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SELECTION FOR VIRULENCE IN THE FISH PATHOGEN *AEROMONAS SALMONICIDA*, USING COOMASSIE BRILLIANT BLUE AGAR

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ABSTRACT: Coomassie Brilliant Blue Agar was used to quantify the frequency of the A-layer phenotype in different isolates of *Aeromonas salmonicida*. Hydrophilic, non-clumping isolates of *A. salmonicida* consisted predominantly of the A-layer minus phenotype. These bacteria were avirulent by intraperitoneal injection into susceptible brook trout (*Salvelinus fontinalis*) and could not be reisolated from infected fish. By contrast, hydrophobic, clumping isolates were predominantly of the A-layer positive phenotype, highly virulent in brook trout, and easily recovered from dead or moribund fish. A-layer positive and negative clones of *A. salmonicida* were derived by plating bacteria on Coomassie Blue Agar. The plating showed clearly that Coomassie Blue Agar could be used as a highly selective in vitro screening method to reclaim the virulence of certain isolates of *A. salmonicida* having a relatively low percentage of A-layer positive phenotypes.

Key words: *Aeromonas salmonicida*, virulence, Coomassie Brilliant Blue Agar, A-layer, phenotype.

INTRODUCTION

Research to develop an effective vaccine against furunculosis in fish has often concentrated on understanding the mechanisms of virulence by which *Aeromonas salmonicida* produces disease. Consequently, the role of certain bacterial virulence factors has been examined, including the production of hemolysins (Titball and Munn, 1981), proteases (Shieh and MacLean, 1975), and leucocidin (Fuller et al., 1977). These virulence factors are often associated with the extracellular products (ECP) of the bacterial metabolism and can induce much of the pathology associated with the clinical signs of furunculosis in fish (Cipriano, 1983). In addition to the ECP components, Udey and Fryer (1978) showed that virulent cells of *A. salmonicida* have an additional protein beyond the cell membrane that is lacking in avirulent bacteria. These workers indicated that this "A-layer" protein enhanced certain physical attributes of the bacterium, including increases in cellular hydrophobicity, cell-to-cell aggregation, and cell-to-tissue adhesion. Further studies have shown that the A-layer is a 50 kd protein (Trust et al., 1980; Kay et al., 1981) that is interspersed between the repeating subunits of the O-antigen side chain (Evenberg et al., 1985).

When Coomassie Brilliant Blue, a protein-specific dye, was incorporated into common growth media, Udey (1982) could differentiate A-layer positive (A^+) bacteria from negative (A^-) variants of *A. salmonicida*. The A^+ bacteria absorbed the protein dye and grew as blue colonies, whereas the A^- bacteria grew as white colonies. In the present study, Coomassie Brilliant Blue (CBB) agar was used to select for and reclaim virulence in certain isolates of *A. salmonicida*.

MATERIALS AND METHODS

Originally, 46 isolates of *A. salmonicida* in the culture collection at the National Fish Health Research Laboratory (Route 3, Box 700, Kearneysville, West Virginia 25430, USA) were screened for their ability to clump in broth and their hydrophobicity in salt suspensions. All bacteria were routinely stored at −70 C in 0.3-ml aliquots of Tryptic Soy Broth (TSB; Difco Laboratories, Detroit, Michigan 48232, USA). The ability of bacteria to clump in broth was determined by inoculating each strain into TSB for 48 hr at 20 C. Bacteria were visually classified

TABLE 1. Phenotypic characterization, virulence and isolation of *Aeromonas salmonicida* injected into brook trout.

Isolate	Suspension in broth[a]	Hydrophobicity[b]	Bacterial inoculum[c]	% Phenotypic composition[d]		Number of dead fish/ group	Phenotype reisolated[e] (%)
				A^+	A^-		
3.10	NC	50	4.6×10^3	6.6	93.4	0/10	none (0%)
3.17	NC	50	3.5×10^3	3.8	96.2	0/10	none (0%)
3.18	NC	50	1.0×10^4	3.5	96.5	0/10	none (0%)
3.30	NC	50	3.9×10^3	5.4	94.6	0/10	none (0%)
3.95	NC	50	5.3×10^3	0.0	100.0	0/10	none (0%)
3.118	NC	50	8.1×10^3	0.0	100.0	0/10	none (0%)
3.75	C	10	5.8×10^3	100.0	0.0	10/10	A^+ (100%)
3.123	C	10	5.9×10^3	100.0	0.0	10/10	A^+ (100%)
3.136	C	10	5.8×10^3	100.0	0.0	9/10	A^+ (100%)
3.138	C	10	1.8×10^3	100.0	0.0	10/10	A^+ (100%)

[a] Formation of clumping (C) or non-clumping (NC) suspensions in tryptic soy broth.
[b] Percent concentration of ammonium sulfate required to agglutinate bacteria.
[c] Number of bacteria injected per fish.
[d] Percentage of inoculum classified as A plus (A^+) and A minus (A^-) phenotype.
[e] Phenotype and percent recovery from each of five fish within each challenge group.

as non-clumping if they formed stable suspensions in the medium, and clumping strains if they flocculated to the bottom of the inoculum tubes. The bacterium's hydrophobicity was determined according to the procedure of Lindahl et al. (1981). Briefly, a 30% transmittance of bacteria was prepared in phosphate buffer (pH 7.2) at 525 nm in a spectronic-20 colorimeter (Bausch & Lomb, Rochester, New York 14625, USA). Bacteria were centrifuged in 1.5-ml aliquots at 10,000 *g* for 5 min and resuspended in 0.1 ml of 0, 10, 20, 30, 40, and 50% concentrations of $(NH_4)_2SO_4$. Bacteria were then incubated for 24 hr at 20 C in V-bottom microtiter plates (Costar, Cambridge, Massachusetts 02139, USA) and visually examined for agglutination.

Selected strains (Table 1) were then incubated on CBB agar to determine the frequency of the A-layer on individual colony forming units (cfu). CBB agar, which was prepared as described by Udey (1982), simply involved adding Coomassie Brilliant Blue R250 (BioRad Laboratories, Inc., Richmond, California 94547, USA) to a final concentration of 0.01% in Tryptic Soy Agar (TSA; Difco Laboratories, Detroit, Michigan 48232, USA). To quantify the frequency of the A-layer within specific strains, bacteria were incubated for 24 hr on TSA slants. The bacteria were washed from each slant with phosphate buffered saline and brought to a 60% transmission in the phosphate buffer at 525 nm on a spectronic-20 colorimeter as described previously. These standardized suspensions were then quantified by a standard drop plate counting technique as described by Miles and Misra (1938) except that CBB agar was used as the plating medium. Plates were incubated for 48 hr at 20 C and total bacterial counts in addition to the numbers of white (A^-) and the blue (A^+) cfu were determined.

Virulence tests were conducted using yearling brook trout *Salvelinus fontinalis* (average weight = 84.2 g) that were obtained from the National Fish Hatchery (White Sulphur Springs, West Virginia 24986, USA). Experimental trout were determined to be specific-pathogen-free on the basis of hatchery records and subsequent inspection for *A. salmonicida*, as recommended by the American Fisheries Society (Amos, 1985). Fish, divided into 10 groups of 10, were maintained in 30-liter aquaria receiving a constant 1-liter flow of 12.5 C spring water/min. Prior to inoculation with test bacteria, brook trout were anesthetized in a 1:1,000 dilution of tricaine methanesulfonate (Argent Chemical Laboratories, Redmond, Washington 98052, USA). Bacteria used in these initial virulence studies are listed in Table 1. The 60% standardized suspensions of bacteria discussed previously were also used in these virulence tests. Three $\log_{10}$ dilutions of the standardized suspensions were prepared. The last of these dilutions, determined by drop plate techniques to yield about 1×10^4 colony forming units/ml, was used as a standard inoculum. Individual trout were injected intraperitoneally with 0.1 ml of the prepared inoculum and replaced in holding aquaria for 14 days; mortality was recorded during the period and the presence of *A. salmonicida* was confirmed in the kidneys of dead fish by direct fluorescent antibody microscopy (Cipriano, 1982). In addition, the number of bacteria per

gram of kidney tissue was determined in both dead and surviving brook trout by using the CBB agar drop plate technique previously described.

Later studies were conducted with isolate 3.10. This isolate was selected because it was previously shown to be avirulent and caused no mortality in brook trout injected intraperitoneally with as many as 1×10^6 bacteria per fish (Cipriano et al., 1981). Bacteria were streaked onto CBB agar and incubated at 20 C for 48 hr. Individual white and blue colonies were picked and then cloned on additional CBB agar plates. This procedure was repeated three times to ensure the phenotypic purity of a given clone. As a final check for purity, the frequency of A-layer phenotypes was determined by the drop plate method and virulence studies were then conducted with each of the clones as described previously.

A soluble whole cell protein extract and proteinase K (PK) digest of each clone was subjected to sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to determine the nature of both A-layer protein and lipopolysaccharide (LPS) in each clone. PK-treated samples for the electrophoretic analysis of LPS were prepared according to the procedure of Hitchcock and Brown (1983). Soluble whole cell protein preparations were processed similarly, except for the omission of PK. SDS-PAGE was conducted as described by Laemmli (1970) using acrylamide concentrations of 4% in the stacking gel and 10% in the resolving gel. Samples of 20 μl were loaded into gel slots and electrophoresis was conducted as described by Cipriano and Pyle (1985). Proteins were stained with 0.01% Coomassie Brilliant Blue and LPS bands were stained with a modified silver-stain as described by Hitchcock and Brown (1983). For some experiments, A-layer protein was detected by Western blot immunoassays as described by Cipriano and Pyle (1985). Rabbit antiserum to the A-layer protein that was used in the immunoblotting experiments was provided by Dolf Evenberg (University of Utrecht, Transitorium 3, Padualaan 8 3584 CH Utrecht, The Netherlands).

RESULTS

Ten isolates were selected for virulence tests on the basis of their ability to clump in broth and precipitate with $(NH_4)_2SO_4$ (Table 1). In these tests, isolates 3.10, 3.17, 3.18, 3.30, 3.95, and 3.118 formed stable suspensions in TSB and were very hydrophilic; 50% $(NH_4)_2SO_4$ was required to agglutinate the bacteria. These isolates were collectively characterized as non-clumping, hydrophilic bacteria. Isolates 3.75, 3.123, 3.136, and 3.138 clumped in broth and were hydrophobic, requiring only 10% $(NH_4)_2SO_4$ to agglutinate the bacteria. These isolates were referred to as clumping, hydrophobic bacteria. Results of growth on CBB agar plates indicated that the hydrophobic isolates of *A. salmonicida* consisted entirely of blue colony forming units, representing a 100% frequency of the A^+ phenotype. However, the hydrophilic isolates were represented by two categories. Two of these isolates, 3.95 and 3.118, were composed entirely of white cfu, indicating a complete absence of A^+ phenotype. The other strains (isolates 3.10, 3.17, 3.18, and 3.30) grew as both white and blue colonies on the CBB agar. Although these bacteria were primarily of the A^- phenotype, a small percentage of the colony forming units (4 to 7%) were A^+. None of the hydrophilic isolates induced mortality in juvenile brook trout injected with about 1×10^3 cfu/fish. However, each of the hydrophobic isolates injected at similar concentrations was highly virulent for brook trout (Table 1).

In addition, bacteria were easily reisolated from the kidneys of dead fish injected with the hydrophobic isolates of *A. salmonicida*. Post mortem analysis revealed the presence of about 1×10^7 to 1×10^9 bacteria per gram of kidney tissue, composed entirely of the blue A^+ phenotype. Similar tests were conducted at 15 days after challenge on fish injected with the hydrophilic isolates of *A. salmonicida*, but no bacteria were reisolated.

Isolate 3.10 was used in an attempt to reclaim virulence in an isolate having a low percent composition at A^+ phenotypes. Four white (A^-) and four blue (A^+) colonies were cloned from an original inoculum of this isolate and determined to be phenotype-pure. In virulence tests, none of the brook trout injected with about 1×10^4 cfu of the white A^- clones died during 14 days of observation; however, the brook trout injected with about 1×10^3 cfu of

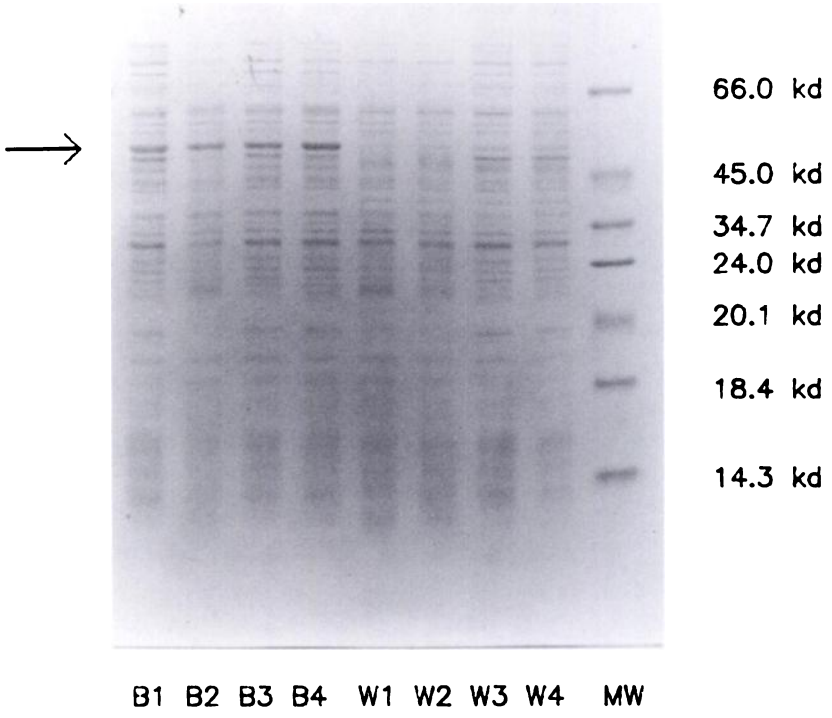

FIGURE 1. SDS-PAGE of soluble whole cell protein sonicates from A^+ (B1–4) and A^- (W1–4) phenotypes of *Aeromonas salmonicida*, isolate 3.10, with molecular weight (MW) markers. Note the presence of the 50 kd A-layer protein (arrow) in the A^+ phenotype.

the blue A^+ clones suffered significant mortality. Within 7 days after injection, all of the fish injected with three of these clones and nine of 10 fish injected with the fourth clone were dead.

SDS-PAGE showed that the most significant difference between the blue and white clones of isolate 3.10 was the presence or absence of the A-layer. Protein profiles of the blue and white cloned bacteria were similar, but the blue clones had a 50 kd protein that was lacking in the white clones (Fig. 1). The molecular weight of this protein and its absence in the avirulent white clones suggested that the band in question was the A-layer protein. Additional evidence to support this hypothesis was provided by Western blot experiments, in which antiserum prepared to the A-layer protein reacted with only the 50 kd protein present in the blue clone types (Fig. 2). Differences were not observed between LPS patterns of the blue and white clones of *A. salmonicida* (Fig. 3).

DISCUSSION

Results of the present study indicated that CBB agar is a highly effective medium for the detection and quantification of the frequency of the A-layer phenotype, that the medium provided an effective in vitro screen for virulence, and that CBB agar could be used as a selective medium to reclaim virulence in isolates containing a low frequency of the A^+ phenotype. When Udey (1982) first described the CBB medium, he demonstrated, by electron microscopy, a correlation between blue colonies and the presence of A-layer. SDS-PAGE experiments conducted in our study corroborated his findings and indicated that the major structural difference in blue and white colonies is indeed the presence of

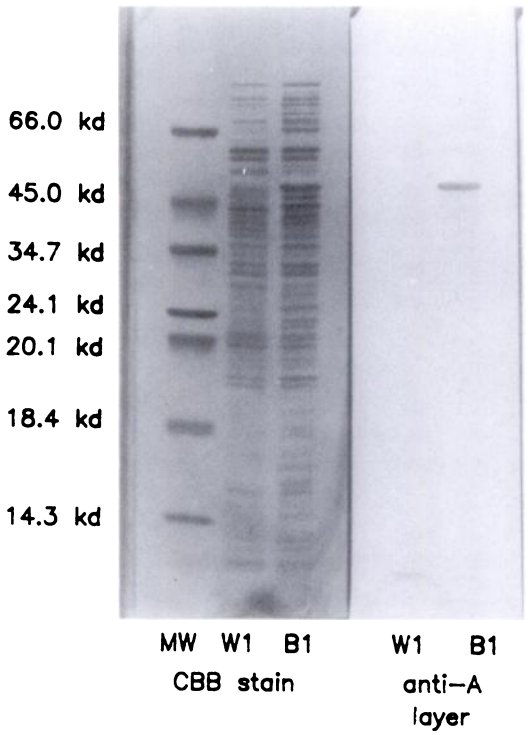

FIGURE 2. Western blot analysis of representative A^- (W1) and A^+ (B1) phenotypes of *Aeromonas salmonicida*. On the left, molecular weight (MW) markers and the two phenotypes were visualized by staining the acrylamide gel with Coomassie Brilliant Blue (CBB). On the right, the two phenotypes were electroblotted onto nitrocellulose and reacted with a rabbit antiserum to the A-layer protein. Note the presence of the 50 kd A-layer protein in only the A^+ phenotype (B1), irrespective of whether CBB or specific antibody was used to visualize the reaction.

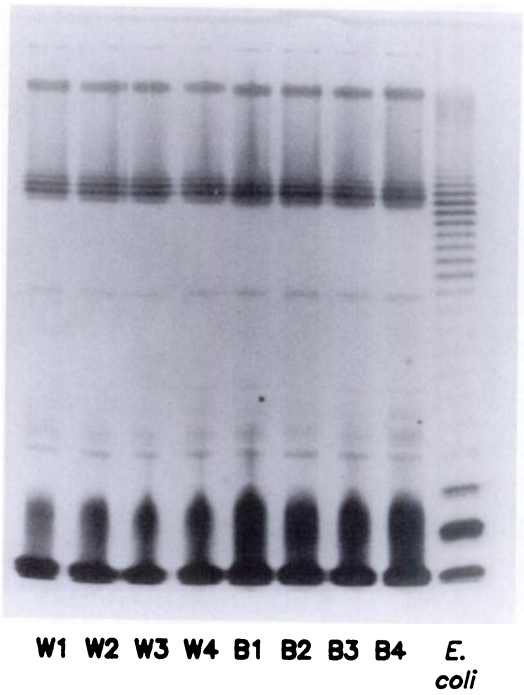

FIGURE 3. SDS-PAGE of proteinase K treated preparations of the A^- (W1–4) and A^+ (B1–4) phenotypes of *Aeromonas salmonicida*, isolate 3.10. Commercial preparations of purified lipopolysaccharide (LPS) from *E. coli* were used as references. LPS was visualized by a modified silver stain procedure as discussed in the text.

the additional 50 kd A-layer protein in the blue colonies. Because Evenberg et al. (1985) proposed that there is a close association between A-layer protein and the repeating O-antigen sub-units of the lipopolysaccharide, LPS electropherograms of pure A^+ and A^- phenotypes of isolate 3.10 were also examined, but no differences were observed. This information suggested that the differences in colony types discussed throughout the present study could be attributed to the selection for the A-layer protein.

The A-layer has received prominent attention as a significant virulence factor of *A. salmonicida*. Duff (1937) was historically the first to demonstrate that a correlation existed between virulence and the ability of an isolate to clump in saline; namely, its relative hydrophobicity. This physical characteristic of virulent isolates was later attributed to the presence of the A-layer protein (Udey and Fryer, 1978; Trust et al., 1983). These observations formed our original criteria for selecting the hydrophobic-clumping and hydrophilic-non-clumping groups of isolates used in the present study.

Examination of hydrophobic isolates by growth on CBB agar showed that they were totally composed of the A^+ phenotype and that all such isolates were virulent in brook trout. However, some hydrophilic isolates, which required a minimum concentration of 50% $(NH_4)_2SO_4$ to precipitate the bacteria from suspension, contained $<10\%$ of the virulent A^+ phenotype. Consequently,

these isolates did not produce mortality in any of the injected fish nor did they induce any of the gross clinical signs of disease associated with furunculosis. Although no strains were studied that had an intermediary level of the A^+ phenotype, the results suggested that a graded state of virulence may exist depending on the frequency of the A^+ phenotype.

Other researchers have noted that certain conditions, including passage on culture media or increased incubation temperatures, can cause a loss of virulence that coincides with a decrease in autoagglutination (Phipps et al., 1983). Ishiguro et al. (1981) associated these phenomena with a decreased ability of the bacterium to produce the A-layer.

By contrast, virulence may be enhanced in *A. salmonicida* by passing bacteria in a susceptible host (McCarthy, 1976). Such passage reverses the trends toward avirulence that were mentioned above, and upon reisolation from a dead or moribund fish, the bacteria generally show increased autoagglutination indicative of a selection for the A^+ phenotype. In our study, we observed that only isolates with a high percentage of the A^+ phenotype produced disease and could be reisolated from dead fish. The avirulent isolates used in this study had such a low percentage of the A^+ phenotype (0 to 7%) that the bacteria were apparently overwhelmed by the host's immunological defenses and of little consequence to the injected fish. This was indicated by a complete inability to reisolate such organisms from challenged hosts. Anderson (1972) also noted such an inability to reisolate non-clumping isolates of *A. salmonicida* from injected fish. Because of this rapid clearance from the fish, it would not be possible to enhance the virulence of such isolates by passage in a susceptible host. Furthermore, if the A-layer is the principal virulence determinant in *A. salmonicida*, our data suggest that a certain threshold level of the A^+ phenotype must already be present to enhance virulence by passage through fish.

Challenge work performed with *A. salmonicida* isolate 3.10 in this study and in other work (Cipriano et al., 1981) demonstrated that the isolate was totally avirulent for fish and could not be reisolated from a challenged host. Because CBB agar could first be used to detect a relatively small number of the A^+ phenotype in this isolate and then be used as a selective agent to clone that phenotype, it was possible to reclaim the virulence of the bacterium. Our experiments with this isolate therefore identified a hitherto undescribed means of reclaiming virulence in isolates of *A. salmonicida* that expressed an extremely low percentage of the A^+ phenotype.

Much information is available on the equivocal nature of producing efficacious vaccines against furunculosis, and recent studies have addressed the importance of the A-layer as an immunogen (McCarthy et al., 1983; Olivier et al., 1985). The drift in the nature of the A-layer within an isolate depends especially on conditions of culture (Ishiguro et al., 1981). When nonselective media (e.g., trypticase soy agar, brain heart infusion agar) are used in the establishment of bacterin seeds, the researcher may be totally unaware of the frequency and disposition of the A-layer inherent within the isolate at hand. Using CBB agar, the researcher can completely remove such obstacles when pure A^+ clones of *A. salmonicida* are required.

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