Journal of General Microbiology (1983), **129**, 2739–2747. *Printed in Great Britain*

Growth Temperature-dependent Variation in the Bacteriophage-inactivating Capacity and Antigenicity of *Yersinia enterocolitica* Lipopolysaccharide

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(*Received 30 November 1982; revised 25 March 1983*)

Growth temperature affected the structure of *Yersinia enterocolitica* Ye 3827 lipopolysaccharide (LPS). Although *Y. enterocolitica* Ye 3827 synthesized smooth LPS when grown at a low temperature (25 °C), partial smooth-rough transition occurred when the bacteria were grown at the physiological temperature (37 °C). The structural alteration was detected by bacteriophage-inactivation assay and chemical and immunological analyses. LPS prepared from bacteria grown at 25 °C inactivated a number of bacteriophages that recognize the O-antigenic polysaccharide portion of LPS, whereas more than 3000 times the amount of LPS from bacteria grown at 37 °C was required for the same degree of inactivation. The antigenic determinant(s) responsible for the major reaction between 25 °C-LPS and anti-25 °C-bacteria was located on the O-antigenic polysaccharide portion of LPS, but those responsible for the major reaction between 37 °C-LPS and anti-37 °C-bacteria were located on the R-core or inner portion of LPS.

INTRODUCTION

Yersinia enterocolitica infection causes a variety of syndromes in man: acute gastroenteritis and other forms of abdominal illness, arthritis, and erythema nodosum. Although this organism grows at an unusually broad range of temperature (Maruyama, 1973), the bacteria grown at 25 °C and those grown at 37 °C are quite different in biological and biochemical characteristics. For example, bacteria grown at 25 °C show bacteriophage sensitivity (Mollaret & Nicolle, 1965), adherence to mammalian epithelial cell lines (Okamoto *et al.*, 1980), motility (Schleifstein & Coleman, 1939), and indirect haemolysin (Tsubokura *et al.*, 1979) and enterotoxin (Pai & Mors, 1978) production which those grown at 37 °C do not show.

In previous reports, we have shown that the insensitivity of the bacteria grown at 37 °C to bacteriophages I, IV, VIII, and XI correlated with a smaller amount of bacteriophage receptors on the cell surface of the 37 °C-grown bacteria than of the 25 °C-grown bacteria (Kawaoka *et al.*, 1982*b*, *c*). The bacteriophage XI receptor was partially purified and seemed to be a glycoconjugate other than lipopolysaccharide (LPS). The receptor molecule was synthesized at 25 °C but not at 37 °C (Kawaoka *et al.*, 1982*c*). Thus, we have shown a growth temperature-dependent structural alteration of a cell-surface component by bacteriophage-inactivation assay.

Acker *et al.* (1980) found a growth temperature-dependent difference in thickness of the outer membrane of *Y. enterocolitica* and examined LPS preparations from the bacteria grown at different temperatures. They showed that LPS from cells grown at higher temperatures had a reduced content of an O-specific sugar, 6-deoxy-L-altrose (Acker *et al.*, 1980). They also reported

Abbreviations: LPS, lipopolysaccharide; O-PS, O antigenic polysaccharide; PCP-LPS, LPS prepared by the phenol-chloroform-petroleum ether method; PW-LPS, LPS prepared by the phenol water method; KDO, 2-keto-3-deoxyoctonate.

0022-1287/83/0001-0944 $02.00

that smooth bacteria grown at 40 °C resembled rough bacteria grown at 22 °C in their reactivity with antibody to the enterobacterial common antigen (Acker *et al.*, 1981).

In the present paper, we show a growth temperature-dependent variation in the bacteriophage-inactivating capacity of LPS and examine the structure and antigenicity of purified LPS from organisms grown at different temperatures.

METHODS

Bacteria and bacteriophages. *Yersinia enterocolitica* strain Ye 3827 (biovar 4, serovar O:3) and phages I through IX used routinely for phage typing of *Y. enterocolitica* were obtained from Dr H. H. Mollaret (National Centre of *Yersinia*, Pasteur Institute, Paris, France).

A bacteriophage IV-resistant strain was isolated after spotting drops of a high-titre bacteriophage suspension on freshly prepared lawns of strain Ye 3827. After incubation for 48 h at 25 °C, colonies growing within the lytic areas were picked and streaked on to nutrient agar plates. The isolated colonies were further tested for sensitivity to the homologous bacteriophage.

Bacteriophages were propagated on *Y. enterocolitica* Ye 3827 in nutrient broth, and the lysates were used without further purification.

Medium. Bacteria were cultivated on nutrient agar plates (pH 7·2). No supplementation with any exogenous cation was made.

Antisera. Antisera to smooth bacteria, Ye 3827, grown at 25 °C (25 °C-bacteria) or at 37 °C (37 °C-bacteria) and to rough bacteria, Ye 3827 IV-3, grown at 25 °C (R-bacteria) were prepared with heat-killed bacteria. Rabbits were immunized intravenously four times at intervals of 7 d. Rabbits were bled 7 d after the last injection.

Extraction of LPS. Bacteria were grown on nutrient agar plates for 48 h. LPS from 25 °C-bacteria (25 °C-LPS) and 37 °C-bacteria (37 °C-LPS) was obtained by the hot aqueous phenol method of Westphal & Jann (1965). LPS from R-bacteria (R-LPS) was obtained by the phenol-chloroform-petroleum ether (PCP) method of Galanos *et al.* (1969). Since the yield of LPS was very low by the PCP method, a second extraction was made by the hot aqueous phenol method of Westphal & Jann (1965). LPS was purified by repeated sedimentation in a preparative ultracentrifuge.

Column chromatographic analysis of acetic acid hydrolysed LPS. LPS in an amount of 100 mg was partially hydrolysed with 1% acetic acid at 100 °C for 1·5 h (Davies *et al.*, 1955). The hydrolysate was neutralized with sodium hydroxide and centrifuged at 3000 *g* for 30 min. The supernatant was concentrated by evaporation under vacuum. The concentrate was applied on a Sephadex G-50 column (1·2 × 111 cm) and eluted with pyridine acetate buffer (pH 5·4) containing 10 ml pyridine and 4 ml acetic acid in 1 l distilled water at a flow rate of 6 ml h^{-1}. The effluent was collected in 3 ml fractions, which were each analysed for neutral sugar (Dubois *et al.*, 1956).

Bacteriophage-inactivation assay. Bacteriophage-inactivation assay was performed as reported previously (Kawaoka *et al.*, 1982*c*). A 0·1 ml portion of a suspension of bacteriophage (10^3 p.f.u.) was incubated with the same volume of LPS for 1 h at 25 °C. Free p.f.u. were determined by plating on nutrient agar plates with Ye 3827 cells.

Immunological methods. Double immunodiffusion tests in 1% (w/v) agarose gel were performed as described previously (Kawaoka *et al.*, 1979). Each antigen was dissolved in phosphate-buffered saline containing 0·1% (w/v) sodium taurocholate.

In immunodiffusion inhibition tests, an antiserum (30 µl) was incubated with an inhibitor for 3 h at 37 °C.

Passive haemagglutination inhibition (HI) tests were performed according to Lindberg & Hellerqvist (1980) with sheep erythrocytes sensitized with LPS. Agglutination was read after incubation for 1 h at 37 °C and after an additional 18 h in the cold.

Chemical analyses. Neutral sugar and phosphorus were determined by the methods of Dubois *et al.* (1956) and Ames *et al.* (1960). Protein was estimated by the Lowry method. Amino groups and 2-keto-3-deoxy-octonate were determined by the 2,4,6,trinitrobenzenesulphonic acid method of Goldfarb (1966) and the thiobarbituric acid method of Weissbach & Hurwitz (1959), respectively. Fatty acid was determined as methyl esters with docosanoic acid as an internal standard by GLC as described previously (Tsuji *et al.*, 1981). Sugar was determined as trimethylsilyl derivatives by GLC as described previously (Kawaoka *et al.*, 1982*a*). Briefly, samples and standard monosaccharides containing 100 to 200 µg carbohydrate and 10 µg mannitol as an internal standard were hydrolysed with 5% (v/v) hydrochloric acid/methanol at 80 °C for 18 h. Each trimethylsilylated sugar derivative was analysed in a glass column (4 × 2000 mm) of 2% OV-1 on Chromosorb W (60/80 mesh). O-PS of *Y. enterocolitica* strain Ye 128 (serotype O:2, O:3), being a homopolymer composed of (1 → 2)-linked 6-deoxy-β-L-altropyranosyl residue (Hoffman *et al.*, 1980), was hydrolysed completely with 0·5 M-sulphuric acid for 16 h at 100 °C. The hydrolysed material was purified by paper chromatography, and the purified material was used as standard 6-deoxy-L-altrose. A gas chromatography apparatus, GC-8A (Shimazu Seisakusho, Kyoto) was used for the analyses.

Table 1. *Bacteriophage inactivation with LPS prepared from Ye 3827 cells grown at 25 °C and 37 °C*

LPS was assayed for receptor activity as described in the text.

Phage	50% inactivation dose (μg) of LPS prepared from the cells grown at: 25 °C	37 °C
I	0·0076	>100
II	0·32	>100
III	0·01	90
IV	0·004	14
V	65	>100
VI	0·0025	29
VII	0·01	30
VIII	0·4	>100
IX	0·5	>100

Table 2. *Chemical composition of LPS and its derivatives prepared from different sources*

Except where indicated the values are expressed as percentage (dry weight) of LPS.

Material examined	Yield	Neutral sugar (%)	Protein (%)	Fatty acid (%)	Phosphorus (%)	Amino group (μmol mg^{-1})	KDO (%)
Ye 3827 cells grown at 25 °C							
LPS	2·6*	42·0	0·9	10·2	1·6	0·08	3·6
O-PS	31	72·3	ND	ND	ND	ND	ND
R-core	18	40·8	ND	ND	ND	ND	10·2
Ye 3827 cells grown at 37 °C							
LPS	1·0*	34·8	1·1	10·7	1·3	0·016	4·2
O-PS	20	79·4	ND	ND	ND	ND	ND
R-core	30	40·8	ND	ND	ND	ND	33·2
Ye 3827 IV-3 cells grown at 25 °C							
PCP-LPS	0·2*	42·5	7·0	14·0	1·7	0·19	34·4
PW-LPS	1·9*	35·8	ND	9·7	2·1	0·17	34·4

ND, Not detectable.
* Figures are in percentage of dry cells.

RESULTS

Growth temperature-dependent variation in the bacteriophage-inactivating capacity of LPS

Bacteriophages II, III, V, VI, VII, and IX adsorbed to 25 °C-bacteria but not 37 °C-bacteria, as had previously been shown for the phages I, IV, VIII, and XI (Kawaoka *et al.*, 1982*b*, *c*). The receptor(s) for phages I through IX is located on the LPS (Table 1). 25 °C-LPS inactivated phages I through IX more efficiently than did 37 °C-LPS (Table 1). 25 °C-LPS required for 50% phage inactivation of all these phages except phage V was 0·5 μg or less, whereas more than 3000 times as much of 37 °C-LPS was required for the same degree of inactivation.

A spontaneous mutant resistant to phage IV was isolated from strain Ye 3827. The resistant strain, Ye 3827 IV-3, did not adsorb phage IV nor was it agglutinable in slide tests with a 1:10 dilution of the antiserum to 25 °C-bacteria. The strain appeared to be a rough mutant. Although the PCP method was applied, the yield of Ye 3827 IV-3 LPS was very low (Table 2). Cell residue was subsequently extracted with phenol-water. The yield of LPS by the phenol-water method was similar to that of other enterobacterial LPS (Table 2). Neither LPS preparation inactivated any of the bacteriophages.

Table 3. *Sugar composition of LPS and its derivatives prepared from different sources*

The results are expressed as a molar ratio to heptose.

Material examined	6-Deoxy-L-altrose	Rhamnose	Galactose	Glucose	Heptose	Glucosamine
Ye 3827 cells grown at 25 °C						
LPS	2·20	0·28	0·21	0·84	1·00	0·26
O-PS	11·60	1·50	0·23	0·56	1·00	ND
R-core	ND	ND	0·35	0·90	1·00	ND
Ye 3827 cells grown at 37 °C						
LPS	1·24	0·13	0·25	0·79	1·00	0·26
O-PS	9·10	1·20	0·23	0·63	1·00	ND
R-core	ND	ND	0·26	0·47	1·00	ND
Ye 3827 IV-3 cells grown at 25 °C						
PCP-LPS	ND	ND	0·33	0·90	1·00	0·28
PW-LPS	ND	ND	0·26	0·49	1·00	0·30

ND, Not detectable.

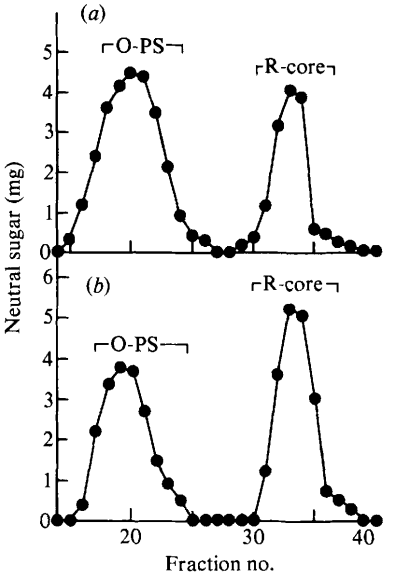


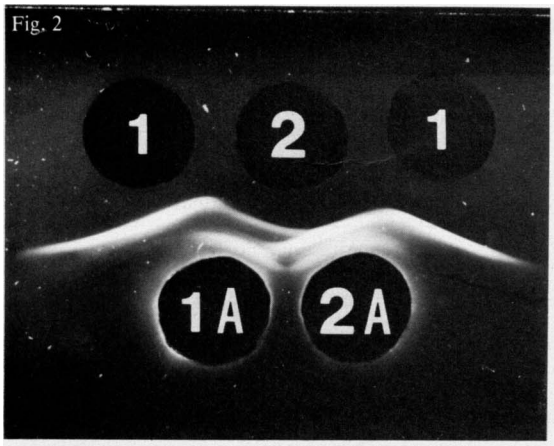
Fig. 1. Sephadex G-50 gel filtration of acetic acid-hydrolysed LPS prepared from Ye 3827 cells grown at 25 °C (*a*) or 37 °C (*b*). Each fraction was analysed for neutral sugar by the method described in the text. Blue dextran was eluted at fraction 14.

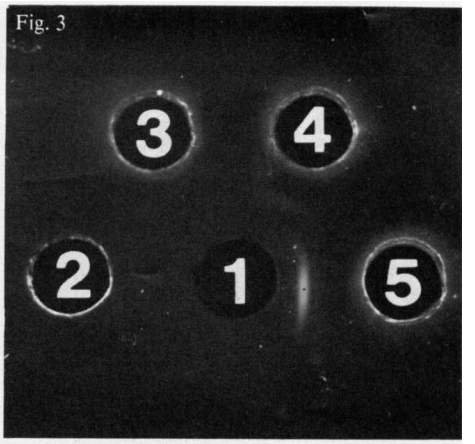
Chemical composition of LPS

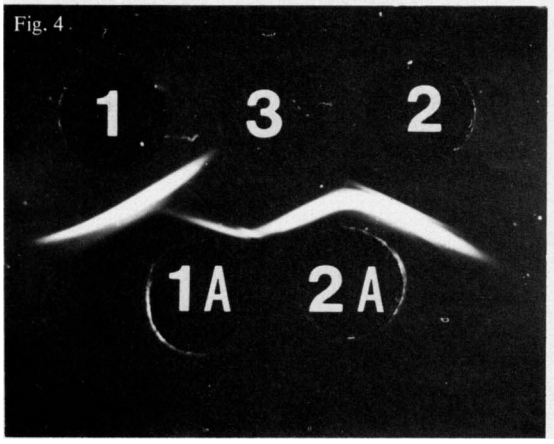
25 °C- and 37 °C-LPS were compared in their chemical properties. As reported previously with *Y. enterocolitica* O:3 LPS (Ellwood & Kirk, 1971), 6-deoxy-L-altrose was found in both (Table 3). The 2-keto-3-deoxyoctonate contents and the ratio of glucose to heptose were almost the same in each (Tables 2 and 3). However, the ratio of 6-deoxy-L-altrose to heptose in 37 °C-LPS was much lower than that in 25 °C-LPS, as reported previously (Acker *et al.*, 1980). Another difference was in the amount of free amino groups. The 25 °C-LPS molecule contained five times as many amino groups as 37 °C-LPS (Table 2).

To investigate the polysaccharide portions of LPS, we hydrolysed LPS preparations with dilute acetic acid. Hydrolysed materials prepared from 25 °C- and 37 °C-LPS were fractionated on a Sephadex G-50 column into O-PS fractions and R-core fractions (Fig. 1).

The yield of O-PS was higher than that of R-core in 25 °C-LPS and lower in 37 °C-LPS (Table 2). Sugar compositions of the O-PS preparations were very similar and consisted mainly of 6-deoxy-L-altrose.







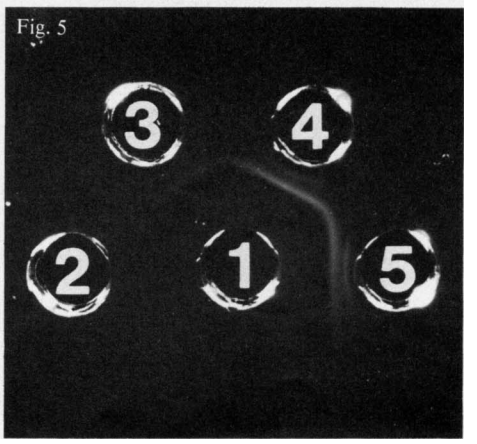


Fig. 2. Immunodiffusion in agarose gel of antiserum against Ye 3827 cells grown at 25 °C (1A) and 37 °C (2A) with LPS prepared from Ye 3827 cells grown at 25 °C (1) and 37 °C (2). Each antigen was dissolved at 3 mg ml^{-1}.

Fig. 3. Immunodiffusion inhibition test in agarose gel of antiserum against Ye 3827 cells grown at 25 °C with LPS (3 mg ml^{-1}) prepared from Ye 3827 cells grown at 25 °C (1). Antiserum (30 µl) was preincubated for 3 h at 37 °C with 3 (2), 0·3 (3), 0·03 (4), and 0 (5) mg of O-PS from the 25 °C-LPS preparation, before being added to the wells.

Fig. 4. Immunodiffusion in agarose gel of antiserum against Ye 3827 cells grown at 25 °C (1A) and 37 °C (2A) with LPS prepared from Ye 3827 cells grown at 25 °C (1) and 37 °C (2) and from Ye 3827 IV-3 cells grown at 25 °C (3) (R-LPS). Each antigen was dissolved at 3 mg ml^{-1}.

Fig. 5. Immunodiffusion inhibition test in agarose gel of antiserum against Ye 3827 cells grown at 37 °C with LPS (3 mg ml^{-1}) prepared from Ye 3827 cells grown at 37 °C (1). Antiserum (30 µl) was preincubated for 3 h at 37 °C with 100 (2), 10 (3), 1 (4), and 0 (5) µg R-LPS, before addition to the wells.

The LPS preparations from the phage-resistant strain (Ye 3827 IV-3) grown at 25 °C obtained by the different methods were somewhat different in composition. However, the most prominent difference in comparison with the parent LPS was that the preparations from the phage-resistant cells did not contain 6-deoxy-L-altrose or rhamnose (Table 3). Thus, the phage-resistant strain, Ye 3827 IV-3, was a rough mutant.

Growth temperature-dependent antigenic variation

In immunodiffusion tests, the major antigenic determinants were shared by both 25 °C- and 37 °C-LPS (but their amount was different) (Fig. 2). The major reaction between 25 °C-LPS and anti-25 °C-bacteria was inhibited by O-PS (Fig. 3) but not by R-LPS from Ye 3827 IV-3 (data

Table 4. *Passive HI tests with LPS prepared from various sources*

Passive HI tests were performed in the micro-HI system using sheep erythrocytes sensitized with LPS. The inhibitor (25 μl) was preincubated with the antiserum (25 μl) containing 4 haemagglutination units for 3 h at 37 °C, and then a 0·5% erythrocyte suspension (50 μl) was added to the reaction mixture.

Origin of LPS for coating sheep erythrocytes	Inhibitor	Amount of inhibitor necessary for complete inhibition of haemagglutination (ng) Antiserum against: Ye 3827 (25 °C)	Ye 3827 (37 °C)	Ye 3827 IV-3 (25 °C)
Ye 3827 (25 °C)	Ye 3827 (25 °C)			
	LPS	6	390	195
	O-PS	1560	—	—
	R-core	—	—	—
	Ye 3827 (37 °C)			
	LPS	12	390	390
	O-PS	1560	—	—
	R-core	—	—	—
	Ye 3827 IV-3 (25 °C)			
	LPS	—	—	97
Ye 3827 (37 °C)	Ye 3827 (25 °C)			
	LPS	97	6	97
	O-PS	—	—	—
	R-core	—	—	—
	Ye 3827 (37 °C)			
	LPS	195	24	97
	O-PS	—	—	—
	R-core	—	—	—
	Ye 3827 IV-3 (25 °C)			
	LPS	—	6250	1·5
Ye 3827 IV-3 (25 °C)	Ye 3827 (25 °C)			
	LPS	780	3125	1560
	O-PS	—	—	—
	R-core	—	—	—
	Ye 3827 (37 °C)			
	LPS	1560	6250	780
	O-PS	—	—	—
	R-core	—	—	—
	Ye 3827 IV-3 (25 °C)			
	LPS	390	390	195

—, > 12500 ng.

not shown). R-LPS gave a line against the antiserum to 25 °C-bacteria that fused with the minor line between this antiserum and 25 °C-LPS (Fig. 4). Against antiserum to 37 °C-bacteria, R-LPS gave a line that fused with the major line formed between this antiserum and 37 °C-LPS (Fig. 4). The major portion of the major reaction in 37 °C-LPS was inhibited by R-LPS (Fig. 5) but not by O-PS from either 37 °C- or 25 °C-LPS (data not shown). Both LPS preparations from R-bacteria showed the same reaction irrespective of the method they were prepared.

The antigenic variation of LPS was further examined by the HI test in the LPS/homologous or heterologous anti-bacterial serum system (Table 4). Both O-PS from the 25 °C- and 37 °C-LPS preparations inhibited only the reaction between 25 °C-LPS and the homologous antiserum with the same inhibitory activity (1·56 μg of the polysaccharide was required), indicating that the reaction between O-PS and its antibody was the predominant one in the system. This agreed with the results in the immunodiffusion inhibition test. 25 °C- and 37 °C-LPS inhibited all the reactions. R-LPS was the most potent inhibitor of all reactions between the antiserum to R-bacteria and erythrocytes coated with 25 °C- or 37 °C-LPS or with the homologous R-LPS (Table 4, the right-hand column). However, it did not inhibit the reaction between the Ye 3827

LPS preparations and their homologous or heterologous antiserum with the exception of the reaction between 37 °C-LPS and its homologous antiserum. Both R-LPS preparations again behaved similarly. R-core fractions prepared from Ye 3827 LPS preparations did not inhibit any reaction.

DISCUSSION

A growth temperature-dependent structural alteration of *Y. enterocolitica* Ye 3827 LPS was revealed by bacteriophage-inactivation assay and by immunological and chemical analyses of purified LPS and its derivatives. 37 °C-LPS contained relatively more of the R-core fraction and less of O-PS than did 25 °C-LPS (Fig. 1 and Table 2). Therefore, 25 °C- and 37 °C-LPS differed in the relative amount of LPS molecules lacking O-PS, indicating a partial transition from smooth to rough when *Y. enterocolitica* was cultivated at 37 °C. This would agree with the indirect observation by Acker *et al.* (1981) with antibody against the enterobacterial common antigen.

It is, however, difficult to explain the several thousand-fold decrease in the phage inactivation rate observed in 37 °C-LPS on the basis of only partial smooth-rough transition. The tremendous drop in phage sensitivity suggests that a qualitative change took place in LPS. Such qualitative change would most probably be located on the O-PS portion, because a rough mutant and its LPS were totally devoid of bacteriophage-inactivating capacity. However, O-PS prepared from 25 °C- and 37 °C-LPS by hydrolysis with dilute acetic acid were similar in their inhibitory activity in the HI tests (Table 4), in their composition (Tables 2 and 3), and in the amount of periodate consumed in periodate oxidation (unpublished data). However, the drastic decrease in the HI potency when comparing O-PS to the homologous LPS (Table 4), suggests that acid-labile determinant(s), destroyed during hydrolysis with acetic acid, may be located on the O-PS portion. As alkaline treatment somewhat altered the reactivity of 25 °C-LPS in the immunodiffusion test (unpublished observation), the determinant might be a substituent such as an O-acetyl group. Therefore, acid-labile determinant(s) might be involved in the qualitative change in LPS responsible for the change in bacteriophage sensitivity.

The antigenic determinant(s) responsible for the major precipitin line between 25 °C-LPS and anti-25 °C-bacteria seems to be located on the O-PS portion since (i) this line was not found with R-LPS (Fig. 4), (ii) 37 °C-LPS formed a weaker but identical precipitation line (Fig. 2), and (iii) O-PS inhibited the reaction (Fig. 3). On the other hand, the determinant(s) responsible for the major portion of the major line between 37 °C-LPS and anti-37 °C-bacteria seems to be located on R-LPS also, since the precipitation line formed by R-LPS against anti-37 °C-bacteria fused with the major line against 37 °C-LPS (Fig. 4) and R-LPS inhibited the major reaction (Fig. 5). However, R-core fractions obtained by mild acid hydrolysis did not show inhibitory activity in HI tests (Table 4) or in immunodiffusion-inhibition tests (data not shown). The reason is uncertain. Acid-labile determinant(s) such as phosphate might be involved, since alkaline treatment altered the reactivities of R- and 37 °C-LPS in the immunodiffusion tests (unpublished observation).

Immunogenicity of *Y. enterocolitica* cells differed depending on whether the cells were grown at 25 °C or at 37 °C. The immunodiffusion and HI tests indicate that the rabbit antiserum against 37 °C-bacteria contained mainly antibody against R-core or inner portion of LPS. The poor anti-O response in rabbits immunized with 37 °C-bacteria can not be explained by the structural difference of isolated LPS. The reduced amount of O-PS in 37 °C-LPS seems not to be a sufficient explanation, because usually O-PS is a better immunogen than R-core. The different immune response might be due to other factors rather than the structural difference in LPS, such as covering up of LPS by capsular-like material.

Structural heterogeneity of LPS is present in smooth Gram-negative bacteria under normal culture conditions (Goldman & Leive, 1980; Jann *et al.*, 1975; McIntire *et al.*, 1969; Nowotny, 1966). The culture condition also affects the structure (Fromme & Schlecht, 1973; Nikaido, 1970). McConnell & Wright (1979) showed that the sensitivity of *Salmonella anatum* A1 cells to the rough-specific bacteriophage Felix O-1 increased at low growth temperature (20 to 25 °C) due to partial smooth-rough transition occurring at lower temperatures. In *S. anatum*, the

proportion of LPS molecules substituted with O-PS is larger at 37 °C than at 25 °C; in *Y. enterocolitica*, the reverse is the case. However, the extent of the structural change, as indicated by chemical analyses, was much higher in *Y. enterocolitica* than in *S. anatum*. Furthermore, a qualitative change in the O-PS portion was suggested by bacteriophage-inactivation assay.

Attempts to elucidate the mechanism of temperature-dependent changes have been made mainly in *Y. pestis* (Darveau *et al.*, 1980; Straley & Brubaker, 1981, 1982; Zahorchak *et al.*, 1979). *Yersinia pestis* requires 2·5 mM-Ca^{2+} for growth at 37 °C but not at 25 °C. Zahorchak *et al.* (1979) showed that a shift of cultivation temperature from 26 °C to 37 °C in a Ca^{2+}-deficient medium caused termination of net RNA synthesis prior to that of protein synthesis in *Y. pestis*. As distinct from *Y. pestis*, *Y. enterocolitica* can grow at 37 °C, although at lower growth rate than at 25 °C. Therefore, termination of net RNA synthesis observed in *Y. pestis* can not explain the temperature-dependent features in *Y. enterocolitica*. In *Y. enterocolitica*, Portnoy *et al.* (1981) reported that three major outer membrane polypeptides appeared during growth at 37 °C but not at 25 °C and the addition of 2·5 mM-Ca^{2+} to the growth medium completely repressed the expression of these polypeptides. By contrast, the partial smooth-rough transition described here occurred at 37 °C regardless of the addition of Ca^{2+} (unpublished observation). Therefore, there seem to be at least two kinds of temperature dependency; one is related to Ca^{2+} and the other not.

We thank Dr H. H. Mollaret for providing the bacteriophages and the indicator cell. This work was partly supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

REFERENCES

ACKER, G., WARTENBERG, K. & KNAPP, W. (1980). Zuckerzusammensetzung des Lipopolysaccharids und Feinstruktur der äusseren Membran (Zellwand) bei *Yersinia enterocolitica*. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene (Abteilung I, Originale A)* **247**, 229–240.

ACKER, G., KNAPP, W., WARTENBERG, K. & MAYER, H. (1981). Localization of enterobacterial common antigen in *Yersinia enterocolitica* by the immunoferritin technique. *Journal of Bacteriology* **147**, 602–611.

AMES, B. N. & DOBIN, D. T. (1960). The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid. *Journal of Biological Chemistry* **235**, 769–775.

DARVEAU, R. P., CHARNETZKY, W. T. & HURLBERT, R. E. (1980). Outer membrane protein composition of *Yersinia pestis* at different growth stages and incubation temperatures. *Journal of Bacteriology* **143**, 942–949.

DAVIES, D. A. L., MORGAN, W. T. J. & RECORD, B. R. (1955). Studies in immunochemistry. 15. The specific polysaccharide of the dominant 'O' somatic antigen of *Shigella dysenteriae*. *Biochemical Journal* **60**, 290–303.

DUBOIS, C. S., GILLES, K. A., HAMILTON, J. K., REBERS, P. A. & SMITH, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* **38**, 350–356.

ELLWOOD, D. C. & KIRK, G. R. A. (1971). The occurrence of 6-deoxy-L-altrose in the lipopolysaccharide of *Yersinia enterocolitica*. *Biochemical Journal* **122**, 14.

FROMME, I. & SCHLECHT, S. (1973). Influence of aeration conditions in fermentation cultures on the chemical composition of *Salmonella* lipopolysaccharides. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene (Abteilung I, Originale A)* **224**, 331–344.

GALANOS, C., LUDERRITZ, O. & WESTPHAL, O. (1969). A new method for the extraction of R lipopolysaccharides. *European Journal of Biochemistry* **9**, 245–249.

GOLDFARB, A. R. (1966). A kinetic study of reactions of amino acids and peptides with the TNBS. *Biochemistry* **5**, 2570–2577.

GOLDMAN, R. C. & LEIVE, L. (1980). Heterogeneity of antigenic side chain length in lipopolysaccharide from *Escherichia coli* O111 and *Salmonella typhimurium* LT2. *European Journal of Biochemistry* **107**, 145–153.

HOFFMAN, J., LINDBERG, B. & BRUBAKER, R. R. (1980). Structural studies of the O-specific side-chains of the lipopolysaccharide from *Yersinia enterocolitica* Ye 128. *Carbohydrate Research* **78**, 212–214.

JANN, B., RESKE, K. & JANN, K. (1975). Heterogeneity of lipopolysaccharides. Analysis of polysaccharide chain lengths by sodium dodecylsulfate-polyacrylamide gel electrophoresis. *European Journal of Biochemistry* **60**, 239–246.

KAWAOKA, Y., NAIKI, M. & YANAGAWA, R. (1979). Radioimmunoassay system using a serovar-specific lipopolysaccharide antigen of *Leptospira*. *Journal of Clinical Microbiology* **10**, 313–316.

KAWAOKA, Y., NAIKI, M. & YANAGAWA, R. (1982*a*). Isolation of the antigen-active components from leptospiral serovar-specific lipopolysaccharide antigen by mild acid hydrolysis. *Japanese Journal of Veterinary Science* **44**, 473–478.

KAWAOKA, Y., OTSUKI, K. & TSUBOKURA, M. (1982*b*). Characteristics of *Yersinia enterocolitica* bacterio-

phages. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene (Abteilung I, Originale A)* **253**, 102–109.

KAWAOKA, Y., OTSUKI, K. & TSUBOKURA, M. (1982*c*). Temperature-dependent variation in the synthesis of the receptor for *Yersinia enterocolitica* bacteriophage XI. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene (Abteilung I, Originale A)* **253**, 364–369.

LINDBERG, A. A. & HELLERQVIST, C. (1980). Rough mutants of *Salmonella typhimurium*: immunochemical and structural analysis of lipopolysaccharides from *rfaH* mutants. *Journal of General Microbiology* **116**, 25–32.

MARUYAMA, T. (1973). Studies on biological characteristics and pathogenicity of *Yersinia enterocolitica*. I. Comparison of growth temperature range between *Y. enterocolitica* and other enteropathogenic bacteria. *Japanese Journal of Bacteriology* (in Japanese) **28**, 343–348.

MCCONNELL, N. & WRIGHT, A. (1979). Variation in the structure and bacteriophage-inactivating capacity of *Salmonella anatum* lipopolysaccharide as a function of growth temperature. *Journal of Bacteriology* **137**, 746–751.

MCINTIRE, F. C., BARLOW, G. G., SIEVERT, H. W., FINLEY, R. A. & YOO, A. L. (1969). Studies on a lipopolysaccharide from *Escherichia coli*. Heterogeneity and mechanism of reversible inactivation by sodium deoxycholate. *Biochemistry* **8**, 4063–4067.

MOLLARET, H. H. & NICOLLE, P. (1965). Sur la frequence de la lysogenie dans l'espece nouvelle *Yersinia enterocolitica*. *Comptes rendus hebdomadaires des séances de l'Academie des sciences* **260**, 1027–1029.

NIKAIDO, H. (1970). Structure of cell wall lipopolysaccharide from *Salmonella typhimurium*. Further studies on the linkage between O-side chains and R-core. *European Journal of Biochemistry* **15**, 57–62.

NOWOTNY, A. (1966). Heterogeneity of endotoxic bacterial lipopolysaccharides revealed by ion-exchange column chromatography. *Nature, London* **210**, 278–280.

OKAMOTO, K., INOUE, T., ICHIKAWA, H., KAMAMOTO, Y., HARA, S. & MIYAMA, A. (1980). Adherence of *Yersinia enterocolitica* to mammalian epithelial cell lines. *Microbiology and Immunology* **24**, 1013–1022.

PAI, C. H. & MORS, V. (1978). Production of enterotoxin by *Yersinia enterocolitica*. *Infection and Immunity* **19**, 908–911.

PORTNOY, D. A., MOSELEY, S. L. & FALKOW, S. (1981). Characterization of plasmid and plasmid associated determinants of *Yersinia enterocolitica* pathogenesis. *Infection and Immunity* **31**, 775–782.

SCHLEIFSTEIN, J. & COLEMAN, M. B. (1939). An identified microorganism resembling *B. ligieresi* and *Pasteurella pseudotuberculosis*, pathogenic for man. *New York State Journal of Medicine* **39**, 1749–1753.

STRALEY, S. C. & BRUBAKER, R. R. (1981). Cytoplasmic and membrane proteins of yersiniae cultivated under conditions simulating mammalian intracellular environment. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 1224–1228.

STRALEY, S. C. & BRUBAKER, R. R. (1982). Localization in *Yersinia pestis* of peptides associated with virulence. *Infection and Immunity* **36**, 129–135.

TSUBOKURA, M., OTSUKI, K., SHIMOHIRA, I. & YAMAMOTO, H. (1979). Production of indirect hemolysin by *Yersinia enterocolitica* and its properties. *Infection and Immunity* **25**, 939–942.

TSUJI, M., KAWAOKA, Y., NAIKI, M. & YANAGAWA, R. (1981). Isolation of antigenically active components from leptospiral serovar-specific lipopolysaccharide antigen by alkaline treatment. *Microbiology and Immunology* **25**, 949–957.

WEISSBACH, A. & HURWITZ, J. (1959). The formation of 2-keto-3-deoxyheptonic acid in extracts of *Escherichia coli* B. I. Identification. *Journal of Biological Chemistry* **234**, 705–709.

WESTPHAL, O. & JANN, K. (1965). Bacterial lipopolysaccharide extraction with phenol-water and further application of the procedure. *Methods in Carbohydrate Chemistry* **5**, 83–91.

ZAHORCHAK, R. J., CHARNETZKY, W. T., LITTLE, R. V. & BRUBAKER, R. R. (1979). Consequences of Ca^{2+} deficiency on macromolecular synthesis and adenylate energy charge in *Yersinia pestis*. *Journal of Bacteriology* **139**, 792–799.