CURRENT MICROBIOLOGY, Vol. 10 (1984), pp. 185–190

Current Microbiology

Isolation and Characterization of Serum-resistant Strains of *Pseudomonas aeruginosa* Derived from Serum-sensitive Parental Strains

Neal L. Schiller,* David R. Hackley, and Amy Morrison

Division of Biomedical Sciences and Department of Biology, University of California–Riverside, Riverside, California 92521-0121, USA

Abstract. Six serum-resistant (serR) mutant *Pseudomonas aeruginosa* strains were isolated from six serum-sensitive (serS) parental strains by subculturing the sensitive strains in increasing concentrations of normal pooled fresh human serum (FHS). Although the colonial type of the mutant was similar to that of the parental strains, each of the serR mutants had an altered serotype when compared to its parental counterpart. Three mutant strains and their corresponding parental strains were chosen for further examination. The lipopolysaccharide (LPS) preparations from the serR strains were found to be heterogeneous, containing LPS with varying degrees of O-side-chain substitution, whereas the LPS of the serS strains contained primarily lipid A-core polysaccharide components. Although two of the serR mutant strains had an outer membrane protein (OMP) profile analogous to their serS parental counterparts, one serR strain differed from its parental strain by the absence of a 32,000 dalton major OMP. These studies suggest that the susceptibility of *P. aeruginosa* to the bactericidal activity of FHS may be related to either or both LPS structure or OMP content.

The bactericidal activity of antibody and complement in fresh normal human serum (FHS) is regarded as a significant component of host defenses against Gram-negative bacteria, since bacilli isolated from localized infections are usually serum-sensitive (serS), whereas those isolated from cases of bacteremia are serum-resistant (serR) [18, 24]. The susceptibility of *Pseudomonas aeruginosa* strains to FHS appears to be quite heterogeneous. Whereas most strains isolated from patients with bacteremia or endocarditis are serR [17, 19, 25], mucoid and nonmucoid strains of *P. aeruginosa* isolated from the sputum of cystic fibrosis (CF) patients are much more sensitive to the bactericidal activity of serum than strains isolated from non-CF patients [9, 13, 15, 19, 22]. The serum sensitivity of these strains that colonize/infect the lungs of CF patients may explain their inability to spread systemically and produce disseminated disease in these patients [8].

Although most strains isolated from CF patients are serS, occasional spontaneous serR mutants of these serS isolates occur in culture [19, 22]. Examination of one of these serR mutant strains suggested that this strain had one or more apparently altered, absent, or inaccessible cell surface components compared to its serS parental counterpart [1]. To examine further the nature of serum resistance in *P. aeruginosa* strains, we isolated six additional serR mutants from serS parental strains. The isolation and characterization of these strains are described below.

Materials and Methods

Bacterial strains. Strains Mc 19, Mc 30, Mc 85, Mc 117, 144M, WcM, and ByM were isolated from the sputum of cystic fibrosis (CF) patients. Non-CF strains were obtained from sputum samples (Mc 145, Mc 207), urine (Mc 192), a surface wound (Mc 84), and the skin of a burn patient (Mc 170). These strains were identified as *P. aeruginosa* by Gram stain, oxidase reaction, pigment production, and growth at 42°C. Where required, strains were further identified using Flow Laboratories, Inc. N/F System (Flow Laboratories Inc., Roslyn, NY). The colonial type of each strain was classified as previously described [19].

Normal pooled fresh human serum (FHS). Blood from four normal, healthy adult male volunteers was obtained by venipuncture and allowed to clot at room temperature for 20 min. After centrifugation at 1000 *g* for 15 min at 4°C, the serum was

* To whom reprint requests should be addressed.

Table 1. Serum sensitivity, colonial type, and serotype specificity of parental and mutant *Pseudomonas aeruginosa* strains

Parental strains				Mutant strains			
Strain	Colonial type	Serum sensitivity[a]	Difco serotype[b]	Strain	Colonial type	Serum sensitivity	Difco serotype
Mc 30	rough	1.56%	1,3,10 (weak)	Mc 30 (SR)	rough	>50%	9 (strong)
Mc 85	entero	3.12%	1,3,14 (weak)	Mc 85 (SR)	entero	>50%	6 (strong)
Mc 170	rough	1.56%	10 (strong)	Mc 170 (SR)	rough	>50%	9 (strong)
144M	mucoid	0.78%	3,14 (weak)	144M (SR)	mucoid	>50%	6 (strong)
ByM	mucoid	0.78%	3,14 (weak)	ByM (SR)	mucoid	>50%	6 (strong)
WcM	mucoid	0.78%	2,16 (moderate)	WcM (SR)	mucoid	>50%	6 (strong)

[a] Percent FHS needed to kill >90% of bacteria in assay.
[b] Serotyping was recorded as strong (immediate reaction and maximal agglutination), moderate, or weak (delayed reaction and minimal agglutination).

removed, pooled, sterilized using a 0.45-μ filter, and either used immediately or stored at −70°C in small aliquots until ready for use.

Isolation of serum-resistant (serR) mutants. After overnight growth on brain–heart infusion (BHI) (BBL Microbiology Systems, Cockeysville, MD) agar plates at 37°C, the bacteria were resuspended in 0.01 *M* phosphate-buffered saline (PBS), pH 7.4, and adjusted spectrophotometrically at 550 nm to a concentration of approximately 2×10^8 bacteria/ml with PBS. This suspension was then diluted 50-fold with PBS and 0.1 ml used as the bacterial inoculum (approximate starting concentration of 4×10^5 bacteria/ml). Experimental tubes contained FHS (at final concentrations of 0.625%–40%), 0.1 ml inoculum, and BHI broth in a final volume of 1.0 ml. (Control tubes contained PBS in place of FHS.) After overnight incubation at 37°C, the tube with the highest serum concentration in which visible growth was observed was selected, the organisms were diluted 100-fold with PBS, and 0.1 ml of this suspension was used to inoculate a second set of tubes. A third subculture was similarly prepared. Organisms that grew in the tube with 40% FHS were presumed to be serR and used for additional studies.

Serum bactericidal assay. The sensitivity of *P. aeruginosa* strains to the bactericidal activity of FHS was determined as described previously [19]. For these assays, the lowest concentration of FHS that caused a >90% reduction in bacterial survival was recorded.

Serotyping. *Pseudomonas* strains were serotyped by the slide-agglutination technique using the Bacto-*Pseudomonas aeruginosa* antiserum set (Difco Laboratories, Detroit, MI).

Outer membrane isolation and SDS-polyacrylamide gel electrophoresis. Each strain was grown overnight at 37°C with shaking in 500-ml BHI broth lots. A total of 4.5 liters of bacteria was harvested by centrifugation at 10,000 *g* for 20 min at 4°C, washed twice with saline, and the pellet was resuspended in distilled water (dH_2O) and lyophilized. The protocol for isolation of outer membranes and their analysis by electrophoresis on sodium dodecyl sulfate (SDS) polyacrylamide slab gels has been previously described [19]. The protein content was determined using the Folin phenol method of Lowry et al. [10].

Lipopolysaccharide isolation and SDS-polyacrylamide gel electrophoresis. For these studies, each strain was grown overnight at 37°C with shaking in 500-ml BHI broth lots, and a total of 12 liters was harvested, washed, and lyophilized as described above. The lipopolysaccharide (LPS) was isolated from each strain according to a recently described protocol of Darveau and Hancock [3], which has been proven useful for isolation of both smooth and rough LPS forms. Isolated LPS preparations were then mixed 1:1 with buffer containing 0.1 *M* Tris-hydrochloride (pH 6.8), 2% SDS (wt/vol), 1% 2-mercaptoethanol (vol/vol), 0.001% bromophenol blue (wt/vol), 20% sucrose (wt/vol), and 0.1 *M* tetrasodium EDTA at a final concentration of 1 mg/ml LPS. After the samples were heated for 10 min at 100°C, 15 μl were applied per lane to a 15% SDS-polyacrylamide slab gel, 0.8 mm thick, prepared according to the protocol of Lugtenberg et al. [11] with the exception that 4 *M* urea was added to both stacking and separating gels as recommended by Tsai and Frasch [23]. Electrophoresis was carried out at room temperature using a constant current of 20 mA until the tracking dye migrated to the bottom of the slab gel (usually about 3–4 h). Gels were fixed by incubation in a 40% ethanol–5% acetic acid solution in a clean dish overnight without agitation.

The gels containing LPS were then stained according to the silver stain procedure described by Tsai and Frasch [23] with a few modifications, as detailed below. After overnight fixation, the fixing solution was replaced with 1.4% periodic acid in 40% ethanol–5% acetic acid and allowed to oxidize for 20 min with agitation. The gel was then washed three times for 15 min each using deionized dH_2O (ddH_2O). After draining off the last wash, the gel was stained for 10 min with agitation (using the staining reagent prepared as described by Tsai and Frasch). After three 15-min washes, the gel was developed to the desired intensity (which usually occurred within 10 min) using a solution containing 50 mg citric acid and 0.5 ml of 37% formaldehyde per liter of ddH_2O. The reaction was then stopped by placing the gel in a stop bath consisting of 0.33% acetic acid [7].

Results and Discussion

Isolation of serR mutant strains. For these studies 12 serS *P. aeruginosa* strains were grown overnight at 37°C in BHI broth in the presence or absence of FHS at concentrations ranging from 0.625% to 40% FHS. From these 12 strains, six serR mutants were obtained. For these six strains (Mc 30, Mc 85, Mc 170, 144M, WcM, and ByM), growth initially oc-

curred only in those tubes with low serum concentrations (such as 1.25% FHS using 144M). However, if the organisms that grew in the tube with 1.25% FHS were used to inoculate a second series of tubes, the bacteria appeared more serR after the second subculture. After a third subculture, the bacteria grew in the presence of 40% FHS. Similar growth patterns were obtained for strains Mc 30, ByM, and WcM, while strains Mc 85 and Mc 170 required only two such subcultures. The organisms that grew in the tubes containing 40% FHS were designated serR (SR), and these organisms were so labeled, e.g., 144M (SR), Mc 30 (SR), etc. No serR mutants were obtained from Mc 19, Mc 84, Mc 117, Mc 145, Mc 192, or Mc 207 using this technique.

Does subculturing serS strains in serum induce serR or simply select out a small subset of serR organisms within the serS population? Examination of an occasional survivor of a serS strain that had been incubated with 50% FHS for 60 min at 37°C revealed that the survivors were still serS. Although this would imply that there were no serR organisms within that serS pool, a fluctuation test [12] would be needed to determine whether there was induction or selection of serum resistance in these studies.

Serum sensitivity, colonial type, and serotype of parental and mutant strains. Each of the six mutant strains and the six corresponding parental strains was examined for sensitivity to the bactericidal activity of FHS, and the least amount of serum required to kill >90% of the inoculum after 60 min incubation at 37°C was determined. The results are shown in Table 1. Whereas all of the parental strains were sensitive to the bactericidal activity of 3.12% FHS or less, the mutant strains were insensitive to the bactericidal activity of 50% FHS (the highest concentration tested).

Previous studies by Thomassen and Demko [22] and Schiller and Hatch [19] have suggested that a strain's colonial type is not related to its sensitivity to the bactericidal activity of FHS. This was supported by the present investigation, in which each of the serR mutants maintained the same colonial pattern as their parental serS counterparts. Of more interest was the observation that the serotype of the serR mutants differed from that of the parental counterpart strains (see Table 1). While most of the parental strains serotyped weakly or moderately (except Mc 170), the serR strains all serotyped strongly, with a serotype distinct from that of its parental strain. Considering the fact that serotype is based on the O-antigenic side chain of LPS [2], the alteration in serotype configuration in these mutants suggested a variation in the LPS composition that might be related to serum resistance.

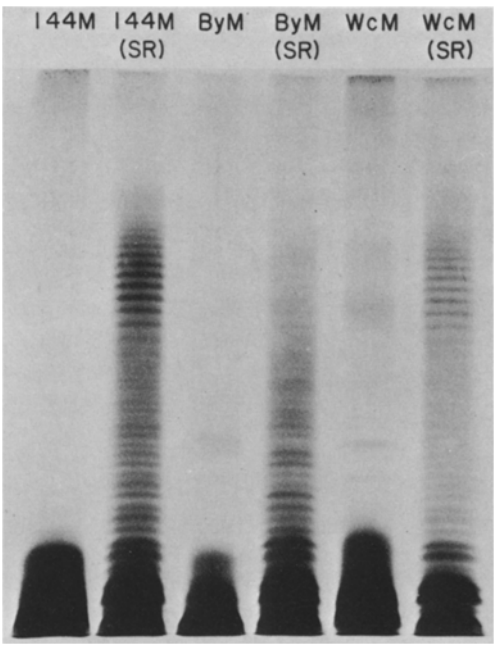

Fig. 1. SDS-polyacrylamide gel electrophoresis of LPS preparations prepared from parental serS *Pseudomonas aeruginosa* strains and their serR mutants. Each lane was loaded with approximately 15 μg of bacterial LPS preparation.

Examination of LPSs from the parental and mutant strains. It has often been noted that smooth strains of Gram-negative bacteria, which synthesize LPS with a high degree of substitution of core units by O-specific side-chain moieties, are more serum-resistant than rough isolates or mutants that have lost the ability to either synthesize or attach the O-antigen component of LPS [20]. To examine this, LPSs from 144M (SR), ByM (SR), and WcM (SR), as well as from each of the parental strains, were obtained and electrophoresed on a SDS-polyacrylamide slab gel and the patterns developed using a periodic acid-silver stain. The mobility of the LPS bands correspond to the degree of "roughness" of the strain with increases in mobility correlating to the shortening of the oligosaccharide core [3, 5, 7, 16]. The results, presented in Fig. 1, indicate that the LPSs of the parental serS strains are mainly "rough" or short LPS, since the dark-staining material near the gel bottom is believed to be mainly lipid-A-core polysaccharide. In contrast, the LPSs from the serR mutant strains display size heteroge-

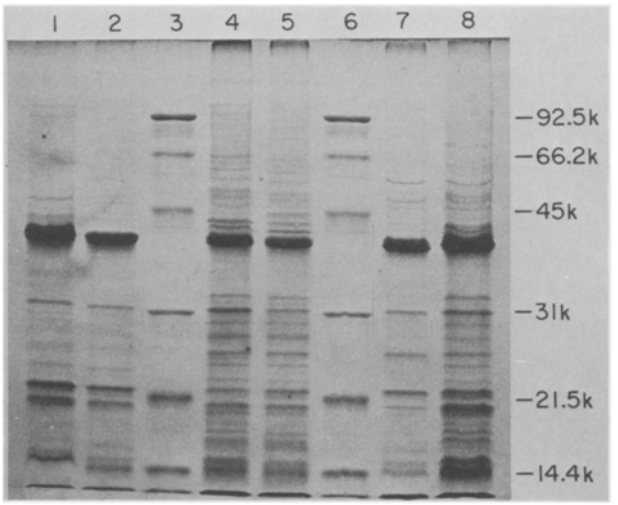

Fig. 2. SDS-polyacrylamide gel electrophoresis of outer membranes of serR *Pseudomonas aeruginosa* mutants and their serS parental counterparts: lane 1, ByM (SR); lane 2, ByM; lane 4, 144M (SR); lane 5, 144M; lane 7, WcM (SR); and lane 8, WcM. Lanes 3 and 6 contain Bio Rad molecular weight standards: phosphorylase B, 92.5K; bovine serum albumin, 66.2K; ovalbumin, 45K; carbonic anhydrase, 31K; soybean trypsin inhibitor, 21.5K; and lysozyme, 14.4K. Each lane was loaded with approximately 30 μg of bacterial outer membrane preparation.

neity as evidenced by the various bands in the middle to upper regions of the gel, which are thought to represent LPSs with varying numbers of side chain lengths, including some with highly substituted O-antigenic side chains comparable to those seen in other smooth Gram-negative organisms.

The role of long-chain LPSs in serum resistance is presumably due to their interference with either the formation, attachment, or subsequent activity of the membrane attack complex of the complement system [20]. We have noted that two serR derivative strains, 144NM [1] and 144M (SR), were unable to absorb the bactericidal antibody in FHS responsible for killing the parental serS strain 144M [1, and N. L. Schiller, unpublished observation]. This might suggest that the LPS on these serR strains simply inhibits the ability of bactericidal antibody from reaching its binding site on the outer membrane surface. This reasoning is supported by the study of Feingold [4] who reported that serR *E. coli* and *P. aeruginosa* strains could be made serS if the amount of O-antigen in the LPS was reduced by culturing these bacteria in the presence of diphenylamine. However, preliminary studies in this laboratory were unable to duplicate these findings for 144M (SR) (N. L. Schiller, unpublished observation).

Examination of the outer membrane protein (OMP) profile of parental and mutant strains. An alternative explanation for the inability of serR strains to absorb out bactericidal immunoglobulins is that these strains are missing the antigenic determinant to which bactericidal antibody binds. Therefore, we isolated the outer membranes from three serR strains and their parental counterparts and analyzed their OMP profiles by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 2, the most prominent OMP had a molecular weight of approximately 41,000 daltons (41K) and was detected in all strains. When each of the mutant OMP profiles was compared with that of its particular parental type, both 144M (SR) and ByM (SR) were observed to have OMP profiles almost indistinguishable from their parental strains. However, a 32K-dalton protein present in the serS strain WcM was either absent or present in much lower concentration in WcM (SR). We are presently attempting to determine whether this 32K OMP is the bacterial target for FHS bactericidal antibody. It should be noted that other investigators have detected alterations in OMP profile that correlated with bacterial serum resistance [6, 14, 21].

In summary, we have isolated six serR mutant *P. aeruginosa* strains from their serS parental counterparts. Using three pairs of strains, we have determined that the amount of O-side chain substitution is greater in the serR strains than in the serS parental counterparts, implying that LPS side-chain length is related to strain sensitivity to the bactericidal activity of FHS. Changes in LPS composition also explain the altered serotype specificity of the mutant strains. In addition, in one serR strain, we have noted the absence of a 32K OMP that was present in the serS parental type. These studies suggest that either or both LPS structure or OMP composition may be related to a strain's susceptibility to serum. Further investigations are in progress to characterize the target site for serum bactericidal immunoglobulin.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the excellent technical assistance of Richard Hatch and Patricia Balazs, and the advice and assistance of Robert S. Borowski. The authors particularly appreciate the help and generosity afforded by Drs. Richard P. Darveau and Robert E. W. Hancock, Department of Microbiology, University of British Columbia, Vancouver, who shared their protocol for LPS isolation prior to its publication.

This investigation was supported in part by Public Health Service Grant AI-17072 from the National Institutes of Allergy and Infectious Diseases, Public Health Service Biomedical Support Grant RR 05816 from the National Institutes of Health, and

grants from the University of California Academic Senate and Cancer Research Coordinating Committees.

Literature Cited

1. **Borowski, R. S., Schiller, N. L.** 1983. Examination of the bactericidal and opsonic activity of normal human serum for a mucoid and non-mucoid strain of *Pseudomonas aeruginosa*. Current Microbiology **9**:25–30.
2. **Chester, I. R., Meadow, P. M., Pitt, T. L.** 1973. The relationship between the O-antigenic lipopolysaccharides and serological specificity in strains of *Pseudomonas aeruginosa* of different O-serotypes. Journal of General Microbiology **78**:305–318.
3. **Darveau, R. P., Hancock, R. E. W.** 1983. Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. Journal of Bacteriology **155**:831–838.
4. **Feingold, D. S.** 1969. The serum bactericidal reaction. IV. Phenotypic conversion of *Escherichia coli* from serum-resistance to serum-sensitivity by diphenylamine. Journal of Infectious Diseases **120**:437–444.
5. **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.
6. **Hildebrandt, J. F., Mayer, L. W., Wang, S. P., Buchanan, T. M.** 1978. *Neisseria gonorrhoeae* acquire a new principle outer-membrane protein when transformed to resistance to serum bactericidal activity. Infection and Immunity **20**:267–273.
7. **Hitchcock, P. J., Brown, T. M.** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. Journal of Bacteriology **154**:269–277.
8. **Hoiby, N.** 1977. *Pseudomonas aeruginosa* infection in cystic fibrosis. Acta Pathologica et Microbiologica Scandinavica, Section C, Supplement No. **262**:1–96.
9. **Hoiby, N., Olling, S.** 1977. *Pseudomonas aeruginosa* infection in cystic fibrosis: bactericidal effect of serum from normal individuals and patients with cystic fibrosis on *P. aeruginosa* strains from patients with cystic fibrosis or other diseases. Acta Pathologica et Microbiologica Scandinavica, Section C **85**:107–114.
10. **Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J.** 1951. Protein measurement with the folin phenol reagent. Journal of Biological Chemistry **193**:265–275.
11. **Lugtenberg, B., Meijers, J., Peters, R., van der Hoek, P., van Alphen, L.** 1975. Electrophoretic resolution of the major outer membrane protein of *Escherichia coli* K12 into four bands. FEBS Letters **58**:254–258.
12. **Luria, S. E., Delbruck, M.** 1943. Mutations of bacteria from virus sensitivity to virus resistance. Genetics **28**:491–511.
13. **Meshulam, T., Verbrugh, H., Verhoeff, J.** 1982. Serum-induced lysis of *Pseudomonas aeruginosa*. European Journal of Clinical Microbiology **1**:1–6.
14. **Munn, C. B., Ishiguro, E. E., Kay, W. W., Trust, T. J.** 1982. Role of surface components in serum resistance of virulent *Aeromonas salmonicida*. Infection and Immunity **36**:1069–1075.
15. **Muschel, L. H., Ahl, L. A., Fisher, M. W.** 1969. Sensitivity of *Pseudomonas aeruginosa* to normal serum and to polymyxin. Journal of Bacteriology **98**:453–457.
16. **Palva, E. T., Makela, P. H.** 1980. Lipopolysaccharide heterogeneity in *Salmonella typhimurium* analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. European Journal of Biochemistry **107**:137–143.
17. **Reyes, M. P., El-Khatib, M. R., Brown, W. J., Smith, F., Lerner, A. M.** 1979. Synergy between carbenicillin and an aminoglycoside (gentamicin or tobramycin) against *Pseudomonas aeruginosa* isolated from patients with endocarditis and sensitivity of isolates to normal human serum. Journal of Infectious Diseases **140**:192–202.
18. **Roantree, R. J., Rantz, L. A.** 1960. A study of the relationship of the normal bactericidal activity of human serum to bacterial infection. Journal of Clinical Investigation **39**:72–81.
19. **Schiller, N. L., Hatch, R. A.** 1983. The serum sensitivity, colonial morphology, serogroup specificity, and outer membrane protein of *Pseudomonas aeruginosa* strains isolated from several clinical sites. Diagnostic Microbiology and Infectious Diseases **1**:145–157.
20. **Taylor, P. W.** 1983. Bactericidal and bacteriologic activity of serum against gram-negative bacteria. Microbiological Reviews **47**:46–83.
21. **Taylor, P. W., Parton, R.** 1977. A protein factor associated with serum resistance in *Escherichia coli*. Journal of Medical Microbiology **10**:225–232.
22. **Thomassen, M. J., Demko, C. A.** 1981. Serum bactericidal effect on *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. Infection and Immunity **33**:512–518.
23. **Tsai, C.-M., Frasch, C. E.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Analytical Biochemistry **119**:115–119.
24. **Vosti, K. L., Randall, E.** 1970. Sensitivity of serologically classified strains of *Escherichia coli* of human origin to the serum bactericidal system. American Journal of Medical Science **259**:114–119.
25. **Young, L. S., Armstrong, D.** 1972. Human immunity to *Pseudomonas aeruginosa*. I. In vitro interaction of bacteria, polymorphonuclear leukocytes, and serum factors. Journal of Infectious Diseases **126**:257–276.