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Congo Red Agar, a Differential Medium for *Aeromonas salmonicida*, Detects the Presence of the Cell Surface Protein Array Involved in Virulence

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Strains of the fish pathogen *Aeromonas salmonicida* which possess the cell surface protein array known as the A-layer (A^+) involved in virulence formed deep red colonies on tryptic soy agar containing 30 μg of Congo red per ml. These were readily distinguished from colorless or light orange colonies of avirulent mutants lacking A-layer (A^-). The utility of Congo red agar for quantifying A^+ and A^- cells in the routine assessment of culture virulence was demonstrated. Intact A^+ cells adsorbed Congo red, whereas A^- mutants did not bind Congo red unless first permeabilized with EDTA. The dye-binding component of A^+ cells was shown to be the 50,000-M_r A-protein component of the surface array. Purified A-protein avidly bound Congo red at a dye-to-protein molar ratio of about 30 by a nonspecific hydrophobic mechanism enhanced by high salt concentrations. Neither A^+ nor A^- cells adsorbed to Congo red-Sepharose columns at low salt concentrations. On the other hand, A^+ (but not A^-) cells were avidly bound at high salt concentrations.

Aeromonas salmonicida is the causative agent of the fish disease known as furunculosis (11). The typical strains of this organism are primarily pathogens of salmonid species. The so-called atypical strains differ from the typical strains in a variety of taxonomic criteria and also have more fastidious growth requirements (11). They also appear to cause disease in a wider variety of fish species than typical strains.

A cell surface protein array designated as A-layer is absolutely required for virulence since isogenic mutants lacking A-layer are avirulent (4, 17). The principal component of the A-layer is a 50,000-M_r protein (A-protein), which has been purified and characterized (3, 6, 14). Comparative studies indicate a high degree of conservation in the primary structures of A-proteins from typical and atypical strains of diverse origins (7). The A-layer is contiguous and appears to shield the outer membrane completely, and this may be the basis for its role as a virulence factor. Thus, the A-layer protects cells from the bactericidal activity of serum in in vitro assays (12). It is also notable that cells endowed with A-layer (A^+) exhibit a markedly enhanced ability to associate with in vitro-cultured fish and mouse macrophages as compared to isogenic mutants (A^-), which lack A-layer (16). It is not yet known whether this enhanced ability of A^+ bacteria to associate with macrophages is significant in the disease process, but it is possible that *A. salmonicida* is a facultative intracellular pathogen capable of surviving within phagocytes. Therefore, on the basis of available data, it would appear that A-layer confers virulence by providing the bacterium with a protective barrier against the defense mechanisms of the fish host.

A. salmonicida readily loses virulence during routine laboratory maintenance and culture. For example, growth temperature is an important factor in the loss of virulence of *A. salmonicida* because A^- mutants have higher maximum growth temperatures than their A^+ parent strains (4). The maximum growth temperatures of numerous A^+ strains from diverse sources studied in our laboratory range from 18 to 26°C (4; unpublished data). In contrast, A^- mutants derived from these strains grow optimally at 30°C. Thus, it was of practical necessity to devise quantitative methods for routinely differentiating A^+ and A^- bacteria to assess the virulence of cultures used for experimentation. We have already demonstrated the efficacy of using bacteriophages which specifically adsorb to *A. salmonicida* outer membrane receptors beneath the A-layer for this purpose (4, 5). Thus, the proportion of A^+ cells in a culture could be estimated by determining the proportion of phage-resistant cells in the culture. Although accurate estimations are obtained by this technique, we have devised a more convenient method and sought to elucidate the molecular basis of it. Here we report that colonies of A^+ bacteria can be directly distinguished from those of A^- bacteria on Congo red agar and that the 50,000-M_r A-protein is the A-layer component responsible for binding Congo red.

MATERIALS AND METHODS

***A. salmonicida* strains and growth conditions.** A^+ strains A449, A450, A451, and A464 and spontaneous A^- mutants derived from them (strains A449-3, A450-3, A451-3, and A464-3, respectively) were typical *A. salmonicida* strains from our laboratory collection. All of the A^- mutants were selected by growth at 30°C as previously described (4). Atypical A^+ strains A400 and A419 and A^- strains A401 and A403 were also from our collection. Bacteria were grown at 20°C in tryptic soy broth (TS broth; Difco Laboratories, Detroit, Mich.).

Use of Congo red agar. Tryptic soy agar (TS agar, Difco) containing 30 μg of Congo red (MCB Manufacturing Chemists, Inc., Cincinnati, Ohio; lot no. CX1905) per ml was used to differentiate A^+ and A^- strains. Bacteria were grown as indicated in the text. Dilutions of the cultures were plated on TS-Congo red agar. The plates were incubated at 20°C, i.e., at a temperature which permitted colony formation by both A^+ and A^- cells.

Phage selection of A^+ bacteria. A^+ cells were selected from mixtures of A^+ and A^- cells using *A. salmonicida* phage strain 37. The details of this experiment are described

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elsewhere (4). Phage 37 adsorbs to a multicomponent outer membrane receptor which is masked by the A-layer (unpublished data). Thus, A^+ cells are phage 37 resistant whereas A^- cells are phage sensitive. Briefly, flasks containing 10-ml quantities of TS broth were inoculated with A^+ strain A451 at initial cell densities of 5.5×10^7 cells per ml. The flasks were incubated in gyratory water bath shakers at the indicated temperatures for 48 h to final cell densities which ranged from 1.4×10^9 to 4.3×10^9 cells per ml. The number of A^+ cells and the total number of viable cells (i.e., A^+ and A^- cells) in these stationary-phase cultures were determined by plating dilutions of the culture on TS agar with and without phage 37 (2×10^8 PFU per plate), respectively.

Outer membrane preparation and purification of A-protein. Outer membranes were prepared by differential solubilization of cell envelopes with sodium lauryl sarcosinate as previously described (6). A-protein was purified as described by Phipps et al. (14).

Congo red binding to intact cells. TS broth stationary-phase cultures were harvested by centrifugation at $4,000 \times g$ for 10 min at 0°C and suspended in sterile saline. Indicated amounts of cells were incubated in 3 ml of saline containing 15 μg of Congo red per ml. After 5 min of incubation at room temperature, the cells and bound Congo red were removed by centrifugation at $27,000 \times g$ for 5 min. The unbound Congo red in the supernatant fluid was quantified spectrophotometrically at 480 nm.

Congo red binding to purified A-protein. Indicated amounts of A-protein were incubated in 200 μl of 20 mM Tris hydrochloride (pH 7.5) containing 10 μg of Congo red per ml. After 10 min at room temperature, 1 ml of saturated $(NH_4)_2SO_4$ was added, and the mixture was kept at 0°C for 60 min to precipitate the dye-protein complex. The precipitate was removed by centrifugation at $5,000 \times g$ for 3 min. The unadsorbed dye was then measured at 480 nm. Protein-free blanks were routinely included. The $(NH_4)_2SO_4$ precipitation step was omitted in one set of experiments to minimize the effect of hydrophobic interactions. In this case, the protein-dye complexes were separated from unbound dye on Sephadex G-25 (Pharmacia) columns (1-ml bed volume).

Preparation of Congo red-Sepharose. Congo red was cross-linked to CNBr-activated Sepharose 6 MB (Pharmacia). The gel (2 g, dry weight) was swollen and washed several times with 200-ml volumes of 10^{-3} M HCl. Congo red (20 mg) dissolved in 0.1 M $NaHCO_3$ was coupled to the gel for 2 h at room temperature and overnight at 4°C. The coupled gel was then washed with 0.1 M $NaHCO_3$, and unreacted groups were blocked with 1 M ethanolamine for 2 h. Noncovalently bound dye was removed by several washes with 95% ethanol, and the gel was washed once more with 50 mM Tris hydrochloride (pH 7.0), leaving a red-colored matrix.

RESULTS AND DISCUSSION

Colonies formed by A^+ and A^- derivatives of typical *A. salmonicida* strains can be clearly differentiated on TS agar containing Congo red (Fig. 1). The four unrelated A^+ strains tested all formed deep red colonies on this medium. In contrast, colonies of A^- derivatives of these strains were colorless or, occasionally, light orange. This distinction was obvious after 48 h of incubation at 20°C and remained so for at least 2 weeks if the plates were stored under refrigeration. Prolonged incubation also enhanced the color intensity. The optimum concentration of Congo red was 30 μg/ml. Most of the atypical strains in our collection failed to grow on TS agar. However, in the few cases where growth occurred (strains A400, A401, A403, and A419), the results were identical to those obtained with the typical strains. Thus, it is likely that, in appropriate media, Congo red could be used to differentiate A^+ and A^- derivatives of atypical strains as well, and work on this theory is continuing.

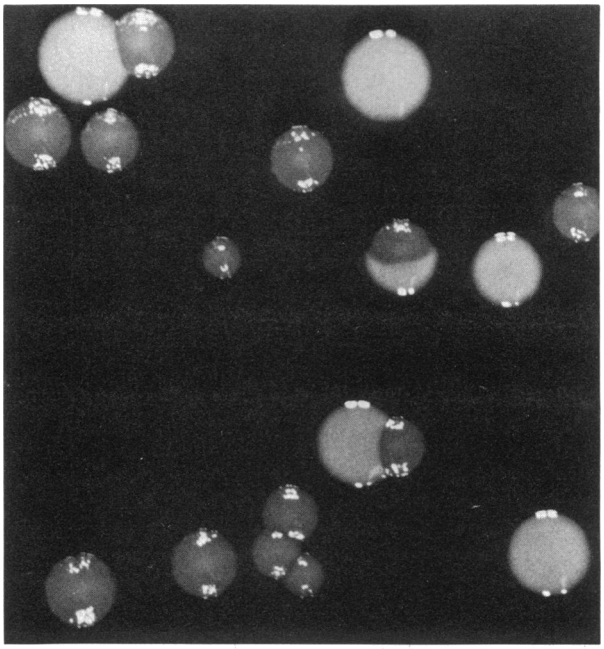

FIG. 1. Colonies of *A. salmonicida* A^- mutant strain A451-3 and its A^+ parent strain, A451, on TS-Congo red agar after 3 days of incubation at 20°C. The colonies of A451 are dark and generally smaller than the light colonies of A451-3.

The Congo red medium and the phage selection technique (4) were compared as means of differentiating A^+ and A^- bacteria. For this purpose the effect of growth temperature on the selection of A^- mutants was determined. As already mentioned, growth temperature is important in the loss of virulence of *A. salmonicida* because A^- mutants have higher maximum growth temperatures than their A^+ parent strains (4). TS broth cultures of strain A451 (A^+) were grown to stationary phase at various temperatures, and the proportions of A^+ and A^- cells in these cultures were determined by plating dilutions on TS-Congo red agar. In addition, the dilutions were plated on TS agar with and without *A. salmonicida* bacteriophage strain 37 (2×10^8 PFU per plate). The data in Fig. 2 confirm previous results (4) indicating that the culturing of *A. salmonicida* at high temperature resulted in the selection of avirulent A^- mutants. In fact, a single subculture of strain A451 at 28°C or higher caused essentially complete loss of virulence and of the A-layer. Figure 2 also shows that the results with TS-Congo red agar were in close agreement with those obtained with the phage selection technique. Thus, the virulence of *A. salmonicida* cultures ascribed to the A-layer can be simply and very reliably assessed on TS-Congo red agar.

Preliminary experiments indicated that A^+ bacteria rapidly adsorbed Congo red from solution. Dye adsorption was essentially instantaneous, and the time course could not be easily followed. The cell density dependence of Congo red adsorption by A^+ and by A^- bacteria was compared (Fig. 3). Strain A451 (A^+) clearly adsorbed Congo red more effectively than its A^- derivative, strain A451-3.

The adsorption of Congo red by outer membrane preparations from A^+ strains A449, A450, and A451 and from A^-

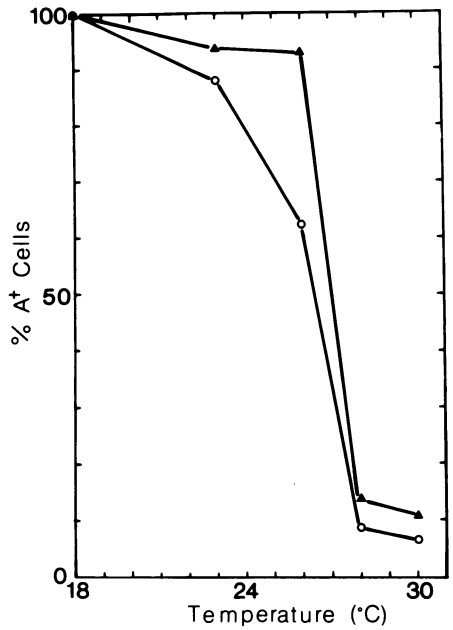

FIG. 2. Effect of growth temperature on the selection of A^- mutants. The proportions of A^+ cells in stationary-phase cultures of A^+ strain A451 grown at the indicated temperatures were determined by use of TS-Congo red agar (▲) and by the phage selection method (○) as outlined in the text.

derivatives of these strains (A449-3, A450-3, and A451-3, respectively) was also determined. All A^+ and A^- cell outer membrane preparations bound Congo red more or less equally (data not shown), suggesting that the dye readily partitions into these disrupted membranes. Furthermore, there were no obvious differences in the Congo red-binding capacities of A^+ and A^- cell envelopes. Thus cellular integrity appeared to be required for differential Congo red binding. Further support for this idea is shown in Fig. 4, which shows the effect of altering the integrity of the outer membrane of whole cells of A^- strain A451-3 on Congo red binding. Treatment of cells with EDTA resulted in a marked increase in Congo red adsorption. EDTA is known to disrupt the outer membrane structure of gram-negative bacteria and to remove as much as 50% of the lipopolysaccharide (9), rendering the cells permeable to various compounds (8). This treatment is also know to render *A. salmonicida* sensitive to lysozyme (unpublished data). In the Congo red-binding experiments with whole cells described above (Fig. 2), A^- cells were found to still bind small amounts of Congo red. Centrifugation of these cell suspensions resulted in a largely colorless pellet with an overlying thin, dark red layer of cells. Under the same conditions, A^+ cells formed a uniform dark red pellet. Therefore, it appeared that most of the Congo red bound by A^- cells were taken up by a small fraction of the population consisting of relatively buoyant cells. We suspected, but were not able to prove, that this small subpopulation represented cells with damaged or altered outer membranes.

Congo red has been incorporated into growth media to permit differentiation of virulent and avirulent strains of a variety of gram-negative bacteria, including *Yersinia* and *Shigella* species as well as *Vibrio cholerae*, *Escherichia coli*, and *Neisseria meningitidis* (10, 13, 15). In these cases, virulent cells adsorb Congo red from the medium and form red colonies, whereas avirulent cells do not. However, the unique Congo red-binding sites in these bacteria have not

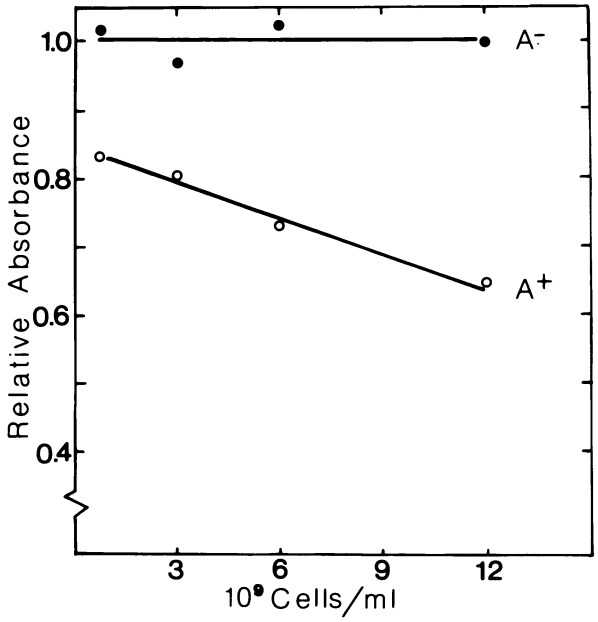

FIG. 3. Adsorption of Congo red by intact cells of A^+ strain A451 (○) and A^- strain A451-3 (●). Indicated amounts of cells were incubated in saline containing Congo red. After removal of the cells by centrifugation, the absorbances of the unbound dye in the supernatant fluids were measured at 480 nm. A relative absorbance of 1 was equal to a sample incubated without cells.

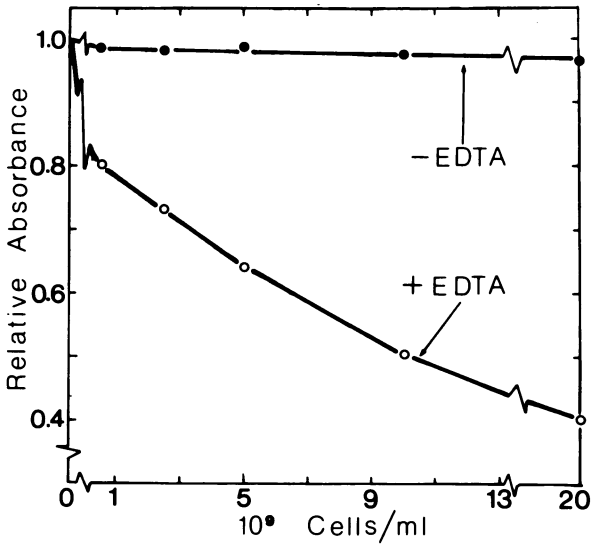

FIG. 4. Effect of alteration in outer membrane permeability on the adsorption of Congo red by A^- strain A451-3. A culture of A451-3 was divided into two equal portions. One portion (○) was assayed for Congo red adsorption as described for Fig. 3. The cells in the other portion were incubated in 50 mM Tris hydrochloride buffer (pH 8.0) containing 50 mM EDTA for 15 min. The cells were collected by centrifugation, suspended in sterile saline, and then assayed for Congo red adsorption as described above (●). A relative absorbance of 1 was equal to a sample incubated without cells.

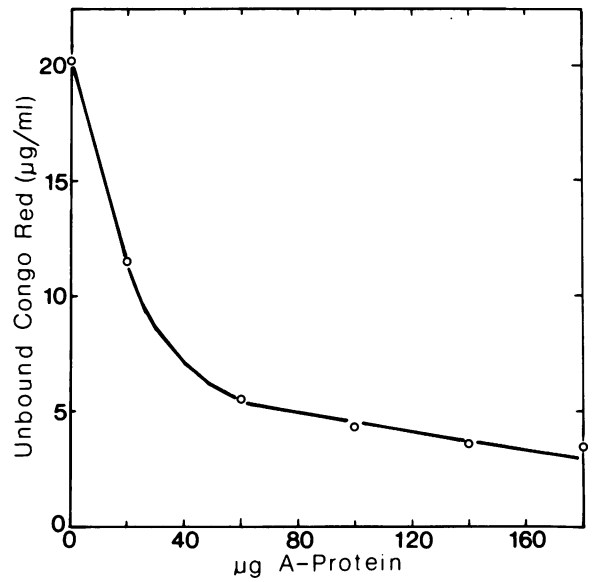

FIG. 5. Congo red binding by A-protein. Indicated amounts of purified A-protein were incubated with Congo red in 20 mM Tris hydrochloride (pH 7.5) for 10 min. The unadsorbed dye was measured at 480 nm after precipitation and removal of the dye-protein complexes with $(NH_4)_2SO_4$.

been identified, although the gene encoding the Congo red-binding component in *Shigella flexneri* has been recently cloned (2). To demonstrate the dye-binding component of A^+ cells, we incubated whole cells of strain A450 (A^+) with 1 μg of Congo red per ml for 10 min, washed the red cell pellet once with saline, and extracted the surface proteins with 0.2 M glycine buffer (pH 2.2). The principal component (~95%) of this fraction was composed of A-protein as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). Under these conditions greater than 90% of the bound Congo red was released along with the A-protein.

To further demonstrate the A-protein was the major protein responsible for dye-binding activity, pure A-protein was incubated with Congo red. The unbound dye was measured after precipitation of the dye-protein complex with $(NH_4)_2SO_4$. Figure 5 demonstrates the binding of Congo red as a function of A-protein concentration. At 20 μg of A-protein per ml, the molar ratio of dye to protein was about 30, indicating multiple dye-binding sites per protein monomer at this dye concentration. When the same experiment was repeated in the absence of $(NH_4)_2SO_4$ (by separating the protein-dye complexes from free dye on Sephadex G-25 columns), the dye-to-protein molar ratio was about 8, indicating that Congo red binding is enhanced by conditions favoring hydrophobic interactions, i.e., high salt concentrations. Congo red binding as a function of $(NH_4)_2SO_4$ concentration was also studied using intact cells (data not shown). In agreement with the results obtained with purified A-protein, Congo red binding by A^+ (strain A451) cells was enhanced at high salt concentrations. On the other hand, no such effect was observed with A^- (strain A451-3) cells.

The salt-dependent binding to Congo red was also demonstrated using a column of Congo red-Sepharose. A^+ (A451) cells suspended in 50 mM Tris hydrochloride (pH 7.0) failed to adsorb to Congo red-Sepharose. However, A^+ cells quantitatively adsorbed to this matrix in the presence of 1 M $(NH_4)_2SO_4$ and could be eluted by washing the column with 50 mM Tris hydrochloride (pH 7.0). In contrast, A^- (A451-3) cells did not adsorb to Congo red-Sepharose even in the presence of 1 M $(NH_4)_2SO_4$. This method has also proved useful in the isolation of A^- mutants by selectively adsorbing A^+ cells of mutagenized cultures to Congo red-Sepharose in the presence of 1 M $(NH_4)_2SO_4$ (unpublished data).

Evidence has been presented suggesting that the A-layer represents a refractile outermost barrier which is permeable to essential solutes but apparently to very little else (4, 6, 12). Some of the *O*-oligosaccharides of lipopolysaccharide extrude through the A-layer, and these appear to be the only other surface-exposed components in A^+ bacteria (1). However, the poor Congo red-binding capacity of A^- cells (which have their entire complement of *O*-oligosaccharides surface exposed) suggested that *O*-oligosaccharides were not involved. The experiments with A^- cells described in Fig. 3 also indicated that although the intact outer membrane had a low affinity for Congo red, dye-binding sites could be exposed by altering the integrity of the outer membrane. This may explain why cell envelopes from both A^+ and A^- bacteria bound Congo red with no obvious differences in binding capacities.

In summary, we have demonstrated that Congo red binding is a useful criterion for the differentiation of A^+ (virulent) and A^- (avirulent) strains of *A. salmonicida* and that the cell surface array A-protein is, for the most part, responsible for the binding characteristics. Furthermore, Congo red binding, or lack of it, is an indicator of cell surface structural integrity.

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