DP-MS-80-107

CONF-810313--1

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A paper prepared for presentation to the American Society of Microbiology in Dallas, Texas, on March 3-6, 1981, and for publication in Current Microbiology.

This paper was prepared in connection with work under Contract No. DE-AC09-76SR00001 with the U.S. Department of Energy.

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ABSTRACT - Detection of Legionella pneumophila by serogroup-specific fluorescent antibodies was combined with a tetrazolium dye (INT) to measure electron transport activity. The biological uptake and reduction of the INT dye was studied in pure cultures and in natural water samples with respect to temperature. Uptake was complete within 60 minutes. Controls inhibited with formaldehyde demonstrated little activity. Both the in vitro and in situ determinations suggested that the electron transport system of Legionella was active over a temperature range of 25° to 60°C.

INTRODUCTION

Autecological studies of the bacterium causing Legionnaires' Disease, Legionella pneumophila, have been hindered by the lack of a selective enrichment medium for isolating the bacterium from habitats heavily contaminated with a mixed microbial fauna. Serogroup-specific fluorescent antibodies provide a highly selective, sensitive, and direct method for studying the bacterium in situ.[1,5]

The direct fluorescent antibody technique permits the simultaneous detection, identification, and enumeration of a particular bacterium in a wide variety of habitats with the disadvantage that dead but intact cells stain equally well.[13] For environmental studies, it is necessary to know not only if a particular habitat is heavily contaminated with L. pneumophila, but whether the bacteria are active. Without the availability of a selective medium, studies have relied on guinea pigs to act as selective agents.[4,5] Such a procedure is expensive, time-consuming, and host-dependent.

Previous work demonstrated that one could combine techniques of autoradiography and specific fluorescent antibodies (FA).[7] Although this method was time-consuming and lacked general utility, it was useful in assessing the incorporation of known compounds into a selected bacterial population under defined environmental conditions. Because of the above limitations, an effective, simple, and useful method was sought to allow one to distinguish metabolically active cells from nonactive ones.

Recent advances by Zimmerman, et al.,[19] using a tetrazolium dye, 2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride (INT), for measuring electron transport system (ETS) activity of procaryotic microorganisms from marine waters, indicated such a technique was applicable to autecological studies of L. pneumophila. The technique makes use of an active ETS which is generally a component of respiring bacteria.[12,19] The oxidized form of the dye

acts at the ubiquinone cytochrome-b complex level in the electron transport system to form the reduced INT compound, formazan.[11,12] Provided the system is active in procaryotic cells, the INT is reduced to an INT-formazan crystal which is deposited as a dark red intracellular spot. Simultaneous viewing of the INT-formazan crystals by transmitted bright field microscopy and the specifically stained fluorescing L. pneumophila by epifluorescence microscopy allows one to observe specific cells. This technique can determine whether the cells are active or not with respect to the electron transport function. The combination of ETS activity and specific fluorescent antibodies for L. pneumophila allows the extension of physiological studies under in situ conditions of a bacterium for which no satisfactory selective or enrichment medium has been developed.

MATERIALS AND METHODS

Legionella pneumophila Cultures

Isolates of L. pneumophila belonging to serogroups 1 through 4 were obtained from lakes as previously described.[4,5] These isolates were stored at -75°C on standard charcoal-yeast-extract (CYE) agar.[2,3] Pure cultures were grown in shaken flasks at 37°C with modified buffered yeast extract broth (BYEB; Feeley, personal communication).

Laboratory Studies

Initial time course incubations for INT uptake were carried out at 37°C using 10.0 mL aliquots of log and stationary phase cultures. These aliquots were transferred into a series of triplicate vials containing 1.0 mL of a 0.2% (aqueous solution) of INT. Incorporation of INT was stopped by the addition of formaldehyde to a final concentration of 0.1%. Formaldehyde-killed negative controls were simultaneously run with each experiment. These time courses were conducted with serogroups 1 through 4 in various stages of growth from early log to late stationary. The data for SRP-36 (serogroup 2), typical of the other three serogroups tested, are reported in Figure 1. Following the designated incubation periods, 10λ sub-samples were appropriately diluted in phosphate buffered saline (PBS), placed on toxoplasmosis slides, heat fixed, stained, and washed as previously described.[4]

Stained L. pneumophila were viewed simultaneously with epifluorescence and bright field microscopy for FA and INT preparations. Although viewing of the ETS active cells was clear, it was difficult to take quality photomicrographs. In order to determine the percentage of L. pneumophila that had active ETS, 300 fluorescing cells were counted per sample and the percent of L. pneumophila containing formazan crystals was calculated.

Temperature Transfer Experiments

Log phase cultures of L. pneumophila were grown at 37°C and transferred without washing into vials and placed in constant temperature water baths. Cells were equilibrated to temperature with a 20 minute preincubation period before INT was added, and subsamples were taken over an 80 minute time course. Incorporation of INT was stopped with the addition of formaldehyde to a final concentration of 0.1%. Subsequent time course experiments were incubated with INT for 60 minutes. The effect of temperature on the recovery of L. pneumophila SRP-22 onto a complex medium was determined. After 40 minutes of incubation without INT at several of the elevated temperatures, serial 10-fold dilutions were prepared with sterile modified BYEB medium, and aliquots plated in triplicate onto CYE agar. All recovery experiments were incubated at 37°C in candle-extinction jars. Colony forming units (cfu) were counted after 48 hours of growth.

FIELD STUDIES

Field studies were conducted at the Savannah River Plant (SRP), a National Environmental Research Park, operated by E. I. du Pont de Nemours & Co. for the Department of Energy. The specific study site was the Par Pond system, which is a 2700 acre lake and canal system for cooling a nuclear production reactor.[6,15]

A man-made thermal gradient at SRP, previously shown to contain a variety of serogroups of L. pneumophila[4,5] was sampled to determine the relationship between the levels of ETS active Legionella and the in situ habitat temperature.

Samples collected in the field were processed in a similar manner to those in the laboratory. Large volumes of water, 8 liters each, were collected in plastic bottles containing 80 mL of the 0.2% INT solution. Incubations were carried out in the field under in situ conditions as described above. All reactions were stopped with formaldehyde at a final concentration of 0.1%. Time course and temperature transfer experiments were carried out in the field. The results indicated that a 60 minute incubation time was adequate for future studies in these habitats.

RESULTS

The growth patterns of four isolates of L. pneumophila cultured on the modified BYEB medium at 37°C are shown in Figure 2. Doubling times for all the serogroups tested were generally 100 minutes or less. Time course experiments for INT incorporation were run with L. pneumophila serogroup 2 (SRP-36). Aliquots (10.0 mL) were taken at various points along the growth curve for both log and late stationary samples. Legionella in all phases of growth demonstrated rapid uptake of INT. Only the very

late stationary cells (72 hours) indicated a reduction in the percentage of cells with active electron transport systems. Formaldehyde at a concentration of 0.1% was an effective control and readily stopped INT incorporation. These results are typical of those seen with the other tested serogroups (1, 3, and 4). All subsequent laboratory studies with INT were incubated for 60 minutes before the reactions were stopped.

Temperature transfer experiments were conducted in the laboratory with L. pneumophila serogroup 4 (Los Angeles). One of the cultures, SRP-22, was isolated from a South Carolina lake,[5] while the clinical Los Angeles culture was obtained through the Center for Disease Control in Atlanta, Georgia. Both isolates were repeatedly grown on the modified BYEB medium and tested during active log growth. The data in Figure 3 demonstrate the wide temperature range within which both the clinical and environmental isolates of serogroup 4 remain active. Experimental data for clinical and environmental isolates of serogroups 1, 2, and 3 are virtually identical (data not shown). Between temperatures of 25° and 60°C, the percentage of Legionella with active electron transport systems is greater than 90%. At temperatures greater than 60°C, the percentage of active cells declines precipitously until background levels of less than 10% are reached at temperatures above 65°C. The data do not demonstrate any differences between

isolates originally obtained from either lakes or clinical samples. Any physiological differences that may have existed before the extensive transfers onto synthetic media are likely to have been minimized because of the similarities in growth conditions. The data (Figure 3) do reflect the adequacy of the formaldehyde controls at both high and low temperatures. The lower temperature transfer data are not well defined; at 25°C, the ETS activity is high (>90%); and at 4°C, the activity is at background levels.

The survival data for L. pneumophila SRP-22 (Table 1) support the rapid drop in ETS activity observed at temperatures greater than 60°C (Figure 3). Although the ETS activity measurements showed no indication of viability reduction between 25°C and 60°C, the recovery onto CYE agar showed a rapid loss of viability above 50°C.

Water samples collected from the Par Pond system at 54°C were transferred to various temperature systems along the man-made thermal gradient, plus a 4°C ice bath. Samples were thermally equilibrated in situ (preincubated) and a time course at each of the temperatures was established for INT incorporation (Figure 4). The water samples were concentrated by centrifugation and stained with polyvalent antisera combining serogroups 1 through 4 of L. pneumophila. Thus, Figure 4 demonstrates the response of all four of these serogroups in the water sample rather than the response of a particular serogroup. Incorporation was fairly well

stabilized by 40 minutes except at one temperature, 37°C. Subsequent samples were incubated 60 minutes before termination of the experiments. Although the water samples were collected at 54°C, the maximum ETS activity was observed at 37°C, where over 85% of the Legionella were active. Control samples remained low, while samples from both the highest and lowest temperatures demonstrated ETS activity of less than 25%.

Water samples collected from a variety of temperatures within the Par Pond system were analyzed with a polyvalent antibody that was specific for serogroups 1 through 4 of Legionella, and their concomitant ETS activity was measured. The majority of Legionella in the Par Pond system are of serogroup 1 followed by serogroup 4.[5] Figure 5 depicts the results of those samples and expresses the percentage of ETS activity for the four serogroups of Legionella at each of the in situ temperatures. No attempt was made to determine the activity of each serogroup with respect to the in situ temperature. L. pneumophila in the Par Pond system demonstrated ETS activity over a wide range of habitat temperatures with an optimum between 40° and 60°C.

DISCUSSION

These findings are significant in that they shed new light on the physiological ecology of Legionella pneumophila. The advancement of ecological studies on Legionella suffers because a selective enrichment medium remains unavailable for isolating the organism from habitats where it generally comprises less than 1%

of the total bacterial population. Further, the relatively slow growth of Legionella allows for rapid and extensive overgrowth of other faster-growing aquatic bacteria. Negative enrichment procedures, whereby Legionella are held static by antibiotics that are lethal to the general population,[16] have theoretical possibilities. However, separate studies by Feeley and Miller (personnal communications) have indicated that the procedure is not useful in samples containing a large number of bacteria as is the case of most ecological habitats.

Initial ecological studies on the temperature relationship of Legionella to its environment are noteworthy. Pure culture work and ecological observations have suggested that Legionella is physiologically related to thermophilic bacteria. Moss, et al.[10] and Moss and Dees[9] demonstrated that L. pneumophila serogroup 1 contained large amounts (>80%) of branched-chain fatty acids. Such findings are similar to those for known thermophiles, i.e., Thermus aquaticus, Bacillus caldolyticus, and B. caldotenax[8,14,18] Tison, et al.[17] showed that Legionella could derive all of its nutritional requirements from the thermophilic cosmopolitan bluegreen algae, Fisherella sp. Additionally, this relationship was observed in situ in algal mats composed primarily of Fisherella. Doubling times for Legionella on minimal salts medium with Fisherella at 45°C were generally 2.5 hours. The relationship between bluegreen algae and Legionella suggests possible reasons

for cooling towers being associated with Legionellosis, since many towers are readily colonized by various types of algae including bluegreens. Berendt (personal communication) has found that Fisherella sp. greatly stabilize Legionella during aerosolization, so that Legionella remains viable for longer than normal periods of time when associated with this bluegreen alga.

Our previous research[5] demonstrated that L. pneumophila could survive and be isolated at temperatures as high as 63°C. The present data corroborate these findings in that a high percentage of Legionella from pure cultures have active electron transport systems between 25° and 60°C (Figure 3). When one repeats these studies in the field, similar results are obtained. Samples taken along the thermal gradient of the Par Pond system show that INT activity among the habitats is widely varied (Figure 5). There appears to be greater ETS activity of Legionella taken from habitats between 40° and 60°C. If an arbitrary level of 50% ETS activity is used as a cut-off point, then of the 16 habitats sampled with temperatures ranging from 40° to 60°C, 88% of the habitats have Legionella with ETS activity of greater than 50% (Figure 6). Habitats sampled from either side of that temperature range contain no more than 35% of stained Legionella with ETS activity greater than 50%.

These data support two conclusions. First, the combining of a specific fluorescent antibody with a dye that measures a metabolic process (such as the electron transport system), allows the advancement of autecological research on microorganisms for which selective enrichment media are not yet available. Secondly, the utilization of this technique has demonstrated that the activity of Legionella can be determined in situ, and that Legionella is more active in moderately thermal environments (40° to 60°C).

This technique provides the opportunity to determine the effects of various physical, chemical, and biological parameters on species and serogroups of Legionella under real world, in situ, conditions.

ACKNOWLEDGMENTS

We thank J. J. Foreman, J. L. Todd, and I. L. Sauls, Jr., for technical assistance. We thank W. B. Cherry for providing helpful discussions and insights. This research was supported by National Institute of Health Grant 1-R01-AI17673-01 and by Contract No. DE-AC09-76SR00001 with the U.S. Department of Energy.

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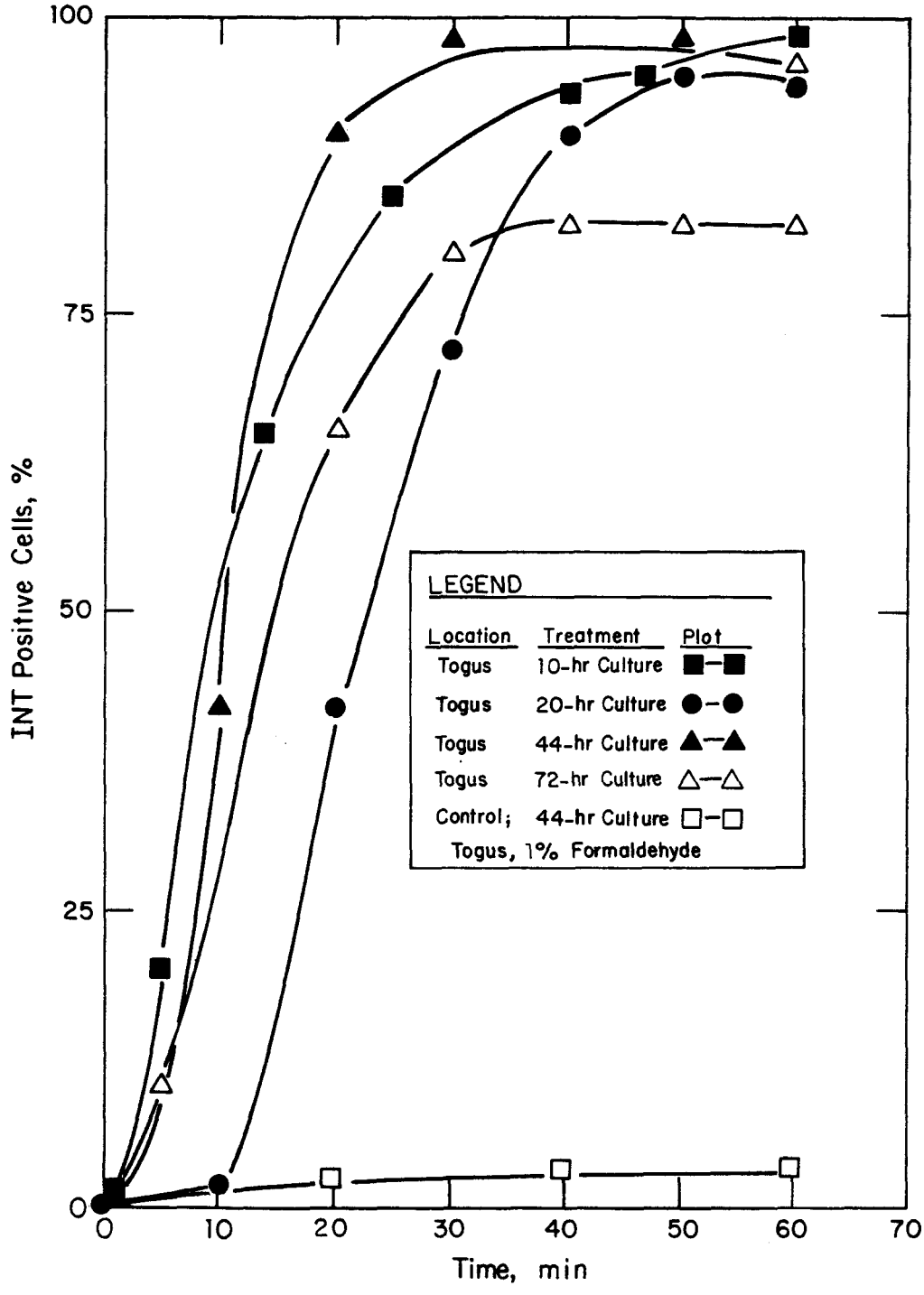

Fig. 1. Studies of pure cultures of SRP-36 grown for various times on liquid BYEB at 37°C, and the percentage of INT positive cells determined over time.

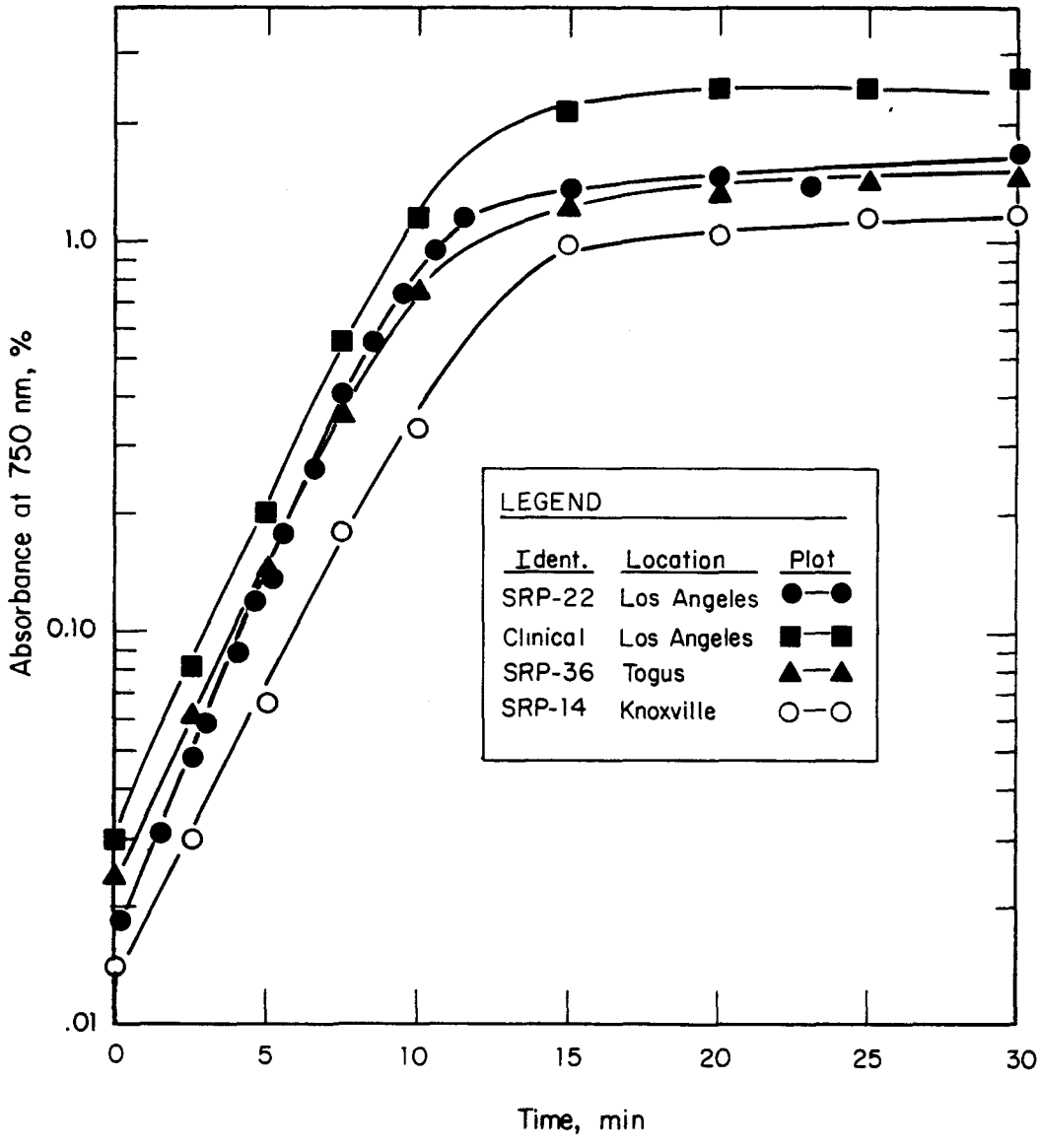

Fig. 2. Growth curve of L. pneumophila on modified liquid BYEB medium at 37°C.

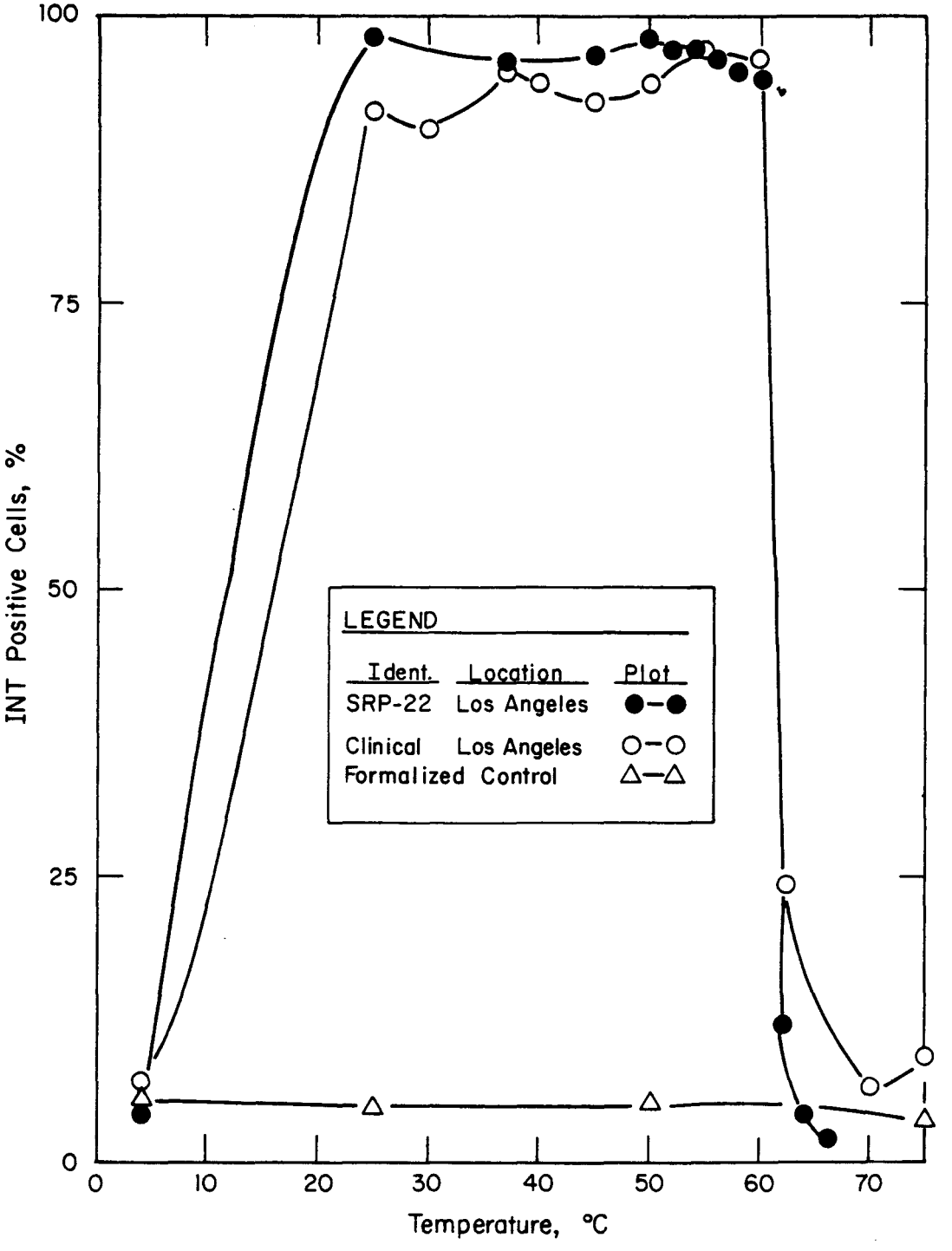

Fig. 3. Temperature transfer experiment of pure cultures of Seragroup 4 clinical and environmental isolates.

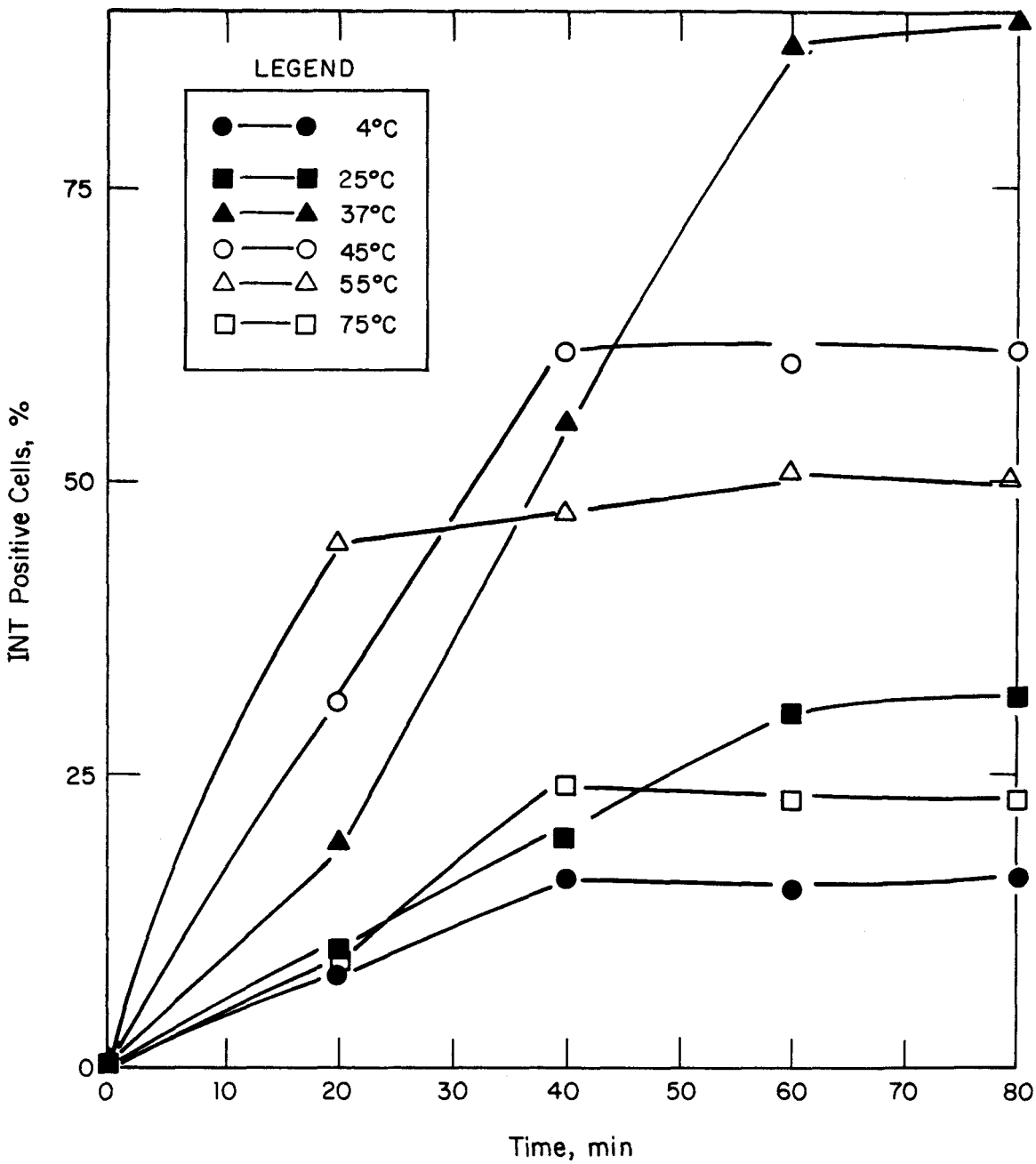

Fig. 4. Temperature transfer and time-course experiment of ETS activity of L. pneumophila in natural waters at 54°C.

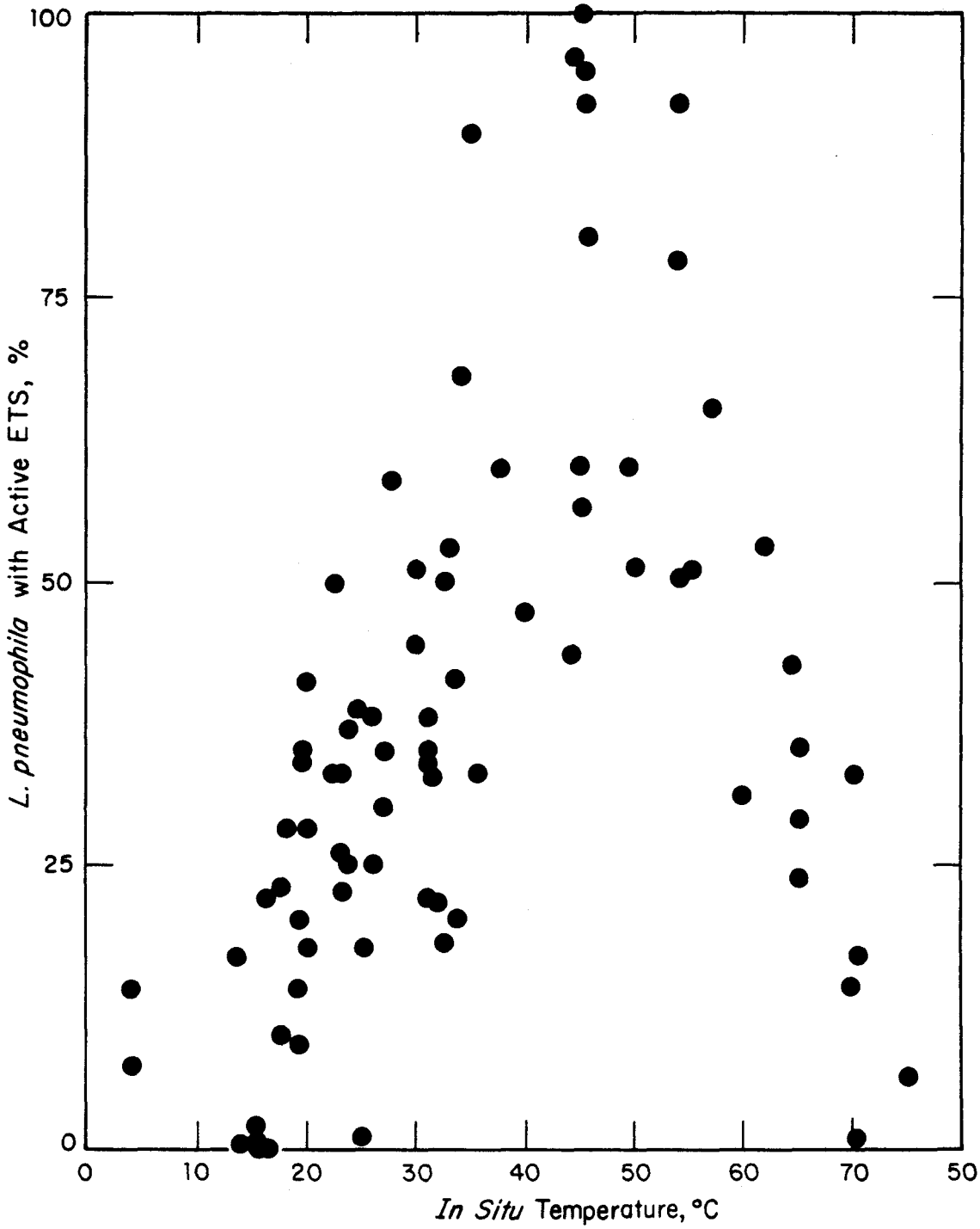

Fig. 5. ETS activity of L. pneumophila in water samples from the Par Pond System at various in situ temperatures.

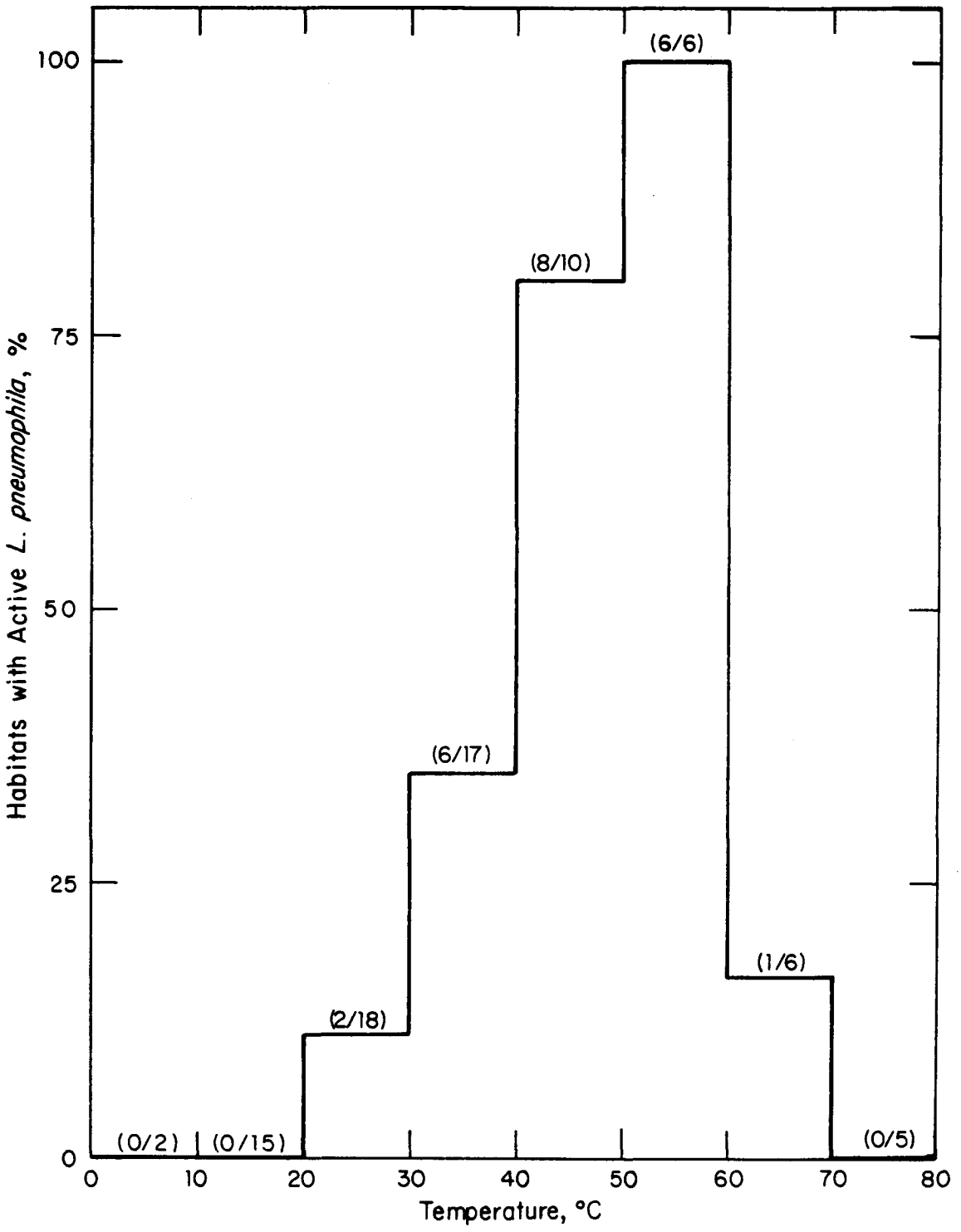

Fig. 6. Histogram of habitats containing L. pneumophila with ETS activity of 50% or higher. Parenthetical numbers are the ratio of habitats containing L. pneumophila with ETS activity of 50% or higher, divided by the total number of habitats sampled within the designated temperatures.

Table 1. Survival of L. pneumophila after a temperature transfer of 40 minutes to temperatures above the culture growth temperature, 37°C.

Temperature of transfer, °C	Colony-forming units (cfu) Before temp. transfer	After 40 min. of temp. transfer	Reduction in cfu, %
37°	3.5×10^8*	3.5×10^8	0
50°	3.5×10^8	2.6×10^8	26
56°	3.6×10^8	1.8×10^8	49
62°	3.5×10^8	N.G.**	100
66°	3.5×10^8	N.G.	100

* Mean of triplicate samples on CYE

** N.G. = no growth.