STUDIES ON *SALMONELLA PULLORUM* PHAGE

IV. PHAGE RECEPTOR OF *S. PULLORUM*

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There are a number of publications concerning the relationship between susceptibility to phage and the somatic antigens of organisms of *Salmonella* and *Shigella*^{8,10,12,13,19,31,33,34}.

It was BURNET¹⁷ who first pointed out that there existed relations between adsorption of phage and bacterial fractions. He and his associate^{2,7} reported that polysaccharide was related to both serological character and phage susceptibility of those organisms, since the bacterial extracts inactivated phage and those actions were avoided by bacterial antiserum. As a result of experiments carried out successively, the culture filtrate^{5,14}, polysaccharide^{7,11,14,15,20-23}, lipopolysaccharide⁶, and the lipocarbohydrate-protein complex²⁴ of susceptible organisms of *Salmonella*, *Shigella*, and *Escherichia* phages were understood as phage receptors.

The author³² reported specific relations between a subtype of *Salmonella pullorum* which had been classified by the serological method and lytic action of group II and III phages. It was difficult, however, to regard antigens 12₂ and 12₃ as phage receptors by which subtypes were determined, since they did not lyse the strains of *S. paratyphi A* and *S. typhi murium* that possessed their antigens. But it will be undeniable that susceptible organisms have some structures or components that may allow the organisms to lyse.

In this investigation, experiment was performed on bacterial fractions, as phage receptors, which had been synthesized in the strains of subtypes, in order to confirm the specific relations between the subtype and the lytic action of phage.

MATERIALS AND METHODS

Phages used: A total of 9 phages were employed. Of them, 3 strains had been selected from types IIa and IIb and group III phages, respectively.

Organisms used: A total of 9 strains were employed. They consisted of one strain of standard (S) type, 2 strains of variant (V) type, and 3 strains of intermediate (I) type of *S. pullorum*, and 3 strains of *S. gallinarum*.

Phage resistant strains used: Five strains were used. They had been prepared by the combination of phages Y-8 (type IIa) and Y-23 (type IIb) with the 9-25 strain of S type, and phages Y-8, Y-23, and LPI-3 (group III) with the L60131 strain of V type. They were designated S-Y-8R, S-Y-23R, V-Y-8R, V-Y-23R, and V-LPI-3R in the order listed.

Separation of bacterial fractions: Fatty substance, protein, polysaccharide, and bacterial residue were prepared in such manner as shown in Fig. 1.

Inhibition of phage action by bacterial extracts: The fractions were mixed with such dilution of phage as containing 200 to 300 particles per ml of broth, so that 0.9 ml of each phage might be mixed with 0.1 ml of 0.1% solution or suspension in broth or distilled water of each fraction which had been sterilized 3 times at 60°C for 30 minutes each. After incubation at 37°C for 4 hours, the amount of active phage remaining was calculated by the plaque counting method on agar layer. Control tubes with added distilled water

Fig. 1. Separation of Bacterial Fractions for Phage-inhibition Test

were set up in the same manner.

Antigenicity of polysaccharide: Polysaccharide was tested for antigenicity by the agglutination test on organisms and rabbit serum immunized against this substance. Precipitation reaction was performed between the solution of polysaccharide and rabbit serum immunized against the chrome alum-treated organism.

Analysis of polysaccharide: Samples of polysaccharides were hydrolyzed with N/10 sulfuric acid at 100°C for 6 hours, and then neutralized with barium hydroxide. The neutral, salt-free hydrolysates were evaporated by drying *in vacuo* and redissolved at a concentration suitable for application to paperchromatography. Tōyō-brand filter paper No. 50 was used. The samples were separated in one-or-two dimensional paperchromatograms with three solvent systems (*n*-butanol-acetic acid-water, acetic ethyl-acetic acid-water, and phenol-water). After drying, the filter paper was sprayed with aniline hydrogen phthalate and heated at 105°C for 5 to 10 minutes to develop color²⁵⁾.

RESULTS

1. Chemical properties of bacterial fractions

Polysaccharide: An 0.5% solution of purified polysaccharide reacted strongly to the Molisch, Fehling, and Benedict reaction, moderately to orcinol, Selivanoff fluid, and aniline-acetic acid, and doubtfully to ninhydrin and Biuret reaction. It did not react to any other protein reagent.

Protein: An 0.5% solution of protein reacted strongly to Biuret, ninhydrin, and Millon reaction, moderately to Hopkins-Cooile, xanthoprotein, and sulfosalicylic acid. It did not react to the Pauly reaction. All the sugar reactions were wholly negative, exception the Molisch reaction.

Table 1. Inhibition of Phages by Bacterial Extracts

Phage \ Fraction	S (9-25)				V (L60131)				I (K-I-8)			
	F	CF	PF	R	F	CF	PF	R	F	CF	PF	R
IIa (Y-8)	0	100.0*	66.0	26.9	0	83.2	36.3	3.4	0	10.0	0	49.2
IIb (Y-23)	0	100.0	65.0	0	0	87.1	8.7	0	0	9.0	0	0
III (LPI-3)	0	0	0	0	0	98.1	0	0	0	0	0	0

F: Fatty substance, CF: Polysaccharide, PF: Protein, R: Bacterial residue.

*Inhibition rate = $\frac{\text{Number of plaques of control} - \text{Number of plaques after mixed with the extracts}}{\text{Number of plaques of control}} \times 100$

Table 2. Inhibition of Phages by Bacterial Extracts of Phage-resistant Strain

Phage \ Fraction	Polysaccharide					Protein				
	S-Y-8R	S-Y-23R	V-Y-8R	V-Y-23R	V-LPI-3R	S-Y-8R	S-Y-23R	V-Y-8R	V-Y-23R	V-LPI-3R
IIa (Y-8)	9.0	4.6	0	18.3	64.0	1.5	6.6	0	11.2	21.3
IIb (Y-23)	0	7.0	0	2.1	73.4	1.0	5.8	0	4.1	29.5
III (LPI-3)	*	*	0	41.5	7.0	*	*	0	0	0

* Not tested.

Fatty substance: The fatty substance was waxy, soluble in ether, and soluble in a small amount of alcohol, but was insoluble in acetone. It was, however, insoluble in water, swollen and suspended after a while.

Bacterial residue: The bacterial residue was insoluble in water. It did not react to the sugar and protein reactions, except the ninhydrin reaction at 1% of the suspension.

2. Inhibitory effect of phage action by bacterial fractions

Fractions of strains of subtypes: The results obtained are shown in Table 1. It was found that the fractions obtained from the polysaccharide synthesized by susceptible organisms had a very strong action to inhibit the phage action. Relations between the effect of the polysaccharide prepared from each strain of the subtypes and the inhibitory effect on phages accorded with relations between the lytic action of phages and living organisms.

Fractions of phage-resistant strains: Tests were made on polysaccharide and protein which showed high activity when the original strain had been used. As shown in Table 2, the polysaccharides of all the strains seemed to have no inhibitory action on phage, except those of strains V-Y-23R and V-LPI-3R which were inhibitory to group III and group II phages, respectively. These results were identical with those of the cross-resistance test.

3. Phage susceptibility of rough variant strains

If polysaccharide is concerned with the adsorption of phage, a strain which has lost such polysaccharide will not be lysed. The rough variant strains isolated from old cultures in broth resisted to phage at as high as 1,000 routine test dilutions.

4. Antigenicity of polysaccharide

As shown in Table 3, the rabbit serum immunized against polysaccharide produced agglutinin titers ranging from 1:800 to 1:1,600. On the other hand, the antigen titers of O-serums prepared from each strain of the subtypes ranged from 1:100,000 to 1:500,000 in the precipitation reaction with polysaccharide.

5. Sugar components of polysaccharide

It became clear that the phage-susceptible determinant was the O-antigenic polysaccharide. Then experiments were performed to examine whether the strains of each subtype

Table 3. Antigenicity of Polysaccharides Prepared from *Salmonella pullorum*

Agglutination									
Immunogen CF	Antigen	Dilution of serum							
		25	50	100	200	400	800	1,600	3,200
9-25	9-25	+++	+++	+++	++	++	+	+	±
L60131	L60131	+++	+++	+++	++	++	+	±	—
K-I-8	K-I-8	+++	+++	+++	++	++	+	+	±

Precipitation							
Antiserum (Agglutinin) titer	Antigen CF	Dilution of antigen					
		10 ³	5×10 ³	10 ⁴	5×10 ⁴	10 ⁵	5×10 ⁵
9-25 (6,400)	9-25	+++	+++	++	+	+	—
L60131 (3,200)	L60131	+++	+++	+++	+	+	+
K-I-8 (3,200)	K-I-8	+++	+++	++	+	+	—

synthesized specific monosaccharide or not. Paperchromatograms of the hydrolysates of the O-antigenic polysaccharides are shown in Fig. 2. Glucosamine, galactose, glucose, mannose, xylose, rhamnose, and tyvelose were found similarly in all strains; that is, one strain of S type, 2 strains of V type, and 3 strains of I type of *S. pullorum*, 5 strains of phage-resistant, and 3 strains of *S. gallinarum*. Ribose, which had been detected in crude polysaccharide, disappeared by the treatment for purification.

DISCUSSION

HADLEY⁸⁾ reported that the strains of *Salmonella*, *S. pullorum*, *S. gallinarum*, and *S. typhi*, were susceptible to the same phage, and that the action of phage on susceptible organisms was determined by the constitution of heat-stable agglutinogens. This observation was confirmed and extended by BURNET^{1,2)} and other investigators^{5-7,11,14,15,20-24)}. In consequence, the opinion became prevalent that the phage-adsorbing substance contained in the Enterobacteriaceae was the O-antigenic polysaccharide.

It is important to make clear not only the lytic phenomenon but the essence of the specificity on the specific relationship between group II and group III phages of the author and the subtypes of *S. pullorum*. It is also necessary to carry out such investigation on

Fig. 2. Paperchromatogram of Hydrolysate of O-antigenic Polysaccharide Prepared from *Salmonella Pullorum*

relations between phage and form variation of antigen 12₂ of *S. pullorum* as the author intended to do.

From the results of these experiments, it is considered that the O-antigenic polysaccharide is a phage receptor. It was found out that the phage action was inhibited specifically by the O-antigenic polysaccharide of susceptible organisms, as well as by many members of the Enterobacteriaceae. Moreover, it was clarified that the polysaccharide of a phage-resistant strain lost its inhibitory action on the related phage.

Many studies have been made on the sugar components of polysaccharide of various organisms, but none of them were concerned with those of strains of the subtype of *S. pullorum*. Only DŻUXYŃSKA and MIKULASZEK⁴⁾ reported the results of comparison of sugar components between smooth and rough strains of *S. pullorum*. According to them, the polysaccharide of the smooth strain contained glucosamine, galactose, glucose, mannose, xylose, rhamnose, and uronic acid?, and that of the rough strain lacked glucosamine, mannose, and rhamnose.

DAVIES³⁾ and STAUB et al.^{29,30)} proved the presence of five monosaccharides, galactose, glucose, mannose, rhamnose, and tyvelose, in *S. gallinarum* and the other strains of Salmonella group D. KAUFFMANN et al.¹⁶⁾ also reported that strains of Salmonella groups D₁ and D₂ synthesized such monosaccharides as glucosamine, heptose (s), galactose, glucose, mannose, rhamnose, and tyvelose. KUROYA et al.¹⁸⁾ detected monosaccharides, such as glucosamine, galactose, glucose, mannose, xylose, rhamnose, and fucose?, from *S. typhi*. From his experiment with the Ikegaki strain of *Pasteurella pseudotuberculosis*, SASAKI²⁸⁾ pointed out that three monosaccharides, galactose, glucose, and rhamnose, might be related to antigen 9 of Salmonella group D.

In the present investigation, it was proved that the sugar components of polysaccharide separated from the subtypes of *S. pullorum*, the phage-resistant strain, and the strain of *S. gallinarum* were identical with one another.

It was known that some strains of Salmonella and Shigella which differed serologically^{3,9,16~18,26,29,30)} from one another were the same in the constitution of monosaccharide. From this point of view, SASAKI^{26,27)} considered that serological specificity was determined by the difference either in quantity or in stereostructure of monosaccharides. STAUB et al.^{29,30)} reported that the type specificity of Salmonella was determined by sugar combination, the terminal monosaccharide of which controlled the antigenic factor. KOGURE and YAMAMOTO¹⁷⁾ and ICHIKAWA and FUJISAWA⁹⁾ proved that this sugar combination acted as determinant on the phage-mediated antigen of Salmonella and Shigella. From the reports which have been published previously^{9,17,26,27,29,30)}, it is considered also about the subtypes of *S. pullorum* that the type specificity can be determined by the sugar combination of the O-antigenic polysaccharide. Moreover, it is supposed that the stereostructure of the sugar combination as phage receptor might have relations between the antigens of strains of S type and group II phage, and the antigens of strains of V type and groups II and III phages.

SUMMARY

In order to confirm the specificity of the lytic action of group II and group III phages on the subtypes of *Salmonella pullorum*, adsorption tests were carried out with fatty substance, polysaccharide, protein, and bacterial residue which had been extracted from strains of standard, variant, and intermediate type, and phage-resistant strains of *S. pullorum*. The results are summarized as follows.

1. Polysaccharide which had been synthesized by susceptible organisms inhibited the

phage action of related phage. On the other hand, polysaccharide separated from a phage-resistant strain had no such action.

2. Monosaccharides which constituted polysaccharide were glucosamine, galactose, glucose, mannose, xylose, rhamnose, and tyvelose. These constituents were found constantly in strains of the subtypes of *S. pullorum*, phage-resistant strains, and *S. gallinarum*.

3. Accordingly, it is supposed that the difference in phage susceptibility may depend upon the combination of monosaccharide.

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ひな白痢菌ファージに関する研究

IV. ひな白痢菌のファージ・レセプターについて

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第II報で、II・III群ファージに対する感受性によって、ひな白痢菌の亜型が同定できることを述べた。これについて、単に溶菌現象の面だけでなく、その特異性の本質を明らかにすることは、ファージ対亜型の関係を一層明瞭にするものと考えられ、著者が意図するファージと、ひな白痢菌 12₂ 抗原の Form variation との関係を究明する上に、重要な資料となる。

本試験では、ファージ・レセプターを明らかにする目的で、各亜型の菌体より分離した脂質、多糖体、蛋白質、およびこれらを抽出した残りの菌体を用いて、溶菌阻止作用を調べ、次の成績を得た。

1) ファージ感受性菌が所有する抗原特異多糖体が、当該ファージの溶菌性を阻止する。すなわち、II群ファージはSおよびV型菌の、III群ファージはV型菌の多糖体によって、溶菌性が阻止された。一方、IIおよびIII群ファージに溶菌されないI型菌より得た分画は、両群のファージに対し、全く活

性を示さない。

2) ファージ耐性菌の多糖体は、ファージに対する溶菌阻止作用を消失した。これは耐性菌交叉試験の所見と良く一致する。

3) 正常な菌体抗原が失われているR型菌は、ファージ感受性をもたない。

4) 各亜型のひな白痢菌、ファージ耐性菌および鶏チフス菌の多糖体を構成する単糖は、定性的にはすべて同一であって、それぞれ Glucosamine, Galactose, Glucose, Mannose, Xylose, Rhamnose および Tyvelose が検出されるが、ファージ感受性を決定する特異的な単糖の存在は認められない。したがって、ファージ・レセプターは、単糖の立体構造の差によって決定するものと考えたい。

本論文は、北海道大学審査学位論文で、規定に基づき公表する。