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SEROLOGICAL SPECIFICITY OF *AEROMONAS HYDROPHILA* AS MEASURED BY IMMUNOFLUORESCENCE PHOTOMETRIC MICROSCOPY

by

C. B. Fliermans and T. C. Hazen

Savannah River Laboratory
E. I. du Pont de Nemours and Company
Aiken, South Carolina 29801

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SEROLOGICAL SPECIFICITY OF *AEROMONAS HYDROPHILA* AS MEASURED BY IMMUNOFLUORESCENCE PHOTOMETRIC MICROSCOPY

C. B. Fliermans and T. C. Hazen[1]

Savannah River Laboratory
E. I. du Pont de Nemours and Company, Inc.
Aiken, South Carolina 29801

Strain specific fluorescent antibodies were prepared for three isolates of *Aeromonas hydrophila*. These antisera were reacted with 255 other *A. hydrophila* isolates, and their immunofluorescence was measured quantitatively by photometric fluorescence microscopy. Only 27.5% of the isolates reacted with the prepared antisera which indicated that other undetected serogroups are present. Statistical analyses indicated that the reactive *A. hydrophila* isolated from aquatic habitats was serologically distinct from the other isolates as a serogroup.

[1]Department of Biology,
Wake Forest University,
Winston-Salem, North Carolina 27109

Introduction

The use of immunofluorescence has significantly expanded identification, enumeration, and localization of specific microorganisms in both aquatic and terrestrial habitats (Fliermans *et al.* 1974; Fliermans and Schmidt 1975 ; Schmidt, *et al.* 1968; and Schmidt 1973). There are two basic approaches to the fluorescent antibody technique; direct and indirect. The direct approach relies on the production of an antibody against a given antigen and the coupling of that antibody with a fluorescent dye forming the fluorescent antibody (FA) complex. The antigen is then directly reacted with the FA under a specific set of conditions and observed by fluorescence microscopy. The fluorescing antigen-antibody complex is scored by a subjective measurement of fluorescent intensity in relation to background, thereby comparing the staining of an unknown antigen to the homologous system.

The indirect approach is a two-stage system whereby the antigen is first complexed with a homologous antibody. This antigen-antibody complex is then reacted with a fluorescently labeled antiglobulin, homologous to the unlabeled antibody used in the initial reaction. The indirect procedure allows the indentification of an unknown antigen as well as the titer of antibodies in an unknown serum. Both approaches require extensive controls to properly evaluate the immunofluorescence reaction (Schmidt 1973).

Immunofluorescence data derived from ecological studies have not been quantified other than by subjectively comparing specimen fluorescence with that of the background and/or other heterologous specimens (Bohlool and Schmidt 1970; Pugsley and Evison 1974 a, b ; Bohlool and Brock 1974). Such measurements of immunofluorescence do not allow the direct assessment of similarity among fluorescing bacteria. Because FA specificity may vary depending on the microorganism (Fliermans *et al.* 1974; Schmidt 1973) antigenically related microorganisms would be separated only when measured objectively.

This paper describes the preparation of antibodies for various strains of *Aeromonas hydrophila*, demonstrates the strain specificity of the antibodies by direct immunofluorescence, and describes the utilization of fluorescence photometry to measure differences and similarities among strains isolated from a variety of natural habitats.

Materials and Methods

Cultures

Stock cultures of *Aeromonas hydrophila* 7966, 19570; *A. liquefaciens* 14715; *A. proteolytica* 15338 and *A. salmonicida* 14174; *A. punctata* 14486, 15468; *A. formicans* 13137; *A. anaerogenes* 15467 were originally obtained from the American Type Culture Collection (ATCC). All axenic cultures were routinely grown in nutrient broth shake cultures at 37°C. Samples from aquatic habitats were filtered onto sterile membrane filters (0.45 μm; Millipore Corp. Bedford, MA) and placed on a medium (RS)

differential and selective for *A. hydrophila* (Shotts and Rimler 1973; Hazen *et al.* 1978). Samples (e.g., fish or alligator tissue, aquatic plants, and sediments) were plated directly on RS medium and incubated at 37°C for 20 hours. Isolates were confirmed as *A. hydrophila* using the profile recognition system for *Enterobacteriaceae* (API 20E) as described by Smith, *et al.* (1972).

Fluorescent Antibody Preparations

Cloned stock culture isolates of *A. hydrophila* 7966 and axenic field isolates from various habitats (*A. hydrophila* HDW1 - water; *A. hydrophila* B323L - lesion of large-mouth bass; *A. hydrophila* GLN1 - lung of an alligator; Gorden *et al.* 1979) were grown to a density of about 10^8 cells/ml for the preparation of individual antisera. Cells were harvested by centrifugation, washed three times in physiological saline, and resuspended in phosphate buffer (pH 7.2) to a Klett turbidity of 180 (green filter). The cell suspension was then heated in a boiling water bath for 60 minutes to inactivate fagellar antigens. Merthiolate (1:100000 final concentration) was added to the antigen suspension, and the suspension was injected intravenously into three New Zealand rabbits. After injections, test bleedings were made, and antibody levels were determined by tube agglutination. When agglutinating antibody titers for the homologous antigen were greater than 1:2560, antisera were harvested by cardiac puncture, pooled from among the triplicate rabbits, fractionated, and conjugated to fluorescein isothiocyanate isomer 1 (BBL, Cockeysville, MD). All procedures were described previously (Schmidt, *et al.* 1968).

Cross reactions between the various strains of *A. hydrophila* were measured using immunofluorescence reactions and tube agglutination titers. Strains B323L, HDW1, and GLN1 were strain specific; 7966 from ATCC was not. No absorption techniques were used to obtain strain specificity of *A. hydrophila* 7966 (Fliermans, *et al.* 1974). A large number of isolates lacking the colonial morphology and biochemical characteristics of *A. hydrophila* when grown on RS medium were reacted with the prepared FA to determine whether bacteria other than *Aeromonas* spp. demonsrated any cross reactions. The reactions were negative.

Photometric Fluorescence Microscopy

The basic fluorescent microscopy equipment used in these experiments was a Zeiss Photomicroscope III equipped with an epifluorescence condenser III RS with both barrier and exciter filters optimized for the excitation and emission of fluorescein isothiocyanate. Light path was from the high pressure 200-watt mercury arc lamp through a KG1 heat-absorbing filter and a BG-38 red supressor filter. Two exciter filters, LP455 and KP500 allowed transmission of light from 455 μm to 500 μm, since the maximum wavelength for absorption of FITC is 490 μm (Nairn 1969). Additionally, the LP510 chromatic filter transmitted light above 510 μm through an LP520 barrier filter to decrease background and enhance the maximum fluorescence of FITC at 520 μm (Nairn 1969). Planachromat oil immersion objectives 40x/1.0, 63x/1.4 and 100x/1.25 with variable numerical apertures were used for viewing specimens. Color photomicrographs were taken with GAF film ASA 200-24 Din with a 30-second exposure.

Immunofluorescence was measured quantitatively with A Zeiss Photometer-Indicator MPM01 connected to a photomultiplier cell capable of measuring fluorescence from a 0.5-μm-diameter particle. Both background fluorescence and FITC fluorescing specimens were measured with a 1-μm-diameter probe. Photometric indicator settings were as follows: damping control for averaging fluorescence display by digital readout, 0.5 (40 individual readings are averaged during 0.5 second); mode selector was in the % transmission mode; high voltage selector was optimized for sensitivity at a setting of 4 (approximately 850 volts); gain control for amplifier sensitivity was set at 100; and the zero suppression and concentration factor dials were in the off position. Individual cells were measured by first measuring the fluorescence intensity of the bacteria cell and dividing it by the background intensity. Simultaneously, cellular intensity was measured by conventional subjective methods (LaBrec, *et al.* 1959; Nairn 1969).

Results

Specificity of Prepared Antibodies

The data in Table 1 indicate that initial tests with the unconjugated antisera generally gave good agglutination titers and each conjugated antisera demonstrated excellent immunofluorescence staining reaction with its homologous antigen although the gross cellular morphology was different (Figure 1). Agglutination titers for *A. hydrophila* 7966 against the tested heterologous antisera lacked specificity both for tube agglutination and

immunofluorescence. Because of this lack of specificity three isolates of *A. hydrophila* (HDW1, B523L, and GLN1) were selected as biochemically representative of the isolates from the studied habitats, i.e., water, largemouth bass, and alligators, respectively. The FA prepared against each of these isolates was strain specific as demonstrated by a high degree of homologous straining with little cross reaction. Although gross cellular morphology was distinct (Figure 1), serotypes were separated by immunofluorescence.

The results from a series of experiments to determine the relationship between subjective fluorescence and photometric fluorescence measurements are shown in Table 2. Fluorescent antibodies prepared for a variety of bacteria with a broad range of specificity were reacted with a number of bacteria giving a variety of immunofluorescent reactions. The intensity of the fluorescence was measured both subjectively and quantitatively (photometric microscopy). The various antigen-antibody complexes demonstrated various degrees of fluorescence, and the distinction among the weak fluorescence (± to 2+) was clear photometrically but not always subjectively. Cells which reacted well with the FA (3+ and 4+) were readily distinguished from the weaker reactions and from each other. An additional level of reactivity (5+) was seen with some of the complexes. Although the number of determinations of highly reactive fluorescence were fewer, the photometric readings demonstrated good separation from the other reactions.

Over 350 different isolates of *A. hydrophila* from across the United States have been tested for differences in biochemistry, antibiotic sensitivity, and immunofluorescence relations (Hazen, *et al.* 1978). Antisera initially prepared against *A. hydrophila* 7966, the neotype strain from ATCC, was not species specific nor reactive with the majority of isolates from water, red-sore infected largemough bass, nor tissue from necropsied alligators (Fliermans, unpublished results). Subsequently, isolates typical of those from water, HDW1; red-sore lesions on largemouth bass, B323L; and from the lung of necropsied alligator infected with *Aeromonas*, GLN1; were used to prepare strain specific antibodies.

Numerous *A. hydrophila* isolates (255) were then tested against the prepared antibodies, and the serological relationships were compared quantitatively (Table 3).

Most of isolates were obtained from Par Pond which has previously been described (Fliermans, *et al.*, 1977). The samples and immunofluorescence reactions for each antigen-antibody complex have been condensed, and only the number of isolates tested, the mean, range, and standard error of the reactions are reported for each group of isolates (Table 3). Analysis of variance and Student Newman-Keul multiple range tests indicated that the mean of the immunofluorescence reaction for *A. hydrophila* from freshwater habitats stained with HDWI-FA were significantly different (0.05 level) from all other isolates. Additionally, all other groups of isolates did not react significantly with FA prepared against

B523L of G1N1. The data in Table 3 further indicate that in each group of isolates, a range of immunofluorescent reactions was observed. While 20.0%(51/255) of the isolates were highly reactive with a single FA, only H2ML3, K1W4 (water isolates) and B6031 (bass intestinal isolate) reacted strongly with all three prepared fluorescent antibodies. Seventy-three percent (185/255) the isolates did not react strongly with any of the prepared antisera.

Discussion

The date in Table 1 indicate the various antisera ~~that~~ were specific for selected strains of *A. hydrophila*. Each recently isolated strain was specific for its homologous system with minimal cross reaction as measured by tube agglutination and immunofluorescence.

Various homologous and heterologous antigen-antibody complexes were prepared and observed both subjectively and quantitatively using fluorescence microscopy and fluorescence photometric microscopy, respectively. Immunofluorescence reactions were quantified on 314 fluorescing antigen-antibody complexes using a variety of conjugated sera that were both specific and non-specific in their homologous staining characteristics (Table 2). Immunofluorescence was grouped initially by subjective observations any then by photometric readings. The antigen-antibody complexes generally were placed in one of six reactive groups. Such grouping was confirmed by quantitative photometry. An additional 5+ group was observed only by photometry and did not appear brighter than the 4+ grouping using subjective

measurements. Standard errors among the groups were low and allowed distinctions between related microorganisms with varying degrees of fluorescence. These initial measurements were used to evaluate 255 isolates of *A. hydrophila* for serological relatedness as measured by fluorescence photometric microscopy.

Previous studies by DeFigueiredo and Plumb (1977) on the strain virulence of *A. hydrophila* to catfish (*Ictalurus punctatus*) suggested that a variety of strains may exist in nature. Although only nine different isolates of *A. hydrophila* were tested, those isolated from water were significantly less virulent than strains from diseased fish, even though all nine isolates were biochemically identical.

A major problem in characterization of a large number of isolates is the relationship of those isolates to the habitat from which they were obtained. We have arbitrarily designated HDW1 as a serotype for a water isolate. The chosen *A. hydrophila* may be transient in the water, and may have been expelled (e.g., from a bass or alligator) before its isolation. Therefore, the three isolates chosen as reference samples from a given habitat may not be, in fact, an intergral part of that habitat. All three of the isolates demonstrated similar biochemical properties as measured by API-20E (Anatabs, Inc.) and similar antibiotic sensitivity. Single linkage cluster analyses of the biochemical and antibiotic characteristics demonstrated a 90% similarity for all the isolates tested (Hazen, unpublished results).

Our data demonstrate that at least three serological groups of *A. hydrophila* do occur and appear simultaneously in natural habitats. These serogroups can be differentiated quantitatively by immunofluorescence even when biochemical or antibiotic sensitivity is the same. Because 72.5% of the *A. hydrophila* isolates did not react with any of the prepared antisera, serogroups other than those described are probably present in the natural habitats. The data further indicate that the large number of water isolates which were tested are significantly different from the isolates taken from bass or the American alligator. The utilization of fluorescence photometric microscopy allows the serological typing of a particular microorganism for the assessment of population dynamics and spatial distribution.

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Table 1. Tube agglutination titers and quantitative fluorescent antibody test reactions of various strains of *Aeromonas hydrophila*

Antigen	Antiserum	Agglutination titer	Immunofluorescence Subjective Reaction	Immunofluorescence Quantitative Reaction[a]
A. hydrophila 7966	7966	> 2,560	4+	9.80
	HDW1	1,280	2-3+	4.40
	B323L	160	±	1.00
	GLN1	640	2+	3.10
	Normal Sera	< 20	neg	1.00
A. hydrophila HDW1	7966	320	1+	1.80
	HDW1	> 2,560	4+	10.30
	B323L	80	neg	1.00
	GLN1	160	+	1.20
	Normal Sera	< 20	neg	1.00
A. hydrophila B323L	7966	80	±	1.10
	HDW1	80	neg	1.00
	B323L	> 2,560	4+	8.90
	GLN1	320	1+	2.00
	Normal Sera	< 20	neg	1.00
A. hydrophila GLN1	7966	320	1+	1.10
	HDW1	160	+	1.30
	B323L	320	1+	2.00
	GLN1	> 2,560	4+	10.70
	Normal Sera	< 20	neg	1.00

a. Ratio of cell fluorescence to background fluorescence.

Table 2. Measurements of immunofluorescence using photometric microscopy

Subjective Fluorescence Reading	Number of Determinations	Quantitative Photometer Reading
±	52	1.41 ± 0.04[a]
1+	53	1.99 ± 0.05
2+	57	2.86 ± 0.06
3+	53	5.03 ± 0.10
4+	58	9.82 ± 0.13
5+	41	20.1 ± 0.44

a. One standard error

Table 3. Composite of *A. hydrophila* isolates reacted with three separate specific fluorescent antibodies

Isolates: Source	Number Stains Tested	Antisera: HDW1	B323L	GLN1
		Photometric Reading		
ATCC	9	2.11±0.75[a] (1.11-5.00)[b]	1.77±0.24 (1.00-3.20)	1.99±0.40 (1.00-4.35)
Surface Slime Largemouth Bass	33	1.96±0.14 (1.05-5.44)	2.72±0.51 (1.00-13.70)	1.81±0.24 (1.00-6.00)
Intestine Largemouth Bass	49	2.91±0.40 (1.07-19.00)	2.58±0.31 (1.00-10.43)	2.61±0.42 (1.00-13.00)
Lesion on Largemouth Bass	35	2.27±0.39 (1.09-11.50)	2.27±0.25 (1.00-6.63)	2.34±0.31 (1.00-8.75)
American Alligator	21	2.20±0.50 (1.00-11.40)	3.13±0.56 (1.05-10.31)	3.86±0.92 (1.02-18.00)
Fresh Water	108	5.31±0.41[c] (1.03-24.29)	2.62±0.23 (1.00-17.18)	2.19±0.22 (1.00-14.20)

a. Mean ± standard error

b. Range of fluorescence measured

c. Antisera significantly different from ~~isolates~~ the two other tested antisera at 0.05 level using Student Newman-Keul test

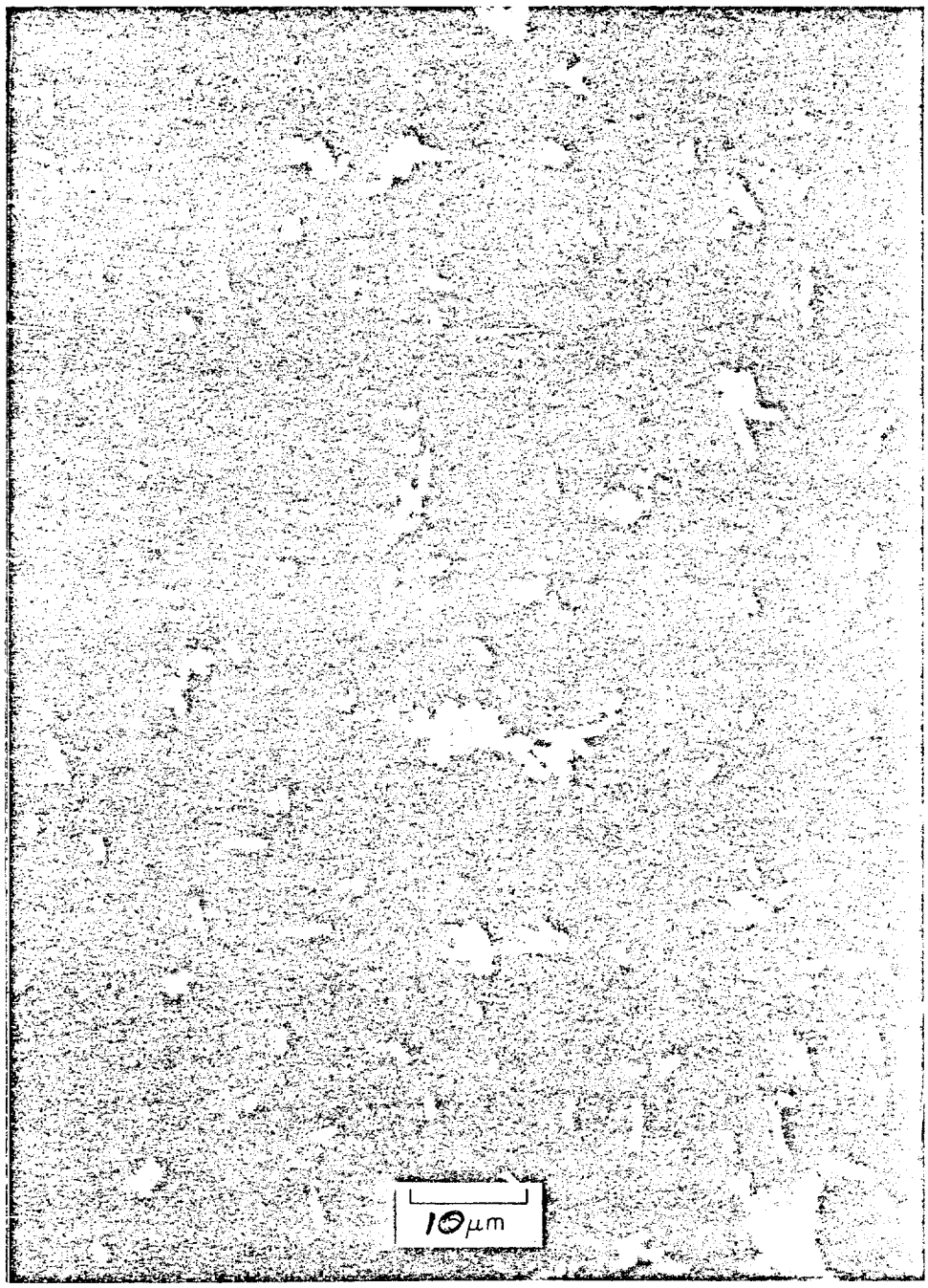

Fig. 1a. Strain specific *A. hydrophila* HDW1 fluorescent antibody staining the homologous system.

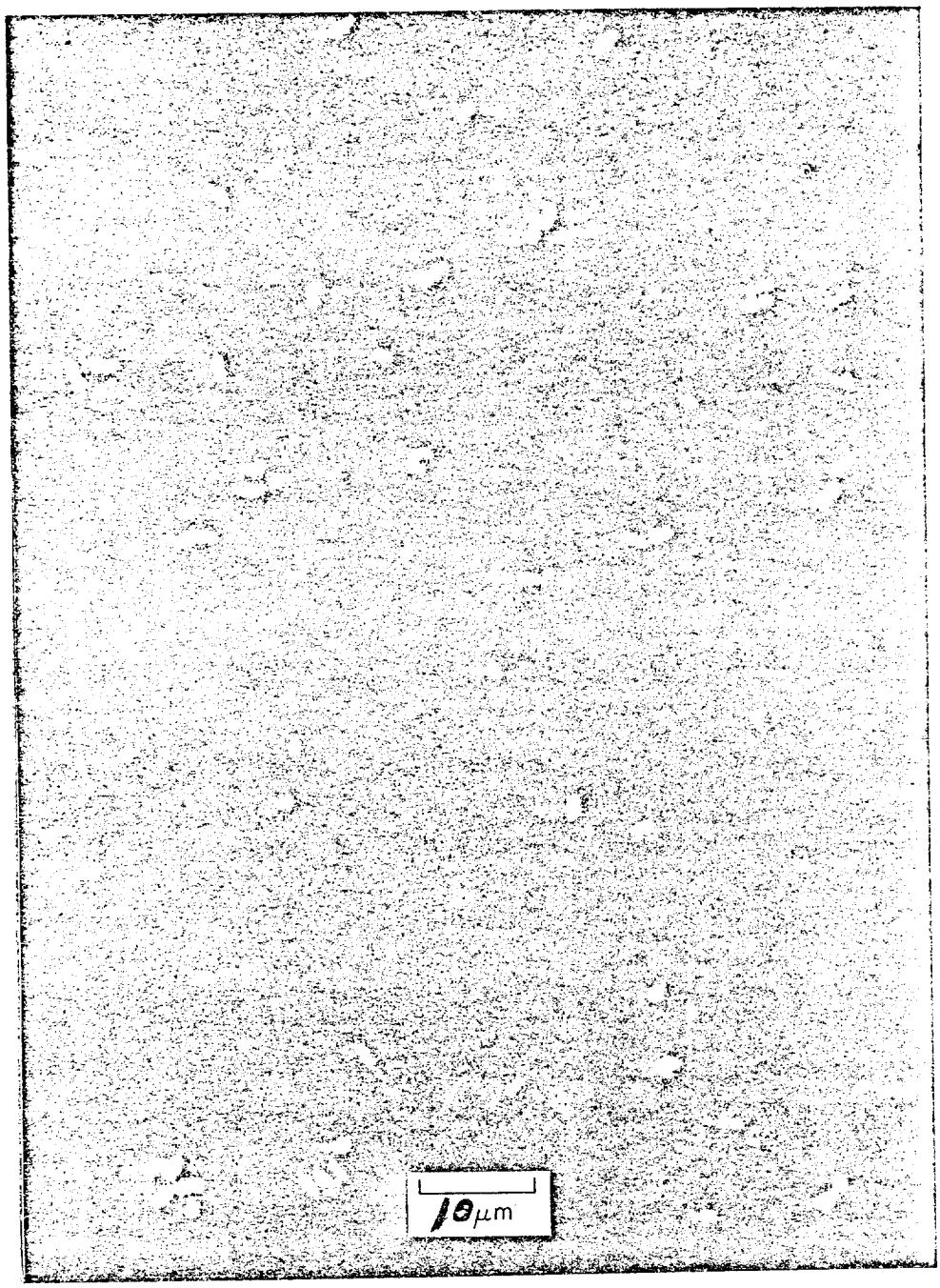

Fig. 1b. Strain specific *A. hydrophila* B323L fluorescent antibody staining the homologous system.

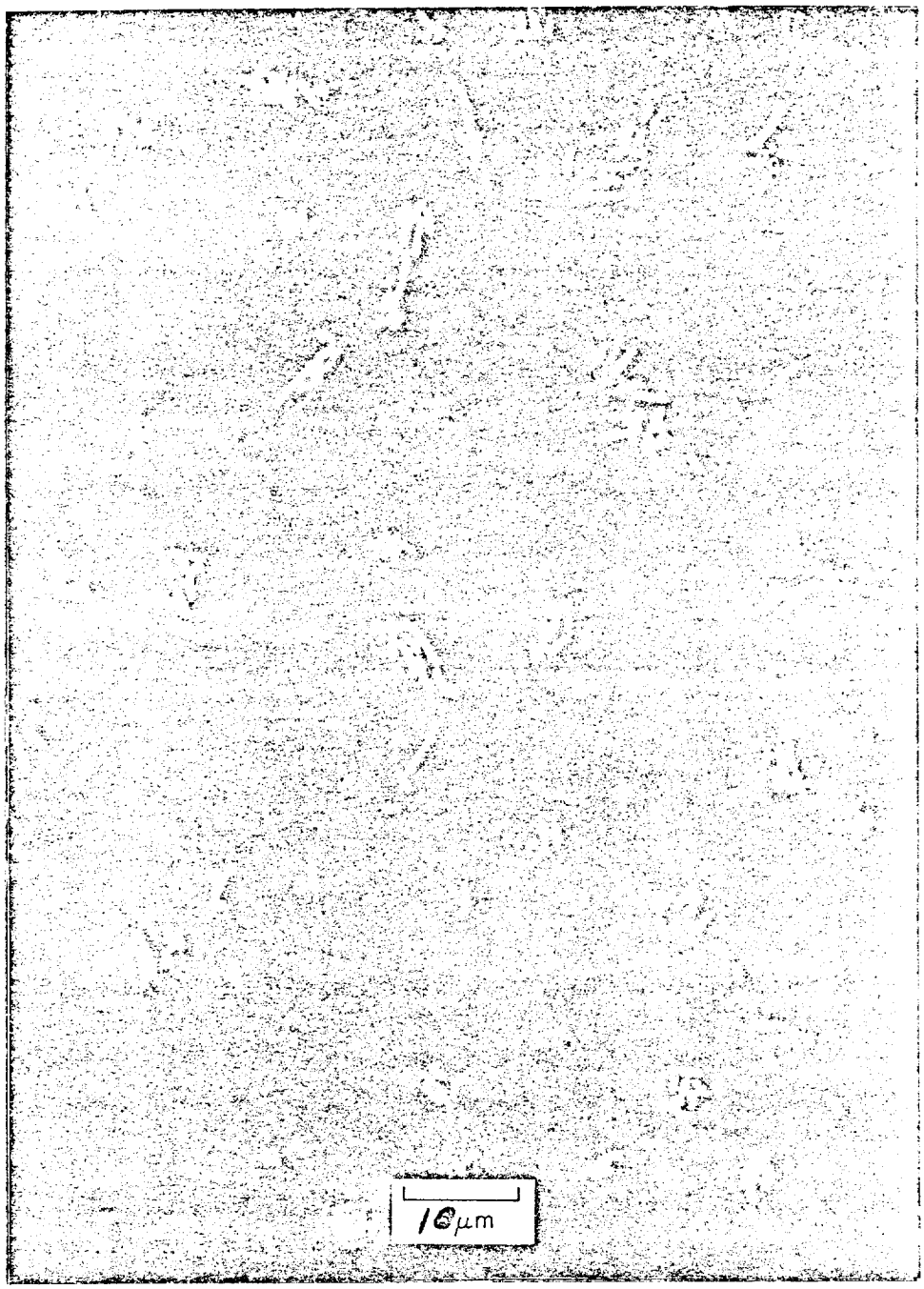

Fig. 1c. Strain specific *A. hydrophila* GLN1 fluorescent antibody staining the homologous system.

CC: D. M. Peek, DOE-SR
R. E. Naylor, Wilm.
J. P. Glas-J. A. Porter, SRL
T. V. Crawford
C. B. Fliermans
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