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Identification of Distinct Lipopolysaccharide Patterns among *Yersinia enterocolitica* and *Y. enterocolitica*-Like Bacteria

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Abstract—The lipopolysaccharide (LPS) of strains representing various serotypes of *Yersinia enterocolitica* and *Y. enterocolitica*-like bacteria was studied by deoxycholate-PAGE and silver staining analysis. Four main types of LPS were detected based on the O-polysaccharide (O-PS): (i) LPS with homopolymeric O-PS, (ii) LPS with ladder-forming heteropolymeric O-PS, (iii) LPS with single-length O-PS, and (iv) semi-rough LPS without O-PS. Within the first three types, several subvariants were detected. Selected serotypes representing all above LPS types are sensitive to bacteriophage ϕR1-37 indicating that they share the phage receptor, a hexasaccharide called outer core in *Y. enterocolitica* O:3. Whereas phage ϕR1-37-resistant mutants of homopolymeric O-PS have lost only the outer core, those of ladder-forming or single-length O-PS have lost also the O-PS suggesting that in the latter ones the outer core is bridging between O-PS and lipid A–core. This work forms a basis of further structural, biochemical and genetic studies of these LPSs.

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The genus *Yersinia* consists of 17 species of which *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* are human pathogens. *Yersinia pestis* causes bubonic and pneumonic plague while *Y. pseudotuberculosis* and *Y. enterocolitica* cause mostly food-borne yersiniosis, usually a diarrheal disease sometimes followed by post-infectious reactive arthritis [1]. The pathogenic potential of these bacteria resides on many essential virulence factors, some of which are encoded by genes located on a 70 kb virulence plasmid of *Yersinia* and others by chromosomal loci [2].

Yersinia enterocolitica and related species have been classified into more than 70 O-serotypes [3]. Human and animal pathogenic strains of *Y. enterocolitica* that carry the virulence plasmid, pYV, belong to certain serotypes, e.g. O:3 and O:9 that are mainly isolated in Scandinavia and Europe, Canada, Japan, and South Africa; and O:8 mainly isolated in the United States. During the last decade, serotype O:3 strains have been isolated also in the United States, and O:8 strains in Europe and Japan [4-6]. Less frequently encountered pathogenic serotypes are O:4,32, O:5,27, O:13a,18, and O:21. Certain *Y. enterocolitica* serotypes do not carry pYV, and these are collectively referred to as non-pathogenic or environmental serotypes. In some cases, however, infection with these strains may cause symptoms. The virulence of the pathogenic serotypes varies; for example, serotype O:8 strains are more virulent than O:3 or O:9 strains [7, 8]. This is most evident in mouse lethality models. Serotype O:9 O-antigen cross-reacts with *Brucella abortus* O-antigen, causing diagnostic problems in serological confirmation of *B. abortus* infections [9-11].

Lipopolysaccharide (LPS), also known as endotoxin, is the major component of the outer membrane of Gram-negative bacteria and a surface structure encountering the surrounding environment. LPS has three main structural components: lipid A; core, a complex oligosaccharide containing up to 15 sugar residues, further divided into inner core (IC) and outer core (OC); and O-polysaccharide (O-PS) chain composed of repeats (O-units) of 1-8 sugar residues. O-PS is highly variable and is the basis of O-serotyping of Gram-negative species (O-antigen). In addition to O-PS, also enterobacterial common antigen can be linked to LPS [12]. The genes directing the

Abbreviations: DOC, deoxycholate; IC, inner core; LPS, lipopolysaccharide; OC, outer core; O-PS, O-polysaccharide.

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biosynthesis of the three structural components of LPS are in operons that usually form clusters mapping to different parts of the bacterial chromosome.

LPS is a heterogenous population of molecules. This is clearly seen when isolated LPS is analyzed by PAGE; a wide range of LPS molecules of different sizes are present [13-15]. The smallest LPS molecules are composed of lipid A and (inner) core moieties, somewhat larger molecules have lipid A and complete core structure, and the largest ones contain lipid A, complete core, and variable numbers of O-PS repeat units. Therefore, these LPS populations appear as a ladder in the stained gel. If the repeat unit is a single sugar, the O-PS is called homopolymeric, and if it is composed of two or more different sugar residues the O-PS is called heteropolymeric.

Biosynthesis of LPS takes place via two converging pathways [16, 17]. In the lipid A–core pathway, lipid A is synthesized on the cytoplasmic leaflet of the inner membrane and the core sugar residues are sequentially transferred on it by specific glycosyltransferases. The completed lipid A–core is translocated to face the periplasmic side of the inner membrane. In the O-PS pathway, both the homo- and heteropolymeric O-PS are synthesized onto a lipid carrier molecule, undecaprenyl phosphate (Und-P). The homopolymeric O-PS synthesized onto Und-P is completed to full length in the cytoplasm, after which it is translocated to the periplasmic space by Wzm and Wzt, an ATP binding cassette transporter system. In contrast, for the heteropolymeric O-PS, the O-units are synthesized on Und-P, a complete O-unit is flipped by Wzx-protein to the periplasmic face of the inner membrane where Wzy (O-antigen polymerase) and Wzz (O-antigen chain length determiner) act in concert to produce O-PS chains with the desired number of oligosaccharide repeats [16, 17]. Both synthesis pathways converge when the O-PS is transferred from the Und-P by the WaaL protein (O-PS ligase) to the lipid A–core molecule [18]. The complete LPS molecule is then translocated onto the outer membrane by a recently resolved mechanism [17].

The LPS of *Y. pestis* and *Y. pseudotuberculosis* has been well characterized in terms of structure and genetics [19-25]. Fewer data have been presented for *Y. enterocolitica* and related species [25-28]. Best known are *Y. enterocolitica* serotypes O:3, O:8, and O:9, and structural data are available also for some other strains. In this work we have characterized the LPS patterns of a collection of *Y. enterocolitica* and related species strains to get an understanding of the kinds of LPS present among these *Yersinia*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in LPS analysis are listed in Table 1. The bacteria were routinely grown on Luria agar plates at 22°C. For LPS analysis, the bacteria were grown in 15-ml Falcon tubes for 14-16 h shaking in 2 ml of Luria broth at 22°C.

Deoxycholate-PAGE analysis of LPS. LPS samples were prepared by the small scale proteinase K method from the different *Y. enterocolitica* bacteria as described earlier [29]. Briefly, the OD_{600} of the 14-16-h cultures was determined, bacteria were pelleted by centrifugation, and the pellet was resuspended in deoxycholate lysis buffer (2% deoxycholate (DOC), 4% 2-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue in 1 M Tris-HCl buffer, pH 6.8) in a volume adjusted according to density of the culture (i.e. 100 µl/OD_{600} = 1). The suspension was heated to 100°C for 10 min, and then 2-4 µl of proteinase K (20 mg/ml) was added and the suspension was incubated 14-16 h at 60°C. An aliquot of 10 µl was loaded on the gel and analyzed in 12% DOC-PAGE, and the LPS bands were visualized by silver staining as described earlier [30].

RESULTS AND DISCUSSION

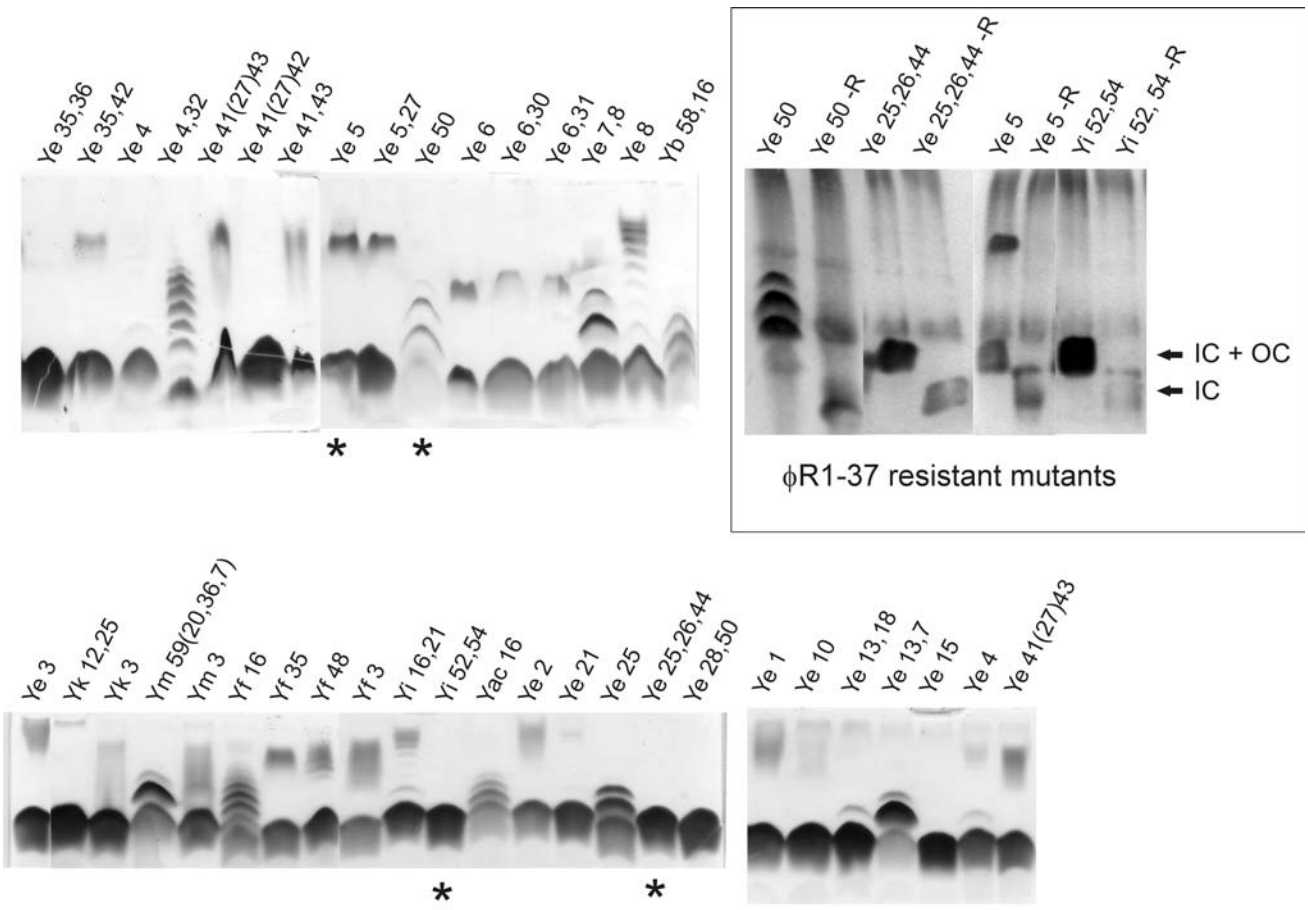
To characterize the LPS diversity within the genus *Yersinia* among *Y. enterocolitica* and closely related species, we used DOC-PAGE and silver staining in analyzing the LPS patterns of a set of 45 strains representing different serotypes of altogether seven species (Table 1). The LPS gave a distinct broad core-band in the low molecular weight region of the gel. The core band position varied a little and is indicated in Table 2 from –2 to 1 (0 indicating mean level and minus below it). The most distinct variation between the strains was seen in the O-PS containing molecules that migrated in the higher molecular weight region of the gel (figure). Based on O-PS differences, we classified the LPSs into four main types: homopolymeric O-PS, heteropolymeric O-PS, single-length O-PS, and no O-PS (semi-rough) (Table 2). Within the three first types, several subvariants were detected.

Homopolymeric O-PS. Altogether 10 strains expressed a clear homopolymeric O-PS as demonstrated by a smear in the high molecular weight region of the gel (figure). The apparent length of the homopolymeric O-PS varied between the strains and allowed division of the O-PSs into short, medium, and long homopolymers (Table 2 and figure). Interestingly, the O:3 serotype has been detected in four *Yersinia* species [31]. The *Y. enterocolitica* O:3 O-PS structure has been elucidated, and it is a homopolymer of β(1→2)-linked 6-deoxy-L-altrose [32, 33] that also functions as the phage ϕYeO3-12 receptor. The serologically identical O:3 strains of *Y. frederiksenii*, *Y. kristensenii*, and *Y. mollaretii* are all sensitive to ϕYeO3-12 [31]. In the DOC-PAGE analysis, they expressed short homopolymeric O-PS, in contrast to the long homopolymer of *Y. enterocolitica* O:3 (compare Ye 3, Yk 3, Ym 3,

and Yf 3; figure). Short homopolymers were also expressed by serotype O:41 strains. Medium length homopolymers were expressed by *Y. enterocolitica* O:9 and O:10 strains (for Ye 10, see figure).

Heteropolymeric O-PS. A majority of strains expressed different types of heteropolymeric ladder-like O-PS. We describe the ladders here by N/X/Z designations where N is the number of steps in the ladder over the core band (if there is an additional weaker band on top it is indicated by +w), X is the distance between steps (S, short; M, medium; L, long), and Z is the modality (numbers indicate which steps in the ladder are strongest; random indicates that the ladder has no modality). Thus, the *Y. enterocolitica* O:7,8 (Ye 7,8; figure) expresses a 2/L/1 ladder (with three well-separated bands, i.e. two steps, of which step 1 is strongest) and *Y. enterocolitica* O:4,32 (Ye 4,32) a 7+w/M/3-6 ladder (with 7+weak medium-separated steps of which steps 3-6 were strongest). The >5/M/7-10 ladders were most frequent, but half of the strains expressed very short ladders with 2-7 steps only. Furthermore, one strain (*Y. frederiksenii* O:16 3400/83) expressed a mixed hetero- and homopolymeric O-PS. While the >5/M/7-10 ladder biosynthesis most likely follows the Wzy-dependent pathway including the Wzz-dependent modality in O-PS chain lengths, there are no structures reported and likewise nothing is known on the genetic basis of the very short, 2-7 step ladders, and it should therefore be further studied.

Single-length O-PS. Interestingly, the *Y. enterocolitica* serotypes O:5 and O:6 expressed a single-length O-PS (figure and Table 2). This phenomenon resembles that reported for bacteria possessing S-layers [34]. To our knowledge, the presence of a single-length O-PS has not been reported earlier for *Yersinia* or the *Y. enterocolitica* O:5 and O:6 serotypes even though chemical structures have been solved for both. For serotypes O:5 and O:5,27, a tetra- or pentasaccharide O-unit structure has been reported consisting of a linear backbone of two (or three) L-rhamnose residues with two β(2→2)-linked branching D-xylulose residues [35, 36]. In our DOC-PAGE analy-



LPS analysis of *Y. enterocolitica* and related species by DOC-PAGE and silver staining. The source of the LPS samples is indicated above the lanes by abbreviated species name and O-serotype number. The species names are abbreviated as follows: *Yac*, *Y. aleksiciae*; *Yb*, *Y. bercovieri*; *Ye*, *Y. enterocolitica*; *Yf*, *Y. frederiksenii*; *Yi*, *Y. intermedia*; *Yk*, *Y. kristensenii*; and *Ym*, *Y. mollaretii*. Asterisks below some lanes indicate serotypes for which the LPS of phage ϕR1-37 resistant derivatives is shown in the framed panel at right. The position of the IC and IC + OC bands are indicated by arrows

Table 1. Bacterial strains used in this work

Species	O-Serotype	Strain code	Isolation country	Isolation source	Reference
1	2	3	4	5	6
Y. aleksiciae	16	404/81	Finland	human stool	Skurnik lab strain collection
Y. bercovieri	58,16	3016/84	–"–	–"–	[42]
Y. enterocolitica	1	132	Netherlands	chinchilla	Institut Pasteur, H. H. Mollaret
–"–	1,2,3	E766	–"–	–"–	[43]
–"–	2	2943	Norway	goat	[44, 45]
–"–	3	6471/76	Finland	stool	[46]
–"–	4	3973-76	Wisconsin, USA	–"–	[47]
–"–	4,32	E701	North America	–"–	[43, 48]
–"–	5	477/78	Finland	–"–	[49]
–"–	5	14779/83	–"–	–"–	[38]
–"–	5	14779/83-ϕR1-37-R	–"–	–"–	[38]
–"–	5,27	gk7500	–"–	coypu	[50]
–"–	6	8841/84	–"–	human stool	[51]
–"–	6,30	769/84	–"–	–"–	[51]
–"–	6,31	155/84	–"–	–"–	[51]
–"–	7,8	1308/83	–"–	–"–	[51]
–"–	8	MCH314	Canada	–"–	[52]
–"–	9	Ruokola/71	Finland	stool	[46]
–"–	10	3788/80	–"–	human stool	[46, 49]
–"–	13	1209-79	North Carolina, USA	blood	[47]
–"–	13a,13b	5074	Canada	–"–	[53]
–"–	13,7	H79B	Norway	healthy carrier	G. Kapperud
–"–	13,18	9312-78	Georgia, USA	stool	[47]
–"–	14	15712/83	Finland	human stool	[51]
–"–	15	IP 614	–"–	–"–	[54]
–"–	20	874-77	New Jersey, USA	stool	[47]
–"–	21	E736	North America	–"–	[43, 48]

Table 1. (Contd.)

1	2	3	4	5	6
Y. enterocolitica	25	431/84	Finland	human stool	Skurnik lab strain collection
–"–	25,26,44	18425/83	–"–	–"–	[51]
–"–	25,26,44	18425/83-ϕR1-37-R	–"–	–"–	[38]
–"–	28,50	5186/84	–"–	–"–	[51]
–"–	34	2139-72	Georgia, USA	–"–	[47]
–"–	35,36	7104/83	Finland	–"–	[51]
–"–	35,52	248/84	–"–	–"–	[51]
–"–	41(27),42	626/83	–"–	–"–	[51]
–"–	41(27),43	647/83	–"–	–"–	[51]
–"–	41,43	264/85	–"–	–"–	Skurnik lab strain collection
–"–	50	3229	–"–	–"–	[51]
–"–	50	3229-ϕR1-37-R	–"–	–"–	[38]
Y. frederiksenii	3	IP 23047	France	human	[31], Institut Pasteur, E. Carniel
–"–	16	3400/83	Finland	human stool	[51]
–"–	35	3317/84	–"–	–"–	[51]
–"–	48	38/83	–"–	–"–	[51]
Y. intermedia	16,21	9/85	–"–	–"–	Skurnik lab strain collection
–"–	52,54	821/84	–"–	–"–	[51]
–"–	52,54	821/84-ϕR1-37-R	–"–	–"–	[38]
Y. kristensenii	3	IP 22828	France	human	[31], Institut Pasteur, E. Carniel
–"–	12,25	119/84	Finland	human stool	[51]
Y. mollaretii	3	IP 22404	France	bovine	[31], Institut Pasteur, E. Carniel
–"–	59(20,36,7)	92/84	Finland	human stool	[42]

Table 2. DOC-PAGE/silver staining patterns of LPS of *Y. enterocolitica* and *Y. enterocolitica*-like strains

Species	Serotype	Strain	Core relative position	Phage ϕR1-37	O-PS
1	2	3	4	5	6
Homopolymeric O-PS					range of smear
Y. enterocolitica	41(27),43	647/83	0	S and R	short
–"–	41,43	264/85	0	S	–"–
Y. frederiksenii	3	IP 23047	–1	nt	–"–
Y. kristensenii	3	IP 22828	0	nt	–"–
Y. mollaretii	3	IP 22404	0	nt	–"–
Y. enterocolitica	9	Ruokola/71	0	S	medium
–"–	10	3788/80	0	R	–"–
–"–	1	132	0	S	long
–"–	2	2943	0	S	–"–
–"–	3	6471/76	0	S	–"–
Heteropolymeric O-PS					steps in ladder/ step distance/ modality
Y. enterocolitica	13,18	9312-78	0	R	1w
–"–	7,8	1308/83	0	R	2/L/1
–"–	13,7	H79B	0	R	2/L/1
Y. mollaretii	59(20,36,7)	92/84	1	R	2/L/1
Y. bercovieri	58,16	3016/84	–2	R	2 + w/M/1
Y. enterocolitica	25	431/84	–2	R	2 + w/M/1
–"–	50	3229	0	S	2 + w/L/1–2
Y. aleksiciae	16	404/81	0	nt	2 + w/S/random
Y. enterocolitica	13	1209-79	0	R	3/L/random
–"–	13a,13b	5074	0	R	3/L/random
–"–	34	2139-72	0	R	4 + w/M/2–4
–"–	20	874-77	0	R	4 + w/M/2–4
–"–	14	15712/83	0	R	5 + w/M/2–4
Y. frederiksenii	16	3400/83	–1	R	6 + w/M/2–4 plus O-PS*
Y. enterocolitica	1,2,3	E766	–2	R	7 + w/M/3–6

Table 2. (Contd.)

1	2	3	4	5	6
Y. enterocolitica	4,32	E701	–2	R	7 + w/M/3–6
–"–	21	E736	0	R	>5/M/7–10
–"–	35,52	248/84	0	R	>5/M/7–10
Y. frederiksenii	35	3317/84	0	R	>5/M/7–10
–"–	48	38/83	0	R	>5/M/7–10
Y. intermedia	16,21	9/85	0	R	>5/M/7–10
Y. enterocolitica	4	3973-76	0	R	>5/M/7–10
–"–	8	MCH314	0	R	>5/M/7–10
Y. kristensenii	12,25	119/84	0	R	very long?
Single-length O-PS					approximate number of residues
Y. enterocolitica	6	8841/84	0	S	15-mer
–"–	6,30	769/84	0		15-mer
–"–	6,31	155/84	0	S	15-mer
–"–	5	477/78	0	S	30-mer
–"–	5,27	gk7500	0	S	30-mer
Semi-rough LPS					
Y. enterocolitica	15	IP 614	0	nt	rough
–"–	25,26,44	18425/83	0	S	–"–
–"–	28,50	5186/84	0	R	–"–
–"–	35,36	7104/83	0	R	–"–
–"–	41(27),42	626/83	0	R	–"–
Y. intermedia	52,54	821/84	0	S	–"–

* A medium-length homopolymeric O-PS was detected above the heteropolymeric O-PS step ladder.

sis, both O:5 and O:5,27 demonstrated a single-length O-PS. The O-PS band migrated at ca. 30-sugar residue position when compared to the pentasaccharide ladder of serotype O:8, suggesting that the O-PS of O:5 and O:5,27 would consist of six pentasaccharide repeats.

Similar but shorter (ca. 15 sugar residues long) single-length O-PS was present in *Y. enterocolitica* serotype O:6, O:6,30 and O:6,31 strains. A →2)-galactose-(β1→3)-6-deoxygulose-(α1→ disaccharide O-unit structure has been reported for serotype O:6,31 [37]. Whether the single-length O-PS in O:5 and O:6 strains is related to S-layers remains to be elucidated.

Presence of phage ϕR1-37 receptor. Phage ϕR1-37 uses the OC hexasaccharide of *Y. enterocolitica* O:3 as the receptor [30, 38-40]. The phage infects also strains of *Y. enterocolitica* serotypes O:2, O:5, O:6, O:6,31, O:9, O:25,26,44, O:41(27)43, O:41,43, and O:50, *Y. intermedia* O:52,54, and *Y. pseudotuberculosis* O:9 [30, 38] indicating that the phage receptor is also present in those strains. LPS analysis of four phage ϕR1-37 resistant

derivatives (Table 1 and figure) gave interesting results. The OC of serotype O:3 has been extensively studied [39, 40]. In DOC-PAGE analysis, due to the loss of the hexasaccharide OC in serotype O:3 and O:9 LPS, the strong and broad fast-migrating LPS band (marked as IC + OC, framed panel in figure) shifts to a faster-migrating band (marked as IC) [30, 41]. A similar shift is also apparent for *Y. enterocolitica* O:25,26,44 and *Y. intermedia* O:52,54 phage resistant mutants (figure). The situation is different for *Y. enterocolitica* O:5 and O:50 ϕR1-37 resistant mutants. While the O:5 parental strain expresses a IC + OC band and a single-length O-PS, the phage resistant mutant has lost both the OC and the O-PS (compare Ye 5 and Ye 5-R; figure). A plausible explanation for such a phenotype is that the O-PS of O:5 would be linked to the OC. This is different from the organization of LPS in serotypes O:3 and O:9 where O-PS is linked to IC [26].

On the other hand, serotype O:50 LPS has a 2+w/L/1-2 O-PS ladder. The phage-resistant strain has lost the weak IC+OC band and gained an IC band. At the same time it has also lost the O-PS ladder bands. Thus, similar to O:5, the O:50 O-PS could be linked via OC to IC. Structural and genetic studies are warranted.

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