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O-Antigen Specificities of the Serotype Strains of *Serratia marcescens*

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O antigens of the 24 O-serotype strains of *Serratia marcescens* were investigated in dot enzyme immunoassay with whole-cell antigens and by immunoblotting with lipopolysaccharide (LPS) antigens. Three pairs of strains, O2/O3, O6/O7, and O12/O14, had indistinguishable LPS antigens, despite having distinct specificities in agglutination tests with whole-cell antigens. Strong cross-reactions were also found in LPS antigens from strains O9/O15, O17/O19, O10/O22, and O16/O20. No high-molecular-weight LPS corresponding to O-side-chain material was detected in strain O11 or O13. A panel of absorbed antisera was prepared to facilitate the detection of a reduced set of LPS antigens in a dot enzyme immunoassay. We conclude that there are discrepancies between the existing serotypes as defined by agglutination tests and the antigenic composition of LPS antigens extracted from the serotype strains and that surface antigens other than LPS make a major contribution to the definition of serotype in the species.

Systematic analysis of the antigenic structure of *Serratia marcescens* began in 1957 with a study of the O antigens by Davis and Woodward (4). O and H antigens have continued to be identified and characterized, and the present system consists of 24 O antigens and 26 H antigens (5, 11–13, 18, 19). Both are useful as epidemiological markers, but serotyping clinical strains can be complicated by the extensive cross-reactions found in agglutination tests. H antigens may be detected by a more specific immobilization test in semisolid agar, but this is a relatively time-consuming technique. Instead, we have relied on the O antigen as the primary epidemiological marker, and phage typing as a secondary system, to characterize clinical isolates (17).

In our hands, many clinical isolates are agglutinated by O14 antiserum and give a variety of cross-reactions with O1, O6, and O12 antisera. The cross-reactions between O6 and O14 are so extensive that it could be questioned whether it is worthwhile distinguishing them in epidemiological investigations (7). In a recent study, we found that lipopolysaccharides (LPS) from clinical strains agglutinated by O14 antiserum exhibited a number of distinct profiles in polyacrylamide gel electrophoresis (PAGE) (6). When tested with O antisera in immunoblotting, only one of these LPS profiles gave a homologous reaction with O14 O antiserum; the other LPS profiles corresponded to O6, O8, and O21 antigens. We concluded that a proportion of O antigens were not correctly identified by the agglutination test, possibly due to a masking surface antigen(s). Therefore, there was a need for a more specific serotyping technique.

Preliminary experiments showed that a dot enzyme immunoassay (dot EIA) could identify the correct LPS O antigen in clinical strains. To establish a set of O-antigen-specific antisera for this assay, we compared the reactions of antisera raised towards the established 24 O-serotype strains in dot-EIA with whole-cell antigens and in immunoblotting with LPS antigens. We have established a set of absorbed antisera that identifies O antigens in the serotype strains, but our results cast doubt on the validity of some of the current O-serotype definitions.

MATERIALS AND METHODS

Strains. The O-serotype strains O1 to O15 used in the study were as described by Edwards and Ewing (5): O1, CDC 866-57; O2, CDC 868-57; O3, CDC 863-57; O4, CDC 864-57; O5, CDC 867-57; O6, CDC 862-57; O7, CDC 843-57; O8, CDC 1604-55; O9, CDC 4534-60; O10, CDC 1289-59; O11, CDC 1914-63; O12, CDC 6320-58; O13, CDC 3607-60; O14, CDC 4444-60; and O15, CDC 4523-60. Strains O16 to O24 were kindly supplied by W. H. Traub (11–13, 18).

Antisera. (i) Preparation. Antisera towards the serotype strains O1 to O24 and experimental strains were prepared as described previously (16).

(ii) Absorption. Antigens for absorption were grown on nutrient agar (Oxoid Ltd., Basingstoke, England) plates overnight at 37°C. Bacterial growth from one 14-cm plate was harvested into 10 ml of phosphate-buffered saline (PBS), pH 7.4, and heated for 1 h at 100°C. Antigen was collected by centrifugation (4,000 × *g*, 20 min) and suspended in 6 ml of PBS. A 0.33-ml portion of antiserum was added, and the mixture was incubated for 1 h at 37°C. Bacterial cells were removed by centrifugation (12,000 × *g*, 20 min), and the supernatant was preserved with 0.1% (wt/vol) sodium azide.

Serotyping. (i) Agglutination. Antigens were prepared from cultures grown overnight in 3 ml of tryptone soya broth (Oxoid). Cultures were heated for 1 h at 100°C; antigen was collected by centrifugation (4,000 × *g*, 20 min) and suspended in 5 ml of PBS. Tests were performed in microdilution trays, as described previously, with both absorbed and unabsorbed antisera (16).

(ii) Dot EIA. Cell growth from cultures incubated overnight at 37°C in 3 ml of tryptone soya broth was collected by centrifugation (4,000 × *g*), suspended in 3 ml of saline, and then heated for either 1 h at 100°C or 30 min at 121°C. Approximately 5 to 10 μl of heated antigen was applied to a nitrocellulose membrane (0.45-μm pore size; Gelman Sciences Ltd., Northampton, England), and the membrane was allowed to dry for 30 min at 37°C. Membranes were stored at room temperature prior to use.

In the assay, all incubations were at room temperature and

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TABLE 1. Major cross-reactions in dot EIA of unabsorbed antisera to *S. marcescens* O serotypes

Antisera[a] to serotype strain	Cross-reactions to antigen
O1	O5, O6, O14, O19
O2	O3, O21
O3	
O4	
O5	
O6	O7, O14
O7	O6
O8	
O9	O15, O17, O19
O10	O22
O11	
O12	O14
O13	O5
O14	O6, O12
O15	
O16	O20
O17	O1, O19
O18	O10, O22
O19	O1, O4, O9, O15, O17
O20	O16
O21	O2, O3
O22	O10
O23	O14
O24	O16, O20

[a] Antisera were assayed at a dilution of 1:500.

TABLE 2. LPS specificities of *S. marcescens* serotype strains

Serotype strain	Serotype strains exhibiting LPS-specific reactions		
	Indistinguishable antigens	Similar but distinguishable antigens	Other immunoblotting reactions
O1		O4, O19	O5, O9 (O17)
O2	O3	O21	
O3	O2	O21	
O4			
O5			
O6	O7		
O7	O6		
O8			
O9		O15	O17, O19
O10		O22	
O12	O14		
O14	O12		
O15		O9	
O16		O20	
O17		O19	O1
O18			
O19		O1, O17	O9, O15
O20		O16	O24
O21		O2, O3	
O22		O10	
O23			O12, O14
O24			O16, O20

were carried out with constant agitation. Nonspecific binding sites were blocked with PBS containing 2% (wt/vol) bovine serum albumin (BDH Chemicals Ltd., London, England) for 20 min. Primary antibody was added to a final concentration of 1 in 300 (absorbed antisera) or 1 in 500 (unabsorbed antisera), and incubation was for 1 h. The membrane was washed twice in tap water and once in PBS and incubated for 1 h with anti-rabbit enzyme-conjugated antiserum (Sigma, Poole, England) diluted 1:1,000 in blocking solution. Excess antibody was removed by washing, and bound antibody was detected with either fast red violet (alkaline phosphatase conjugate) or 4-chloro-1-naphthol (peroxidase conjugate) substrate.

Electrophoresis. Sodium dodecyl sulfate (SDS)-PAGE was carried out by using standard methods (10) with 10% resolving gels. LPS was detected by using a silver stain (20) with carbonate developer (0.28 M sodium carbonate containing 0.05% formaldehyde). Separated components from SDS-PAGE gels were transferred to nitrocellulose as described previously (6). LPS-containing extracts were prepared by proteinase K digestion (9).

RESULTS

Dot EIA. All antisera gave strong reactions with their homologous antigen, and most also gave strong reactions with a number of heterologous antigens (Table 1), together with minor, nonspecific reactions with most antigens. Minor reactions were strongest in antisera towards O8, O10, O11, O13, and O24.

Immunoblotting. All antisera that showed strong cross-reactions in dot EIA were immunoblotted with LPS extracts of the appropriate heterologous strains. The results were compared with the homologous immunoblotting profile and with the LPS profiles obtained in SDS-PAGE. A series of absorption experiments was then carried out to determine the relationships within those strains with similar LPS antigens. Table 2 summarizes the results of these experiments and shows the relationships among the major LPS antigens found in the serotype strains.

Distinct LPS antigens. LPS antigens for serotype strains O4, O5, O8, and O18 were essentially free of any significant cross-reactions.

Indistinguishable LPS antigens. (i) O2 and O3. LPS antigens from strains O2 and O3 were indistinguishable in SDS-PAGE and immunoblotting with O2 and O3 antisera (Fig. 1). Cross-absorption removed the LPS reaction in immunoblotting. Antisera to these strains did not cross-react in agglutination tests.

(ii) O6 and O7. LPS antigens from strains O6 and O7 were indistinguishable by SDS-PAGE and immunoblotting with O6 and O7 antisera (Fig. 1). Cross-absorption removed the LPS reactions in immunoblotting, but the absorbed antisera retained serotype-specific agglutinins (Table 3). Antiserum raised towards O6, but not O7, reacted strongly in dot EIA with O14 antigen but did not react with O14 LPS in immunoblotting.

(iii) O12 and O14. LPS antigens from strains O12 and O14 were indistinguishable by immunoblotting with O12 and O14 antisera (Fig. 1). The LPS profiles of these strains in SDS-PAGE were very similar with respect to band spacing, but their profiles were slightly offset so that they could not be superimposed directly. Cross-absorption removed the LPS reaction in immunoblotting, but the absorbed antisera retained serotype-specific agglutinins (Table 4). Serum raised towards O14, but not O12, reacted strongly in dot EIA with O6 but did not react with O6 LPS in immunoblotting. The O14/O6 cross-reaction was consistently stronger than the O14/O12 reaction in dot EIA.

Similar LPS antigens. (i) O9 and O15. The LPS profiles of strains O9 and O15 in SDS-PAGE were similar with respect to band spacing, but their profiles were offset. Antisera to both strains reacted in immunoblotting with both LPS antigens. Absorption of O15 antiserum with O9 removed anti-

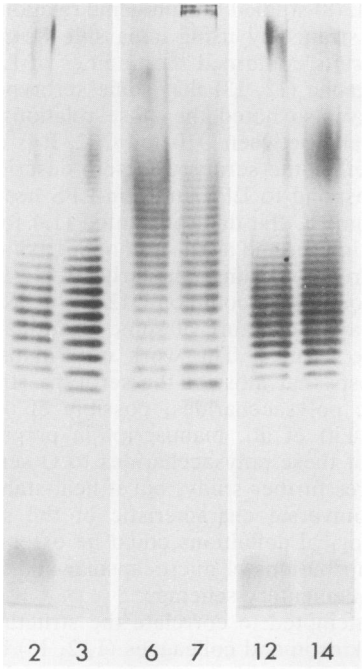

FIG. 1. Immunoblots of closely related O antigens. (Left to right) LPS O2 and O3 reacted with O2 antiserum, LPS O6 and O7 reacted with O7 antiserum, and LPS O12 and O14 reacted with O12 antiserum.

bodies to both LPS antigens, whereas absorption of O9 antiserum with O15 failed to remove O9 LPS-specific antibodies and only reduced the O15 LPS-specific reaction. The precise level of identity between these antigens was therefore not established.

(ii) O10 and O22. The LPS profiles of strains O10 and O22 were similar except for bands in the lower third of the gel, which did not align. The bands also tended to be fainter in extracts of O10. Absorption of antiserum O22 with O10 removed both O22 and O10 specificities but left a medium- to high-molecular-weight "smear" in the O22 LPS track, whereas antiserum O10 absorbed with O22 still reacted with O10 LPS. O10 LPS therefore appeared to contain an additional antigenic determinant.

(iii) O16 and O20. The LPS profiles of strains O16 and O20 were identical, although weaker banding was observed in O16. Extracts of O16 also tended to show a smeared track on silver-stained gels. In immunoblotting with O16 antiserum, O16 LPS gave a smeared profile, although a faint ladder pattern could be discerned. Conversely, the immunoblotting profile of O16 LPS extract with O20 antiserum was a conventional ladder pattern, similar to O20. Antiserum O16 absorbed with O20 removed the ladder pattern entirely but left only the smeared profile for O16. The O16 serotype strain may therefore have expressed two molecular species that gave rise to immunoblotting reactions. Absorption of antiserum O20 with O16 removed both O16 and O20 reactions.

(iv) O1, O17, and O19. LPS from serotypes O1, O17, and O19 formed a cross-reacting complex, and the results of absorption experiments are given in Table 5. Antisera to O17 and O19 failed to agglutinate strain O1 but reacted strongly in immunoblotting with O1 LPS. Absorption of antiserum O17 with O19 removed both O19 and O17 specificities, whereas antiserum O19 absorbed with O17 still reacted with O19 LPS. Similarly, absorption of antiserum O1 with O19 removed both O1 and O19 specificities, whereas antiserum O19 absorbed with O1 still reacted with O19 LPS; i.e., O19 LPS contained an additional antigenic determinant. O1 and O17 cross-reacted in immunoblotting but gave stronger reactions with their respective homologous antisera. Absorption of O19 antiserum with a combination of O1 and O17 cells reduced the strength of the homologous immunoblotting reaction considerably.

Other cross-reactive LPS types. Antiserum to O21 reacted with O2/O3 LPS, but specific antiserum was readily prepared by absorption. Antiserum to O23 reacted relatively weakly with O12/O14 LPS and could be made specific by absorption. Similarly, a cross-reaction between O24 antiserum and O16 and O20 LPS could be removed by absorption with either O16 or O20 antigen.

Strains with poorly expressed O side chain. No ladder profiles could be visualized for the O11 and O13 serotype strains, an indication that these strains express very little O-side-chain-substituted LPS. O13 antisera bound to O5

TABLE 3. Cross-reactions between *S. marcescens* strains O6 and O7

Serum	Absorbing antigen	Agglutination titer		Immunoblotting with LPS	
		O6	O7	O6	O7
O6		80	80[a]	+++[b]	+++
O6	O7	80			
O6	O14	40	40[a]	+++	+++
O7			320	+++	+++
O7	O6		320		

[a] Trailing reactions to titer. Majority of cells apparently unagglutinated at all antiserum dilutions.
[b] +++, Strong reaction in immunoblotting.

TABLE 4. Cross-reactions between *S. marcescens* O12 and O14

Serum	Absorbing antigen	Agglutination titer		Immunoblotting with LPS	
		O12	O14	O12	O14
O12		320		+++	+++
O12	O14	320			
O14			160	+++	+++
O14	O12		80		

TABLE 5. Cross-reactions among *S. marcescens* strains O1, O17, and O19

Serum	Absorbing antigen	Agglutination titer			Immunoblotting with LPS		
		O1	O17	O19	O1	O17	O19
O1		160	80	160	+++	+	+++
O1	O17	40		20			
O1	O19	40					
O17		0	1,280	1,280	+++	+++	+++
O17	O1	0	640	320		+++	+++
O17	O19	0	80	0			
O19		0	2,560	2,560	+++	+++	+++
O19	O1	0	2,560	640		++	+++
O19	O17	20	0	320	++		++

TABLE 6. Absorption scheme to produce *S. marcescens* O antisera with a high degree of LPS specificity

O serotype	Vaccine strain	Absorbing antigen
O1	O1	O14
O2/O3	O2	O21
O4	O4	O14
O5	O5	
O6/O7	O7	O14
O8	O8	O14
O9 (O15)	O9	O1
O10	O10	O14
O12/O14	O12	O6
O16	O16	O20
O18	O18	O14
O19 (O17)	O19	O1
O20	O20	O14
O21	O21	O3
O22	O22	O14
O23	O23	O14
O24	O24	O14

antigen in dot EIA and bound strongly to high-molecular-weight material in O5 corresponding to LPS, although no corresponding reaction was found with the homologous LPS.

Scheme to identify LPS antigens. By using the data summarized in Table 2, a set of absorbed antisera was prepared that reacted with the serotype strains in a pattern that corresponded approximately with their O antigens (Table 6). All strains except O5 were absorbed with either the O14 serotype strain, to remove nonspecific antibodies, or a specific strain, to remove a strong cross-reaction.

Antiserum O2 was used to detect the O2/O3 LPS antigen; similarly, O7 and O12 antisera were selected to detect O6/O7 and O12/O14, respectively. O15 and O17 were excluded on the basis of their similarity to O9 and O19, respectively. O11 and O13 were excluded because we failed to detect any significant amounts of high-molecular-weight LPS in SDS-PAGE or immunoblotting.

DISCUSSION

Pairs of strains within the serotype set of *S. marcescens* have very similar O antigens, yet antisera to these strains can give more or less specific reactions in agglutination tests. Antibodies to surface antigens other than LPS must therefore play an important role in some agglutination reactions. It has been suggested that heat-stable microcapsular antigens may be responsible for some O-like agglutination reactions (3, 6, 14). If this is correct, then it is possible that the current serotype definitions of the species are based on a mixture of microcapsular and LPS antigens, rather than on LPS alone.

Some of the cross-reactions commonly recorded in agglutination tests (5, 16) corresponded to pairs of strains we found to have very similar O antigens, i.e., O2/O3, O6/O7, and O12/O14. It is of particular interest that the O6/O14 cross-reaction did not appear to be due to shared LPS epitopes. This provides circumstantial support to the conclusions of Brigden and Wilkinson (3) that the O6/O14 antigen is an acidic glucomannan, possibly of microcapsular origin. Our finding that the O13 strain had a rough or semirough phenotype also supports Oxley and Wilkinson's conclusion that the O13 antigen is an acidic polymer, rather than an integral part of LPS (14).

Guinée et al. (8) studied the antigenic relationships among the serotype strains by using a passive hemagglutination test. Their results confirmed the findings of Le Minor and Sauvageot-Pigache (12, 13) that some serotypes are multifactorial. They also noted the close relationship between O16 and O20 and between O10 and O22. It is interesting to speculate whether the serotype factors described by these workers correspond to LPS and non-LPS heat-stable antigens. For example, the data presented (13) for Co 2/3 (IP 978) could be interpreted as the common LPS antigen in the O2 and O3 serotype strains. Similarly, Co 12/13/14 (IP 572) could correspond to the common O12/O14 LPS antigen (12).

The microcapsular nature of some agglutinating antigens remains to be verified. The work of Wilkinson and colleagues suggests that most of the serotype strains possess distinct acidic polysaccharides, possibly of microcapsular origin (T. L. Pitt et al., manuscript in preparation). The contribution of these polysaccharides to O serology of the species requires further study, but if heat-stable microcapsules are a universal characteristic of the species, then perhaps serological definitions could be extended by separating the contributions of microcapsular and LPS antigens into two complementary schemes.

We have attempted to correlate the structural data produced by Wilkinson and colleagues (1, 2, 15) for cell envelope polysaccharides with our serological results. There is a high degree of agreement between the two systems. The neutral polymers (putative O-side-chain polysaccharides) isolated from strains O6 (CDC 862-57) and O7 (CDC 843-57) are structurally indistinguishable, composed of a glucorhamnan disaccharide repeating unit. Similarly, strains O12 (CDC 6320-58) and O14 (CDC 4444-60) contained an indistinguishable polymer of ribose and 2-acetamino-2-deoxygalactose. This sort of comparison is necessarily complex because of the sometimes contradictory data available from serological studies. Nevertheless, the available structural data lend support to the argument that the agglutination reactions of antisera raised against the serotype strains do not necessarily represent the true picture of the LPS antigens of the species.

As a typing laboratory, our requirement was for a "workable" serotyping scheme; therefore, Table 6 represents a pragmatic view of the O antigens present in the serotype strains. The serological relationships found in immunoblotting require further characterization before the exact relationships among the O antigens in the serotyping set can be defined. However, with this absorption scheme, the results of the dot EIA are easy to interpret and give a more reliable indication of the O antigen. The test does require more "hands on" time for the operator than the traditional agglutination test, but results can be obtained in 3 to 4 h.

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