimens decreased by approximately half during the 3 h period of observation, but the proportion of A50 to A50(A1/A50) organisms among survivors showed little variation. In similar experiments with the sera after heating, the concentrations of the inoculated bacteria increased approximately eightfold in the 3 h period.

DISCUSSION

Our results show that, as in the case of those isolated in the U.S.A. (Escobar & Edwards, 1968), strains of *Salmonella cholerae-suis* isolated in the U.K. that possessed the O antigens 6₁₇ were latently infected with a phage which, during lysogenization, converted O 6₂₇ strains to O 6₁₇. In the infection experiments, the virulence of the two O 6₂₇ strains, A50 and A58, had been significantly increased by lysogenizing them with the converting phage possessed by the two O 6₁₇ strains, A1 and A3, which were themselves more virulent than the O 6₂₇ strains. Particularly as no increase accompanied the lysogenization of strain A50 with the two non-converting phages, A99/A50 and A102/A50, it seems possible that the increased virulence of the two converted strains was associated with their acquisition of the O 6₁ antigen and that this antigen contributes more to the overall virulence of *Salm. cholerae-suis* than does the O 6₂ antigen. That alteration in mouse virulence can be achieved by replacing the O antigens of *Salmonella* strains by other *Salmonella* O antigens was shown by Valtonen (1970) and Valtonen & Mäkelä (1971). Of particular relevance to the present study was the replacement by conjugation and transduction of the gene locus determining the 4,12 antigens in a strain of *Salm. typhimurium* by the locus determining the 6,7 antigens in a strain of *Salm. montevideo*, a replacement that was accompanied by a reduction in mouse virulence.

Judging from the survival studies *in vivo* and *in vitro* of strains A50 and A50(A1/A50) in the blood and liver, the actual contribution made by the O 6₁ antigen was to make organisms possessing it better able to resist the defence mechanisms posed by the reticulo-endothelial system, because the O 6₁ organisms were removed more slowly from the blood of the infected chickens than were the O 6₂ organisms and survived better in their livers; both kinds of organisms appeared to resist the bactericidal action of serum equally well. Because *Salmonella cholerae-suis* is essentially parasitic, this resistance property of O 6₁ strains may have contributed to their ecological dominance over O 6₂ strains (83 of the 86 *Salm. cholerae-suis* strains examined were O 6₁) although it is, of course, tempting to ascribe their dominance to the infectious nature of the O 6₁ gene. It is noteworthy in this respect that *Salm. cholerae-suis* is not a common organism in nature and consequently opportunity may seldom be presented for an O 6₁ converting phage to lysogenize an O 6₂ strain. It would happen more often if the converting phage commonly parasitized a numerically-superior gut organism such as *Escherichia coli*. This, however, is not the case because none of the 900 *E. coli* strains we examined was either latently infected with or susceptible to the converting phage.

The virulence of the A50 strain, and to a lesser extent the A58 strain, of *Salmonella cholerae-suis* after conversion from O 6₂ to O 6₁ was greater than that of the O 6₁ strains A1 and A3 from which their converting phages were obtained. The reason for this may

be that A1 and A3 differ from A50 and A58 in respects other than lysogenicity and O antigen composition – respects which influence their overall virulence. That wild strains of the same species should so differ is not surprising; it could well be a reflexion of their divergent evolutionary histories. Whatever the reason, the results of the virulence tests on these strains stress the advantage, when studying a bacterial characteristic, of being able to compare strains before and after their acquiring the characteristic (O 6₁ and lysogenicity in this case) rather than having to resort to a comparison of wild strains with and without the characteristic.

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