Early Changes in Bacterial Envelopes after Inhibition of Peptidoglycan Synthesis, as Shown by the Use of a Fluorescent Probe

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The probe 8-anilino-1-naphthalene sulphonate (ANS) showed increasing fluorescence with *Bacillus subtilis* *metC* *lyt-2* cells taken from exponentially growing cultures treated with antibiotics that inhibit cell-wall peptidoglycan synthesis. This increase was due to the probe reaching hydrophobic cell constituents, probably membranes, and started within 30 min of the addition of the antibiotics. This corresponded to the time at which membrane function had been shown to be damaged. The increased fluorescence of the cells with ANS persisted after removal of the antibiotics.

INTRODUCTION

Antibiotics such as β -lactams or cycloserine that inhibit peptidoglycan biosynthesis are bactericidal and lead to the lysis of growing cultures of many bacteria, but for other species of bacteria they are bactericidal yet do not cause perceptible lysis. The relation between the possession and action of autolysins and the death of antibiotic-treated bacteria has been the subject of much work and speculation (Tomasz, 1979; Shockman *et al.*, 1979, 1981; Rogers *et al.*, 1980, 1983; Lopez *et al.*, 1976; Sanchez-Puelles *et al.*, 1986) but the situation is still unclear. Mutants of *Bacillus subtilis* deficient in autolysins were not lysed significantly by β -lactams as measured by optical density, leakage of protein or the formation of soluble wall constituents (Rogers *et al.*, 1983) but the bacteria died, suffering what has been called non-lytic death (Shockman *et al.*, 1981). Early damage to membrane functions was shown but wall changes allowing penetration of cationized ferritin particles could only be seen much later and in rare cells. The present paper reports an early change in β -lactam-treated, autolysin-deficient *B. subtilis* that is best attributed to a change in the permeability of the cell walls.

METHODS

Micro-organisms and growth media. Two autolysin-deficient mutants were used: FJ6 (*metC3* *lyt-2*) (Fein & Rogers, 1976) and 109 (*metC3* *lyt-2* TOL⁺). The latter was one of a number of mutants selected from strain FJ6 after mutagenesis with ethylmethane sulphonate as surviving on beef heart infusion medium (Difco) containing 5 μ g cephalothin ml⁻¹ but not forming colonies on media containing 0.1 μ g ml⁻¹. The MIC for cephalothin was about 0.05 μ g ml⁻¹ when freshly isolated as was that of strain FJ6. This has increased slightly to between 0.05 and 0.1 μ g ml⁻¹ over a period of 4 years. Strain 109 was susceptible to rapid lysis by added purified autolysins or lysozyme. Its rates of lysis when antibiotics were added to cultures were much slower than those of FJ6 as were rates of lysis of suspensions at either pH 9.0 or 5.5. Extracts from cells made with 5 M-LiCl were too low in activity for quantitative measurement. Its rate of cell autolysis in suspension has increased somewhat over the years but it still lysed at about 10 to 20% of the rate of FJ6 when grown on beef heart medium (H. J. Rogers & S. Raytor, unpublished work; Rogers & Wright, 1986). Precautions have been found to be necessary to avoid all measurable lysis of both strain FJ6 and strain 109 when treated with antibiotics. Spore suspensions were carefully freed of vegetative debris by adequate lysozyme treatment and repeated washing with buffer. They were stored at -10 °C and only small samples were thawed for use in the automatic inoculator (Rogers & Wright, 1986). The growth

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Abbreviations: ANS, 8-anilino-1-naphthalene sulphonate; PMSF, phenylmethylsulphonyl fluoride.

media were CHSC and MSM (Rogers *et al.*, 1983; Rogers & Thurman, 1985). The behaviour of FJ6 with β -lactam antibiotics in these media was found to differ. Whereas in MSM medium the concentrations of β -lactams had to be carefully chosen to avoid a very slow lysis subsequent to their addition, in CHSC medium the concentrations could be increased at least twentyfold beyond that necessary just to stop growth without perceptible lysis occurring for at least 8 h. In 24 h the optical density of CHSC cultures treated with cephalothin was reduced by only about 10%. Cultures were grown in side-arm flasks of five times the volume of medium at 35 °C in an orbital incubator.

Measurement of growth and lysis in cultures. OD₆₇₅ was used as described by Rogers & Wright (1986).

Preparation of SDS-walls. Bacterial cell walls free of protein and lipid were prepared as by Fein & Rogers (1976).

Fluorescence measurements. Samples were taken of sufficient volume eventually to provide two 1 ml suspensions of OD₆₇₅ about 0.600. The samples were filtered through 0.45 μ m membranes, the bacteria washed with twice the volume of 0.05 M-sodium potassium phosphate buffer, pH 6.6, and resuspended to give the two 1.0 ml volumes. To one of these was added 2.0 ml and to the other 1.85 ml of the same phosphate buffer together with 150 μ l 1 mM-8-anilino-1-naphthalene sulphonate (ANS). The fluorescence of both samples was measured in a 1 cm glass cuvette at 25 °C in a Perkin-Elmer MPF3 fluorimeter using an excitation wavelength of 370 nm, an emission wavelength of 475 nm, and slit widths of 8 nm. The OD₆₇₅ of the samples was also recorded. The instrument was standardized daily by measuring the fluorescence of 5 μ M-ANS in ethanol and the results corrected for any change in the value obtained. The fluorescence readings for the bacterial sample containing ANS (R_{ANS}) were also corrected for the value obtained for the sample not containing ANS, which measured scattered light (R_s), and for the fluorescence of 50 μ M-ANS in 0.05 M-sodium potassium phosphate buffer pH 6.6 (ANS_{Aq}), itself corrected for light absorption using its inner filter factor (IFF). The fluorescence intensity F was then

$$\left[\frac{[(R_{ANS} - R_s) - \text{ANS}_{Aq}] \text{ IFF}}{\text{OD}_{675} \times 0.5} \right] \cdot \frac{485}{\text{ANS}_{Alc}}$$

where 485 was the value initially obtained as the fluorescence of 5 μ M-ANS in absolute ethanol and ANS_{Alc} the value obtained in the particular experiment. The IFF was measured as

$$\text{antiln} \left[\frac{\text{OD}_{370} + \text{OD}_{475}}{2} \right]$$

Preparation of membranes. Bacteria were incubated for 2 h at 35 °C in 0.05 M-sodium potassium phosphate buffer, pH 6.6, containing 50 μ g lysozyme ml⁻¹, 10 μ g deoxyribonuclease ml⁻¹, 10 μ g ribonuclease ml⁻¹ and, in some experiments, 10 μ g phenylmethylsulphonyl fluoride (PMSF) ml⁻¹. The digest was then centrifuged (100000 g for 30 min at 4 °C) and the pellet taken as the membrane fraction of the cells. The supernatant was called the cytosol.

RESULTS

Fluorescence of exponentially growing FJ6 with ANS

Solutions of ANS in 0.05 M-sodium potassium phosphate buffer, pH 6.6, had a weak fluorescence with an emission maximum of 520 nm. As the concentration of bacteria present in such solutions was increased up to 100 μ g dry wt ml⁻¹ (equivalent to an OD₆₇₅ of 0.2) the fluorescence increased and the emission maximum shifted towards 475 nm. No further shift from 475 nm occurred with higher bacterial concentrations but the fluorescence intensity was increased. Within the range of OD₆₇₅ 0.02 to 0.30 fluorescence was proportional to bacterial mass. It was also proportional to ANS concentration providing the appropriate inner filter corrections were made. The reduced wavelength of the emission spectrum is characteristic of ANS in an hydrophobic environment. Membrane preparations made from strains FJ6 and 168 were suspended in phosphate buffer and 50 μ M-ANS added; the maximum in the emission spectrum was also at 475 nm. Cell wall preparations (SDS-cell walls) did not significantly increase the fluorescence of ANS in phosphate buffer (Table 1) nor shift the emission maximum. The fluorescence intensity of the membranes was 1420 per mg membrane protein and the membrane content per mg dry wt bacterial cells showed that the fluorescence of the bacteria was only 16% of that calculated if the probe had reacted with all the cell membrane. Further evidence that the fluorescence of ANS with the bacteria was due to reaction with membranes was provided by varying the pH of the suspension buffer. The fluorescence of ANS with membrane preparations is known to be greatly influenced by pH and ionic strength (Freedman & Radda, 1969). When the pH was varied from 3.0 to 10.0 using either FJ6 bacteria or

Fig. 1. Increase in fluorescence with ANS during treatment of strain FJ6 with cephalothin. Strain FJ6 was grown on CHSC medium and at time zero $0.2 \mu\text{g}$ cephalothin ml^{-1} was added. At the times indicated samples were withdrawn and the OD_{675} (●) and the fluorescence (F) with ANS (○) measured as described in Methods.

membranes isolated from them, the effects on both samples were identical. The absolute values were five to six times higher for the membranes than for the bacteria.

Effect of antibiotics on the fluorescence of ANS with whole bacteria

A concentration of $0.2 \mu\text{g}$ cephalothin ml^{-1} was added to exponentially growing cultures of strain FJ6 in CHSC medium and samples were taken at regular intervals, filtered, and the bacteria washed and resuspended in phosphate buffer with and without $50 \mu\text{M}$ -ANS as described in Methods. The fluorescence with ANS started to increase about 20–30 min after adding the antibiotic and continued to do so for about 6 h by which time the fluorescence per mg bacterial dry wt was about six to seven times the value for control cells (Fig. 1). Increasing the concentration of cephalothin to $1 \mu\text{g}$ ml^{-1} had little effect on either the rate of development or final intensity of fluorescence. When FJ6 was grown in MSM medium and $0.05 \mu\text{g}$ cephalothin ml^{-1} added the results were similar but the rise in fluorescence started slightly later at 30–40 min after adding the antibiotic. Addition of $0.2 \mu\text{g}$ cephalothin ml^{-1} to strain 109 in CHSC medium led to a much longer period before growth stopped and a much greater increase in OD_{675} , being 2–3 times that obtained with FJ6. Under these conditions fluorescence did not start to increase for 60–70 min and then rose much more slowly than with FJ6 but if the concentration of antibiotic was increased to $1 \mu\text{g}$ ml^{-1} the kinetics of growth and fluorescence increase for FJ6 and 109 were identical. The relationship, if any, between this behaviour of strain 109 and its slight increase in resistance to cephalothin is unknown. Cycloserine added at a concentration of $330 \mu\text{g}$ ml^{-1} inhibited growth of exponential cultures of FJ6 and the increase in fluorescence of ANS was identical to that given by cephalothin. A range of β -lactam antibiotics behaved similarly whereas antibiotics that did not interfere with wall synthesis at concentrations sufficient to stop growth, such as chloramphenicol, erythromycin, spectinomycin and kanamycin, caused no increase of fluorescence with ANS.

Although no reduction occurred in OD_{675} during many hours treatment of the mutants in the CHSC medium with cell-wall-inhibiting antibiotics, this did not exclude actions of autolysins too slight to cause bacterial rupture but sufficient to modify the cell walls so that they were more permeable to ANS. If so, methods which either increased or decreased cell autolysis might have been expected to alter the fluorescence of bacteria with ANS. Both cerulenin and 1-alkanols added to the growth medium for strain 168 reduce cell autolysis (Rogers & Thurman, 1985; Rogers & Wright, 1986). The addition of neither $3 \mu\text{g}$ cerulenin ml^{-1} nor 0.7 M -ethanol to cultures of FJ6 30 min before $0.2 \mu\text{g}$ cephalothin ml^{-1} affected the rate of development or final intensity of fluorescence with ANS.

Table 1. *ANS fluorescence with cell fractions and with strain FJ6 treated with cephalothin*

Strain FJ6 was grown on CHSC medium and treated with $0.2 \mu\text{g}$ cephalothin ml^{-1} . The lysozyme digest and the cell fractions were prepared as described in Methods. The results are expressed as fluorescence per mg dry wt bacteria $\pm$ SD. The number of independent determinations is given in parentheses.

	Fluorescence	
	- PMSF	+ PMSF
Untreated bacteria	25.0 ± 23.5 (19)	
Cephalothin treated bacteria		
3 h	93.4 ± 6.4 (3)	98
5 h	138	130
Lysozyme digest	211 ± 31.5 (10)	336.6 ± 45.9 (4)
Membranes	105.5 ± 16.7 (3)	212
Cytosol	92.8 ± 23.3 (3)	124.2
Cell walls		
Untreated cells	0 ± 2.6 (3)	—
Cephalothin treated cells	12.8, 13.3	—

Persistence of fluorescent effect

A sub-inhibitory concentration of cephalothin ($0.03 \mu\text{g ml}^{-1}$) added to exponentially growing cultures of FJ6 in MSM medium caused a temporary reduction in growth rate after 1 h which lasted for a further 1–2 h. The fluorescence of the bacteria with ANS was measured 2 h after adding cephalothin and the whole culture was then filtered through a $0.45 \mu\text{m}$ pore membrane, the bacteria washed with a large volume of warm medium not containing antibiotic, and resuspended in more of the same medium at 35°C . Growth accelerated within 1 h to the same rate as a culture that had not been treated with antibiotic. Samples were taken at frequent intervals for measurement of fluorescence with ANS. After the period of accelerating growth, the fluorescence intensity declined exponentially but at about half the rate calculated by simple dilution of the original antibiotic-treated cells. The observed half-life for the decay was 175 min whereas the calculated value was 80 min which is longer than the doubling time of the bacteria which was 65 min due to allowance made for the fluorescence of the growing population of new undamaged cells.

Fluorescence of bacterial components with ANS

The fluorescence given with ANS by lysozyme digests, membranes and intracellular soluble components (see Methods) was increased if the protease inhibitor PMSF was present during digestion (Table 1). If membrane preparations were treated briefly with trypsin in the absence of PMSF fluorescence with ANS was greatly reduced. Inclusion of up to 5 mM-PMSF in the culture medium during treatment of FJ6 with cephalothin, however, failed to alter the fluorescence of the cells with ANS. If as seems likely the proteases were only able to act on cell components after disruption, the fluorescence results for the cephalothin-treated cells should be compared with those for cell fractions prepared in the presence of PMSF. Then the value for the cells treated with antibiotic for 5–6 h was lower than that given by the membrane they contained. The cell walls isolated from control cells showed no fluorescence with ANS whereas those from bacteria treated for 3 h with cephalothin showed a just-detectable signal; however, this was too small for exact measurement. As can be seen measurement of the control cells themselves was difficult and subject to high variation because of the small signal emitted.

DISCUSSION

Earlier work (Cramer & Phillips, 1970; Cramer *et al.*, 1973; Phillips & Cramer, 1973; Helgersson & Cramer, 1977; Nieva-Gomez & Gennis, 1977; Nieva-Gomez *et al.*, 1976) with *Escherichia coli* showed that fluorescence with various probes was increased and the emission wave-length peak reduced when the bacteria were deprived of energy source or treated with

colicin E1. The general conclusion from the work was that the change in the fluorescence was due to an alteration in the organization or conformation of the bacterial cell envelope (Nieva-Gomez & Gennis, 1977; Phillips & Cramer, 1973). The present work describes a similar change in fluorescence with ANS when the Gram-positive *B. subtilis* strain FJ6 was treated with antibiotics that inhibit cell-wall synthesis. The increased fluorescence of the probe started within 30 min of the addition of the antibiotics while the biomass in the culture was still increasing and at the same time as membrane damage became apparent (Rogers *et al.*, 1983). Unlike Gram-negative organisms in which the lipopolysaccharides and outer membranes are known to exclude hydrophobic substances (Nikaido & Vaara, 1985) the walls of Gram-positive organisms might have been expected to be permeable to ANS which is a negatively charged amphipathic molecule of M_r 310.5. The fixed negative charges in the walls do not seem to play a major role in its exclusion since wide variations in pH affected fluorescence intensity in the same way as for isolated membranes. Two possibly interrelated processes might have led to increased contact between the probe and hydrophobic cell contents. Some hydrolysis of peptidoglycan bonds by the residual autolysins present in all autolytic-deficient mutants so far isolated might have made the wall more porous. Growth under conditions further depressing cell autolysis by the mutants may still have left enough active enzyme. Additionally, however, hydrophobic cell constituents might have emerged into the altered walls. A number of species which show no loss of optical density when treated with antibiotics inhibiting wall synthesis excrete phospholipids, albeit at very different rates (Horne & Tomasz, 1977; Brissette *et al.*, 1982; Rogers *et al.*, 1983). Moreover, phospholipid turnover increased at the same time as fluorescence with ANS started to increase (Rogers *et al.*, 1983). Indeed, whole membrane vesicles have been claimed to be excreted by autolysin-defective strains of pneumococci treated with benzylpenicillin (Hakenbeck *et al.*, 1978) although this did not occur with the *B. subtilis* mutants. The known peripheral location in membranes of probes like ANS (Azzi, 1975), and the importance of membrane proteins in their fluorescence, makes emergence of membrane proteins rather than phospholipids into the walls a more likely cause of the phenomenon.

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