Binding of the Fluorescent Probes 1-Anilidonaphthalene-8-sulfonate and *N*-Phenyl-1-naphthylamine to Bacteria

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Changes in fluorescence intensities in *Pseudomonas fluorescens*, *Escherichia coli*, *Streptococcus thermophilus*, and *Bacillus subtilis* were investigated by means of the fluorescence probes (1-anilidonaphthalene-8-sulfonate and *N*-phenyl-1-naphthylamine) during a temperature programmed run.

A decrease in fluorescence intensity was observed in all investigated organisms when the temperature was elevated from 4 to about 40 °C. A strong increase in fluorescence intensity was found in gram-negative *Ps. fluorescens* and *E. coli* when the temperature was elevated over 40 °C. On the other hand, only a slight enhancement in fluorescence intensities was observed in gram-positive *Str. thermophilus* and *B. subtilis* when the lipid specific dye NPN was applied at elevated temperatures. Discontinuities in all the fluorescence temperature profiles were found between 20 and 30 °C in the case of both ANS and NPN. With ANS the fluorescence profiles turned upwards and with NPN downwards after the inflection points.

The effects of KCl, NH₄Cl, NaCl and LiCl on ANS binding differed from those detected for NPN. Saturation of probe binding was reached at lower salt concentrations in the case of NPN than ANS, but depended somewhat on temperature.

Location of the inflection points and fluorescence intensities depended on growth temperature and growth phase, as well as thermophilicity of the bacteria. When *Ps. fluorescens* and *Str. thermophilus* were cultured at critical growth temperatures (8 and 37 °C and 20 and 49 °C, respectively) the inflections between 20 and 30 °C were shifted toward higher temperatures with increasing growth temperature. Furthermore, there was a marked enhancement in both ANS and NPN fluorescence between 45 and 60 °C when growth proceeded from the lag to the stationary phase in *E. coli* and *Ps. fluorescens*. In *Str. thermophilus*, on the other hand, there was only a slight binding maximum between 45 and 60 °C; ANS binding increased with growth.

Changes in polarity and hydrophobicity in bacteria as a function of temperature are discussed.

The fluorescent dyes 1-anilidonaphthalene-8-sulfonate and *N*-phenyl-1-naphthylamine have often been used as probes of structural changes in proteins and lipids in cell constituents.¹⁻¹⁰ These dyes are non-fluorescent in aqueous liquids but undergo strong fluorescent enhancement in the presence of various membranes.⁵ ANS reportedly binds at polar-apolar interphases within membranes that contain charged groups and NPN reflects changes in membrane lipids.⁵ It has been suggested that increase in molecular rigidity rather than solvent polarity is the dominant factor in fluorescence intensity enhancement.¹¹ The molecular rigidity of cells changes with melting of the lipid fraction and the denaturation of proteins. Physical methods such as calorimetry,¹² X-ray diffraction,¹³ and spectroscopy,⁵ have been used increasingly to monitor the alterations in membranes induced by various conditions. Lipid compositions from several organisms show an increase in unsaturation with a decrease in growth temperature.¹⁴⁻¹⁶ Furthermore, lipid composition may vary according to medium constituents, aeration and the growth phase.¹⁷ In *Bacillus megaterium* NPN fluorescence has been found to increase with the growth of the bacteria,¹⁸ reflecting the increase in lipid constituents during the growth cycle.

This paper describes changes in ANS and NPN fluorescence intensities in different microorganisms exposed to elevated temperatures. Discontinuities were observed both in ANS and

NPN fluorescence profiles in the temperature ranges 20–30 °C and 40–60 °C. Changes in growth temperatures and the physiological state of the cells resulted in shifts in the inflection points and affected fluorescence intensities.

MATERIALS AND METHODS

Test organisms. The organisms *Escherichia coli* U5/41, *Pseudomonas fluorescens* UK-1, *Streptococcus thermophilus* KQ and *Bacillus subtilis* ATCC 6633 were grown in 1 liter erlenmeyer flasks, each containing 200–500 ml of 0.5 % micro inoculum broth. The flasks were shaken on a rotary shaker (New Brunswick Scientific Co., G 10 Gyrotary Shaker) or aerated by aquarium pumps in water baths. Turbidity of the cultures was measured with a Klett-Summerson colorimeter, employing filter 62. The cells were harvested by centrifugation (5000 *g*, 15 min) when turbidity was about 100, and washed twice with 0.05 M phosphate buffer (pH 6.6). Samples of 20 ml were withdrawn from the different growth phases, centrifuged and washed as described above. The pellets were suspended in 0.05 M phosphate buffer (pH 6.6).

Treatment of the cells. Membrane vesicles were prepared from the cells by the method of Kaback¹⁹ as modified by the author.²⁰ Samples for the fluorimetric measurements were taken after EDTA, lysozyme, and osmotic shock treatments. Freezing and thawing of the cells were carried out in 0.5 ml of 0.05 M phosphate buffer (pH 6.6). After this procedure the cell fractions were separated by centrifugation at 10 000 *g* for 15 min. Both the cell pellets and supernatants were stored for the fluorimetric measurements. The other cell samples were partly disrupted by disintegrating them for short times in a MSE Sonic Oscillator.

Fluorimetric measurements. The fluorescence was measured with a Hitachi-Perkin-Elmer MPF-2A spectrophotofluorimeter. The fluorescence values were corrected for temperature but not for the absorption of the exciting light by the free dye. The fluorimetric measurements were carried out as described earlier by Mäntsälä and Lang⁸ except that a temperature-programmed run was applied. The incubation mixtures (2.5 ml) contained 0.05 M phosphate buffer (pH 6.6), 20 μ M ANS or 10 μ M NPN and $3.0\text{--}4.2 \times 10^9$ cells/ml. The fluorescence profiles were determined without preincubation and temperature was elevated 2 °C/min during the temperature-programmed runs.

RESULTS

Binding of ANS and NPN as a function of temperature in whole cells. Changes in fluores-

cence intensity in *Ps. fluorescens*, *E. coli*, *Str. thermophilus*, and *B. subtilis* were investigated in the temperature range 2–70 °C. Intensity decreased in all the organisms when the temperature was elevated over 5 °C. Several inflection points were observed in the fluorescence profile (Fig. 1). The first one was at 4–5 °C with both ANS and NPN. We can assume that this inflection point reflects crystalline-liquid phase transition or changes in

Fig. 1. Interactions of ANS and NPN with microbial cells during the temperature programmed run. The incubation mixtures of 2.5 ml contained the washed cells ($2\text{--}5 \times 10^9$ cells/ml) in 0.05 M potassium phosphate buffer (pH 6.6) and the fluorescence dye (20 μ M ANS or 10 μ M NPN). ANS fluorescence was excited at 380 nm and emission was measured at 465 nm. Wavelengths of NPN fluorescence were 345 and 405 nm, respectively. Other experimental conditions were the same as described earlier by Mäntsälä and Lang.⁸

A. Intensities of ANS fluorescence in *Ps. fluorescens*, grown at 30 °C, a; in *E. coli*, grown at 37 °C, b; in *Str. thermophilus*, grown at 37 °C, c; in *B. subtilis*, grown at 37 °C, d.

B. Intensities of NPN fluorescence in *Ps. fluorescens*, a; in *E. coli*, b; in *Str. thermophilus*, c; in *B. subtilis*, d.

C. Intensities of ANS fluorescence in *E. coli* (a) and in *Str. thermophilus* (b) when the cells were cultured in anaerobic growth conditions.

D. Intensities on NPN fluorescence in *E. coli* (a) and in *Str. thermophilus* (b) when the cells were cultured in anaerobic growth conditions.

the properties of water and water-membrane interactions. The latter suggestion seems more likely because the same effect was observed in all investigated organisms although growth at 4 °C is a property typical of *Ps. fluorescens* and requires a liquid lipid phase. Inflections between 20 and 30 °C depended on thermosensitivity of the bacteria. For example, in mesophilic *Ps. fluorescens* this inflection point was located at 20–22 °C, perhaps reflecting the “melting” of some lipid fraction in *Ps. fluorescens*. The inflection point was shifted toward higher temperatures in *E. coli* and *Str. thermophilus*, to 26–27 °C and about 28–30 °C, respectively. Several reports indicate that psychrophilic and mesophilic organisms contain more unsaturated fatty acids than thermophilic ones. Although the bacteria studied here are not obligate meso- or thermophiles, they exhibit different thermosensitive properties and the inflection points correlate well with the order of thermosensitivity of the bacteria. If these points represent “melting” points of the lipids, they should also correlate somehow with transport processes in the bacteria, because uptake of solutes and energy sources requires a liquid lipid phase. There are many examples in the literature indicating that initial velocities of transport are negligible below 20–30 °C.^{5,10,21–23} As we see in Fig. 1, the inflection points are located in the same temperature range for both ANS and NPN. However, inflections were not in the same direction, for with ANS the fluorescence profiles turned upwards and with NPN downwards. These results suggest that the “melting” of lipids provides an environment with less rigidity, thus revealing more polar groups which increase ANS fluorescence intensity. Remarkable inflections were located also between 40 and 50 °C. Fluorescence intensities increased strongly especially in gram-negative *Ps. fluorescens* and *E. coli* (at about 40–45 °C and 42–50 °C, respectively). On the other hand, in gram-positive *Str. thermophilus* and *B. subtilis*, the interactions with ANS at elevated temperatures were remarkably less and only a negligible increase in fluorescence intensity at 40–70 °C was observed when NPN was used as the probe (Fig. 1). However, as we will see later, maximal intensities depend on the growth phase, too.

Anaerobic conditions do not markedly change

the binding of the dyes in *Str. thermophilus* and *E. coli* below 30 °C (Fig. 1). Only negligible differences were observed in NPN interactions with the cells, suggesting that the hydrophobic core of the investigated bacteria is stable and that lipid synthesis is virtually independent of oxygen supply.

The effect of salts on fluorescence intensity. The exact role of the different ions remains obscure. Radda and Vanderkooi⁵ have suggested that during the process of binding to membranes there is a displacement of water molecules from the hydration shells. Our results reveal that the interactions of NPN with *E. coli* and *Ps. fluorescens* are more sensitive to increased salt concentrations than the interactions of ANS (Fig. 2). Saturation levels were reached already at concentrations of 0.1–0.4 M NPN, whereas with ANS the maximal intensities were not reached at 1.5 M concentration. All investigated salts had about the same effect on the inter-

Fig. 2. Effect of various monovalent cations on ANS (●) and NPN (○) fluorescence intensities. The reaction mixtures contained 3×10^9 cells/ml and 2×10^9 cells/ml, respectively. Other experimental conditions were the same as in the legend to Fig. 1. *E. coli*: A, NH₄Cl; B, NaCl; C, LiCl; D, KCl. *Ps. fluorescens*: E, NH₄Cl; F, NaCl; G, LiCl; H, KCl.

actions of NPN, whereas with ANS intensities followed the order $\text{NH}_4\text{Cl} > \text{LiCl} > \text{NaCl} > \text{KCl}$.

Effect of various treatments on the interactions of the dyes with the bacteria. Several lines of evidence suggest that hydrophobic interaction between various protein components is the force holding the cell wall together. Gram-negative bacteria characteristically have a cell envelope consisting of two membranes separated by a thin peptidoglycan layer. This layer can be destroyed by lysozyme. To specify critical points in fluorescence behaviour during the programmed run of gram-negative *E. coli* and *Ps. fluorescens*, various destructive treatments were carried out before the cells were exposed to temperature changes. After incubation with ethylenediamine tetraacetate the cells appeared to have lost both protein and lipid material, but still retained an ANS fluorescence profile similar to the untreated cells. When lysozyme was added to the incubation mixture, increases

Fig. 3. Effect of various destructive treatments on ANS fluorescence intensities. Other experimental conditions were the same as in the legend to Fig. 1. A. *E. coli* no treatments, (a); 10 mM EDTA, (b); 10 mM EDTA + lysozyme (0.5 mg/ml), (c); 10 mM EDTA + lysozyme (0.5 mg/ml) + DNase (4 mg/ml) + osmotic shock, (d). B. *Ps. fluorescens* the treatments and the symbols are the same as above. C. *E. coli* freezing and thawing, twice (a); ultrasonic disintegration 10 s (b); 20 s (c). D. *Ps. fluorescens* the treatments and the symbols are the same as in Fig. C.

Fig. 4. Effect of stepwise temperature elevations on ANS fluorescence intensities in *E. coli* (A) and in *Ps. fluorescens* (B). The reaction mixtures (2.5 ml) contained 0.05 M phosphate buffer (pH 6.6), 20 μM ANS and 3.0×10^8 cells/ml of *E. coli* or 4.2×10^8 cells/ml of *Ps. fluorescens*. The temperature of the reaction mixtures was elevated to 32, 40, 50, and 60°C, decreasing to 10°C between these elevations. The arrows show the direction of the temperature change.

in fluorescence intensity were observed at 45–60°C; they were not as strong as before lysozyme treatment. DNase + osmotic shock, ultrasonic or freezing and thawing treatments produced fluorescence profiles with slight or without any increases (Fig. 3). These results suggest that the increase in fluorescence intensity at 40–60°C results from the irreversible denaturation of proteins. This view is further supported by experiments in which the temperature was elevated stepwise to 60°C decreasing the temperature between these steps to 10°C (Fig. 4). As we can see the fluorescence intensities increased strongly when temperature was decreased (arrow direction $\leftarrow$) and the intensities remained higher during the next elevations. When the stepwise temperature elevations reached 60°C there was no increase

in intensity (between 40 and 60 °C) during the next elevations suggesting that the complete denaturation of proteins had occurred.

Effects of growth temperature and growth phase on fluorescence profiles. It has been reported that saturation of fatty acids increases when growth temperature is elevated.¹⁴⁻¹⁶ Using NPN to probe the lipid region of the cells it was seen that the inflection point near 20 °C

shifted to higher temperatures when *Ps. fluorescens* and *Str. thermophilus* were cultured at elevated temperatures. In *Ps. fluorescens* it shifted from 18 to 25–26 °C when the growth temperature was elevated from 8 to 37 °C (Fig. 5). The effect was the same with both NPN and ANS, although the effect of ANS was somewhat unclear. Furthermore, both NPN and ANS interacted at 40–60 °C more effectively

Fig. 5. Effect of growth temperature and the growth phase on fluorescence intensities. Other experimental conditions were the same as in the legend to Fig. 1.

A. ANS fluorescence intensities in *Ps. fluorescens* when the cells were cultured at 8 °C (a); at 15 °C (b); at 30 °C (c); at 37 °C (d). B. ANS fluorescence intensities in *Str. thermophilus* when the cells were cultured at 20 °C (a); at 30 °C (b); at 49 °C (c). C. NPN fluorescence intensities in *Ps. fluorescens*. The symbols are the same as in Fig. A. D. NPN fluorescence intensities in *Str. thermophilus*. The symbols are the same as in Fig. B. E. ANS fluorescence intensities in *Ps. fluorescens*. The cells were taken from the lag ($KU_{62}=26$)^{*} (a), from the acceleration ($KU_{62}=38$) (b), from the logarithmic ($KU_{62}=102$) (c) and from the stationary ($KU_{62}=184$) (d) phases. F. ANS fluorescence intensities in *E. coli* $KU_{62}=18$ (a); $KU_{62}=28$ (b); $KU_{62}=80$ (c); $KU_{62}=161$ (d). G. NPN fluorescence intensities in *Ps. fluorescens*. The growth phases and the symbols are the same as in Fig. E. H. NPN fluorescence intensities in *E. coli*. The growth phases and the symbols are the same as in Fig. F. I. ANS fluorescence intensities in *Str. thermophilus* $KU_{62}=18$ (a); $KU_{62}=26$ (b); $KU_{62}=74$ (c); $KU_{62}=148$ (d). J. ANS fluorescence intensities in *B. subtilis* $KU_{62}=20$ (a); $KU_{62}=27$ (b); $KU_{62}=94$ (c). K. NPN fluorescence intensities in *Str. thermophilus*. The growth phases and the symbols are the same as in Fig. I. L. NPN fluorescence intensities in *B. subtilis*. The growth phases and the symbols are the same as in Fig. J. ^{*} KU_{62} = Klett-reading employing filter 62.

with the cells grown at 8 °C and 15 °C than with those grown at 37 °C.

When a new medium was inoculated with the bacteria withdrawn from the stationary phase of growth, an increase in fatty acids was observed in *B. megaterium* during growth.¹⁸ As we can see from Fig. 5, binding of NPN also increases markedly in *Ps. fluorescens* when the cells are cultured from the lag to the end of the logarithmic growth phase. These results suggest that lipid constituents increase during growth, or that denaturation of proteins or destruction of some protective structural components reveals more lipid groups. The inflection point in ANS profiles of *Ps. fluorescens* was located in the same temperature range as in NPN profiles (40–45 °C). Furthermore, the intensities followed the same order and were at their highest during the late growth phases. These results further support the view that the same factors are responsible for increases in ANS and NPN profiles although the components reacting with the dyes may be different. Maximal intensities were reached at the end of the exponential growth phase, being as much as 2–3 fold compared with the intensities at the beginning.

The profiles of *E. coli* resemble those of *Ps. fluorescens*, and as we can see from Fig. 5 a great many new groups interacting with ANS and NPN have formed during growth, or rigidity has greatly changed due to the denaturation of proteins. The effects are remarkable, suggesting that, for *E. coli* 40 to 45 °C are critical temperatures at which partial destruction of cells occurs.

Fluorescence profiles of *Str. thermophilus* differ somewhat from those of *Ps. fluorescens*. Inflection points shifted from 24–25 to 30–33 °C when the growth temperature was elevated from 20 to 49 °C (Fig. 5). Furthermore, maximal intensities increased and shifted toward higher temperatures when growth proceeded from the lag to the stationary phase. However, only slight increases in fluorescence intensities were observed during the temperature-programmed run when NPN was used as the fluorescent probe. The fluorescence profiles of *B. subtilis* (Fig. 5) resemble those of *Str. thermophilus*, though the first inflection point was located somewhat earlier (at 24–25 °C).

DISCUSSION

The factors responsible for heat resistance in intact bacteria and membranes have been extensively studied by measuring the differences in chemical composition produced by heat treatments.^{24–28} Anilinonaphthalene dyes, which become highly fluorescent in a hydrophobic environment, have also proved to be useful indicators of alterations in cell constituents. There are several examples in the literature of ANS binding to membrane proteins as well as to lipids, making fluorescence data difficult to interpret. We used both ANS and the lipid specific dye NPN as fluorescence probes, because differences in the fluorescence intensities may reveal specific alterations in the protein and lipid constituents. It has been reported that the increased rigidity of the environment enhances fluorescence intensity.^{29,30} This agrees well with our results, and a steeper decrease in NPN fluorescence intensity after the inflection point (between 20 and 30 °C) can result from the “melting” of lipids.

One of the most important physiological functions of cytoplasmic membranes is to transport solutes. Shechter *et al.*³³ have reported that the initial rates of sugar and amino acid transport exhibit sharp activity changes with respect to temperature at 15 to 20 °C and optima at 45 to 50 °C. By means of fluorescence they found two inflections to be functions of temperature. One inflection point was correlated with the transition in transport activity that occurred at around 20 °C and the other at above 35 °C. They explained the first transition as due to the “melting” of paraffin chains of membrane phospholipids and they linked the second one to abrupt increase in passive membrane permeability. On the other hand, “melting” of lipids may allow ANS to transport to an environment with more binding groups. It is also possible that “melting” of lipids changes the conformation of proteins and thus the environment of the binding sites. Trauble³¹ found a considerable enhancement of ANS fluorescence when one of the characteristic transition temperatures of lipids was reached. He attributed this enhancement to a “dispersing” of the polar groups and a greater flexibility of the CH₂-chains of the lipid, thus creating more binding sites for the dye.

Low growth temperatures shifted the inflection points a few degrees downwards in *Ps. fluorescens* and *Str. thermophilus*. On the other hand, no considerable shift to lower or higher temperatures was observed when these bacteria and *E. coli* were cultured in aerobic or anaerobic conditions. It has been reported earlier that relative amounts of unsaturated acids increase with decreasing growth temperature in *Ps. fluorescens*.¹⁸ The results of Cullen *et al.*¹⁶ as well as our own suggest that, although *Ps. fluorescens* is an aerobe and *E. coli* and *Str. thermophilus* are facultative anaerobes, they all have a rather constant lipid composition independent of oxygen supply. This view agrees well with the results of Erwin and Bloch³² who found that all these organisms synthesize unsaturated acids by an anaerobic pathway.

Melchior *et al.*¹² have demonstrated by means of a calorimeter two inflection points of heat absorption in the membranes of *Mycoplasma laidlawi*. The first one is due to reversible transition in the lipid component and the second to an irreversible denaturation of the protein component. The second inflection point in our investigations is likely due to irreversible protein denaturation, too, because when the temperature was elevated by stepwise heating and then returned to the initial temperature, alterations in the cells were partly reversible until 35°C, after which the fluorescence intensity steadily increased (Fig. 4). Furthermore, preincubations of the bacterial cells at elevated temperatures resulted in irreversible increases in fluorescence intensity.⁸ The effective temperatures were also between 40 and 60°C depending on the thermosensitivity of the bacteria. Although the inflection points of fluorescence intensity (between 35 and 60°C) followed the order of bacterial thermosensitivity, the presence or absence of the peptidoglycan layer may affect the fluorescence profile too. When this layer was removed by EDTA-lysozyme treatment the intensities of ANS and especially NPN were considerably reduced.

These results suggest that liquid-crystalline phase transitions as well as membrane damages of the bacterial cells change the rigidity of the environment of naphthalene dyes; thus these dyes can be applied to monitor phase transitions and the starting points of bacterial destructions.

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