1-ANILINO-8-NAPHTHALENE SULFONATE AND *N*-PHENYL-1-NAPHTHYLAMINE AS THE INDICATORS OF BACTERIAL THERMOSENSITIVITY

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1. Introduction

1-Anilino-8-naphthalene sulfonate (ANS) and *N*-phenyl-1-naphthylamine (PNA) have been used as probes for investigation of structures and changes in membranes [1]. Changes in lipid constituents during the growth of bacteria can be monitored by using PNA as the probe [2]. On the other hand, all transitions in the state of membranes, which lead to increased hydrophobicity may be detected with ANS [1]. The observation that extraction of membrane proteins by organic solvents exposes further ANS-binding sites [3] led us to investigate whether fluorescence probes can be used as the indicators of bacterial thermosensitivity. The results show that an increase in temperature produces extra binding sites in *Pseudomonas fluorescens*, *Escherichia coli* and *Streptococcus thermophilus* and that the formation of these extra binding sites is in a good agreement with bacterial thermophilicity.

2. Materials and methods

Ps. fluorescens P-2, *E. coli* U-5/41 and *S. thermophilus*, which were used as the test organisms, were cultured in a glucose-minimal medium at 30°C (control) or at 30°C, 42°C and 50°C, respectively. The cells withdrawn during the lag or exponential phase of growth were washed twice with 0.1 M phosphate buffer (pH 6.6). The final suspension contained about 5×10^9 cells/ml. The membrane vesicles were prepared from the cells by the modification of the method of Kaback [4, 5]. The vesicles were suspended in 0.1 M phosphate buffer (pH 6.6) up to a final concentration of 1 mg protein per ml. The concentrations of ANS and PNA were 20 μM and 10 μM, respectively. PNA was diluted in dimethyl sulphoxide (DMSO)–water (1:3) solution. The cell suspensions and membrane vesicles were incubated at 10°–70°C and fluorescence intensity measurements were carried out after addition of ANS or PNA at 25°C. For some fluorescence measurements, the cells and the membrane vesicles were incubated at the same time with ANS or PNA. ANS fluorescence was excited at 380 nm and emission was measured at 465 nm. Wavelengths of PNA fluorescence were 345 nm and 405 nm, respectively. Fluorescence measurements were performed in a Perkin–Elmer MPF-2A spectrofluorimeter at 25°C.

3. Results and discussion

It has been suggested that the normal effect of temperature on the cells is a reversible denaturation of proteins [6]. However, this mechanism may be limited to the narrow temperature region where growth ceases. This stoppage is reversible so that growth is continued again when damage has been repaired [7]. The changes can be monitored by ANS, which interacts with hydrophobic sites of the cells.

Although the intensity of ANS fluorescence decreases in phospholipid [8] and in protein [9] systems as a function of temperature, increase in ANS fluorescence intensity was observed in all investigated bacteria when the cells were exposed to temperature gradient before and after addition of ANS. Increase in intensity was detected in *Ps. fluorescens* after 30°C, in *E. coli* after 38–40°C and in *S. thermophilus* after 42–44°C (fig. 1). The starting point and the levels of fluorescence increase were not markedly dependent on

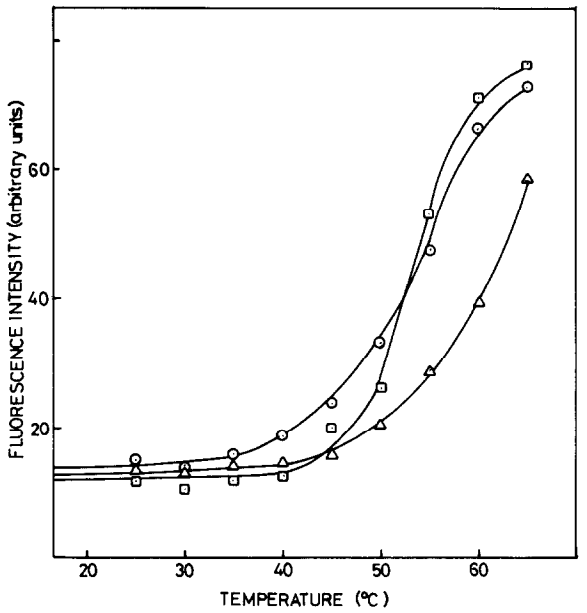

Fig. 1. The effect of temperature on the enhancement of 1-anilino-8-naphthalene sulfonate fluorescence by *Ps. fluorescens* UK-1, *E. coli* U-5/41 and *S. thermophilus*. The cells (5×10^9 cells/ml) were preincubated for 30 min at investigated temperatures. After addition of ANS (up to the final concentration of 20 μM) fluorescence intensity was measured at 25°C. Excitation at 380 nm, emission at 465 nm. (○—○—○) *Ps fluorescens*; (□—□—□) *E. coli*; (△—△—△) *S. thermophilus*.

the temperatures used for the growth of *E. coli* (30°C and 42°C) and of *S. thermophilus* (30°C, 42°C and 50°C). The effect of low molecular weight compounds, which were possibly effluxed during incubations, was eliminated by washing the cells with 0.1 M phosphate buffer before the addition of the probe. The effects found in fig. 1 may reflect the first cell damages, which are detectable, but not yet fatal, because growth was observed at temperatures 5–10°C higher, than at the starting point of fluorescence increase. The higher growth temperatures could be explained by attachment of low molecular weight ligands stabilizing proteins in growth conditions. The high temperatures (above 30°C in *Ps. fluorescens*, 40°C in *E. coli* and about 45°C in *S. thermophilus*) may denature proteins like organic solvents which produce proteins with hydrophobic side chains exposed to solvent, and make them enhance ANS fluorescence

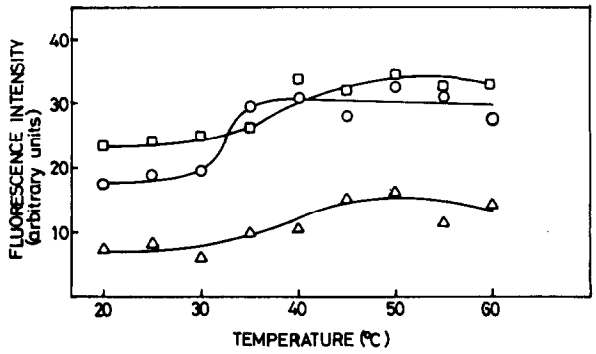

Fig. 2. The effect of preincubation of the cells at different temperatures on *N*-phenyl-1-naphthylamine fluorescence intensity. The concentration of PNA in the cell of the spectrofluorimeter was 10 μM. Excitation at 354 nm, emission at 405 nm. Other experimental conditions were the same as in the legend to fig. 1. (○—○—○) *Ps. fluorescens*; (□—□—□) *E. coli*; (△—△—△) *S. thermophilus*.

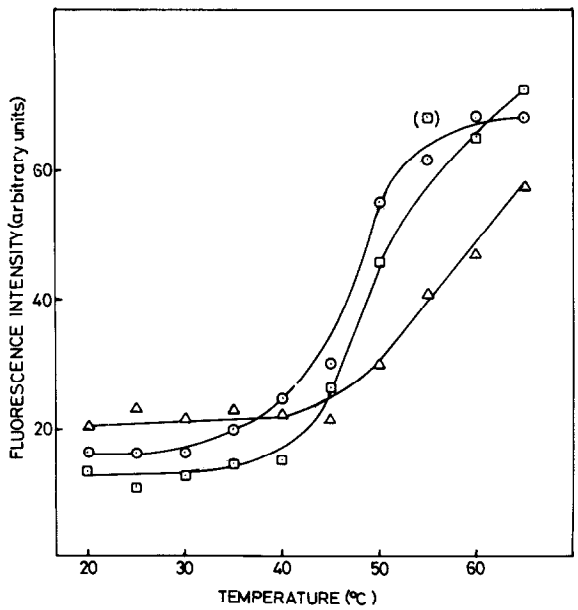

Fig. 3. The effect of preincubation of isolated membrane vesicles at different temperatures on 1-anilino-8-naphthalene sulphonate fluorescence intensity. Experimental conditions were the same as in the legend to fig. 1. (○—○—○) Membrane vesicles from *Ps. fluorescens* ; (□—□—□) from *E. coli*; (△—△—△) from *S. thermophilus*.

intensity more strongly than their native forms [10].

On the other hand, binding of PNA, which is used as a probe for the lipid region of the bacterial membrane, does not change markedly when temperature is elevated (fig. 2). The differences in fluorescence levels may reflect the different amounts of lipids in *Ps. fluorescens*, *E. coli* and *S. thermophilus*. Although dimethylsulphoxide was used as a solvent of PNA, any difference in regard to the water–PNA–membrane system was found only in the higher fluorescence intensity throughout the measured temperature region. This is well understood if we think about the slight solubility of PNA in water and the solubility of lipids in DMSO. When suspended with cells or membranes this lipid-soluble dye interacts with cell lipids.

The isolated membrane vesicles are more sensitive to temperature changes than the intact cells (fig. 3). Otherwise the results agree well with those obtained with whole cells.

These results suggest that ANS (and possibly also other dyes which interact with hydrophobic sites in the cells) can be used as an indicator of meso–, psychro– or thermophilicity of bacteria.

References

[1] Radda, G.K. and Vanderkooi, J. (1972) Biochim. Biophys. Acta 265, 509–549.
[2] Ballard, S.G., Barker, R.W., Barrett Bee, K.J., Dwek, R.A., Radda, G.K., Smith, D.S. and Taylor, J.A. (1972) in: (Azzone, G.F., Carafoli, E., Lehninger, A.L., Quagliariello, E. and Siliprandi, N., eds.), Biochemistry and Biophysics of Mitochondrial Membranes, p. 257, Academic Press, New York.
[3] Randall, R.F., Stoddart, R.W., Metcalfe, S.M. and Metcalfe, J.C. (1972) Biochim. Biophys. Acta 255, 888–899.
[4] Kaback, H.R. (1971) in: Methods in Enzymology (Jacoby, W.B., ed.), Vol. 22, p. 99, Academic Press, London.
[5] Mäntsälä, P. (1974) Acta Chem. Scand. 28, in press.
[6] Johnson, F.H., Brown, D. and Marsland, D. (1942) Science 95, 200–203.
[7] Airas, K. (1972) Biochem. J. 130, 11–119.
[8] Vanderkooi, J. and Martonosi, A. (1969) Arch. Biochem. Biophys. 133, 153–163.
[9] Augustin, J. and Hasselbach, W. (1973) Eur. J. Biochem. 35, 114–121.
[10] Gally, J.A. and Edelman, G.M. (1965) Biochim. Biophys. Acta 94, 175–182.