JOURNAL OF BACTERIOLOGY, Apr. 1973, p. 59-64
Vol. 114, No. 1

Acriflavine Uptake and Resistance in *Serratia marcescens* Cells and Spheroplasts

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Received for publication 5 October 1972

Acriflavine uptake and resistance were investigated in red, sensitive *Serratia marcescens* cells and in orange, resistant mutant cells and their respective spheroplasts. Acriflavine-sensitive cells bound more acriflavine than acriflavine-resistant cells. Spheroplasts from sensitive and resistant cells were both resistant to and bound similar amounts of acriflavine. Sensitive cells were resistant to acriflavine in medium supplemented with 0.01 M $MgSO_4$ and 0.5 M sucrose. In the presence of 0.01 M $MgSO_4$ and 0.5 M sucrose, acriflavine binding by sensitive cells was reduced to the level of binding by resistant cells. Inhibition of metabolism by carbon starvation, chloramphenicol, As_2O_3, nitrosoguanidine, and bromouracil did not affect the uptake of acriflavine by sensitive and resistant cells. Rapid temperature changes did not alter the acriflavine-binding capacity of the cells, and no temperature dependence of acriflavine uptake or release was observed at 0 and 30 C. Acriflavine uptake by both sensitive and resistant cells increased with increase in pH from 5.7 to 8.0. The logarithm of acriflavine uptake was a linear function of the logarithm of the acriflavine concentration in the binding medium.

The binding of acriflavine (AF) by sensitive and resistant *Escherichia coli* cells and the mechanism of AF resistance were described by Nakamura (3, 4) and Silver et al. (5). Nakamura (3) reported that AF binding by *E. coli* cells was affected by metabolic activity and suggested that AF resistance was due to the synthesis of a macromolecule which interfered with the capacity of cells to bind AF. Woods et al. (7) isolated a stable, orange, AF-resistant *Serratia marcescens* mutant by treatment with AF of a wild-type, red, AF-sensitive strain. The mechanism of AF resistance in *S. marcescens* cells and spheroplasts was investigated and shown to be different from that in *E. coli*.

MATERIALS AND METHODS

Strains. A wild-type, red, AF-sensitive strain of *S. marcescens* and an orange, AF-resistant, mutant strain were used (7). Incubation was at 30 C.

Media. Nutrient broth and agar (Difco) were used. Minimal salts solution (3) contained (per liter): 3 g of KH_2PO_4, 1 g of NH_4Cl, 0.6 g of Na_2SO_4, and 0.4 g of $MgCl_2$. The minimal salts solution was supplemented with 0.4% glucose. Spheroplast medium contained nutrient broth supplemented with 0.01 M $MgSO_4 \cdot 7H_2O$ and 0.5 M sucrose. Brain Heart Infusion broth (Difco) was supplemented with 1.8% NaCl, 20% human serum (inactivated at 56 C for 30 min), and 10% yeast extract (Difco). All media were adjusted to pH 7.2 or buffered at pH 7.2 with 0.1 M sodium phosphate buffer.

Preparation of spheroplasts. Overnight nutrient broth cultures of AF-sensitive and AF-resistant cells were diluted 1:10 in prewarmed spheroplast medium containing 5,000 units of penicillin per ml and were incubated with aeration for 5 h. Samples were examined with a microscope, and spheroplasts were counted in a hemocytometer.

AF sensitivity of cells and spheroplasts. Samples (1 ml) containing 10^7 spheroplasts from AF-sensitive and AF-resistant cells were each inoculated into 9 ml of supplemented Brain Heart Infusion broth and 9 ml of spheroplast medium, each containing 5,000 units of penicillin per ml, as well as into 9 ml of supplemented Brain Heart Infusion broth and 9 ml of spheroplast medium, each containing 5,000 units of penicillin per ml plus 35 μg of AF per ml. This was repeated using 1-ml samples of AF-sensitive and AF-resistant cells except that penicillin was omitted from each medium. Spheroplast and cell cultures were then incubated without aeration for 18 h prior to dilution in spheroplast medium and plating on spheroplast agar (spheroplast medium containing 1.5% agar).

Uptake of AF. Overnight AF-sensitive and AF-resistant broth cultures were turbidimetrically adjusted to be equivalent to 10^9 cells per ml. Spheroplast cultures were adjusted to 2×10^7 spheroplasts per ml. Samples (20 ml) of the cell and spheroplast

cultures were sedimented by centrifugation, washed, and suspended in 10 ml of the relevant medium (pH 7.2) containing 5 μg of AF per ml. The suspensions were incubated at 30 C for different time intervals and then centrifuged at 6,500 × *g* for 10 min. The AF concentration in the supernatant fluid was determined from the absorbancy at 450 nm measured with a Unicam SP 800 spectrophotometer. The amount of AF lost from the supernatant fluid was taken as the AF content of the bacteria and used as an index of their AF-binding capacity.

Metabolic activity and AF uptake. Overnight AF-sensitive and AF-resistant cultures were washed, suspended in minimal salts solution (pH 7.2) with and without the addition of glucose (0.4%), and incubated for 3 h. The cells from each suspension were then washed and transferred to minimal salts solution containing 5 μg of AF per ml, with and without glucose. The AF content of the cells was determined after 2.5 h at 30 C. The effects of metabolic inhibitors and mutagens were determined by suspending washed cells in buffered nutrient broth (pH 7.2) containing (per ml): 80 μg of chloramphenicol, 80 μg of As_2O_3, 80 μg of nitrosoguanidine, and 80 μg of bromouracil. After 2 h of incubation, AF was added to these cultures (final concentration of 5 μg/ml), and the AF content of the cells was determined after a further 2.5 h incubation period.

Effect of temperature. The effect of low temperature shock on the AF uptake of the cells was determined by suspending washed cells in buffered nutrient broth (pH 7.2) containing 5 μg of AF per ml at 30 C. The AF content of the cells was estimated at 30, 60, and 90 min. After 90 min of incubation, half the suspension was placed at 0 C. After further 30-, 60-, 90-, and 120-min incubation periods, the AF content of the cells at 0 and 30 C was determined. The effect of increased temperature was investigated by incubating cells at 0 C for 90 min and then at 30 C for a further 120 min. Heat-killed cells were obtained by heating cells at 100 C in a water bath for 10 min.

Effect of pH and AF concentration. Cells were grown overnight in nutrient broth buffered at pH 5.7, 6.2, 7.2, and 8.0 prior to harvesting and washing in buffer at the same pH as the growth medium. The cells were incubated in nutrient broth containing 5 μg of AF per ml and then were buffered at the same pH as the overnight growth medium. The AF concentration of the cells was determined after 1 h at 30 C. Cells were also grown in media buffered at pH 5.7 and 8.0, washed, and suspended for 1 h in AF media buffered at pH 8.0 and 5.7, respectively. The effect of AF concentration was determined on washed cells suspended for 30 min in nutrient broth containing different concentrations of AF.

RESULTS

Sensitivity of cells and spheroplasts to AF. AF-sensitive cells in supplemented Brain Heart Infusion broth were sensitive to 35 μg of AF per ml, but conversion of AF-sensitive cells to spheroplasts rendered them resistant to AF (Table 1). AF-resistant cells and spheroplasts both were resistant to 35 μg of AF per ml. Treatment of AF-sensitive cells in spheroplast medium which contained 0.01 M $MgSO_4$ and 0.5 M sucrose inhibited the action of AF (Table 1).

Uptake of AF. After treatment with 5 μg of AF per ml in nutrient broth, in 1.8% NaCl, and in supplemented Brain Heart Infusion broth, AF-sensitive cells bound more AF than AF-resistant cells (Fig. 1). The amount of AF bound by AF-sensitive cells in spheroplast medium, which contained 0.01 M $MgSO_4$ and 0.5 M sucrose, was reduced and was similar to that bound by AF-resistant cells. Spheroplasts from AF-sensitive and AF-resistant cells bound approximately the same amounts of AF.

Metabolic activity and AF uptake. Carbon starvation did not affect the AF binding capacity of sensitive or resistant cells (Fig. 2). This result differed from that reported for AF binding in *E. coli*.

The metabolic inhibitors, chloramphenicol and As_2O_3, and the mutagens, nitrosoguanidine and bromouracil, did not alter the binding capacity of the *S. marcescens* cells, and AF-sensitive cells bound more AF than AF-resistant cells (Table 2). The results differ from those reported by Nakamura (3) for *E. coli* where these metabolic inhibitors caused an increase in AF uptake. The increased uptake was more marked in resistant than sensitive *E. coli* cells.

Effect of temperature. Nakamura (3) and Silver et al. (5) reported that the AF binding of *E. coli* cells increased rapidly at low temperature (0 C) and that the increase was most marked in the AF-sensitive strain. The increased binding at 0 C was readily reversible as the AF absorbed at 0 C was released when the temperature was raised to 25 or 37 C. In *E. coli* it was possible to distinguish between external and internal binding of AF on the basis of the ready reversibility of the binding to the external receptors and the essentially irreversible binding after the cellular permeability barriers were removed by heating. The effect of rapid temperature decrease and increase on AF binding was studied with sensitive and resistant *S. marcescens* cells, but no temperature-dependent reversible binding was observed (Fig. 3 and 4). The resistant cells took up more AF within 30 min at 0 C than at 30 C. *E. coli* AF-resistant cells also took up more AF within 30 min at 0 C than at 30 C (3). Between 0 and 55 C, heat-killed cells absorbed greater amounts of AF than did living cells (Fig. 5).

TABLE 1. *Percent survival of AF-sensitive and AF-resistant cells and their spheroplasts after treatment for 18 h in supplemented Brain Heart Infusion broth and in spheroplast medium, each containing 35 μg of AF per ml*

Strain	Survival[a] (%)			
	Brain Heart Infusion broth		Spheroplast medium	
	Cells	Spheroplasts	Cells	Spheroplasts
AF-sensitive	15	100	100	100
AF-resistant	100	100	100	100

[a] Percent survival (mean of these experiments) is the percentage of the control in each case without 35 μg of AF per ml.

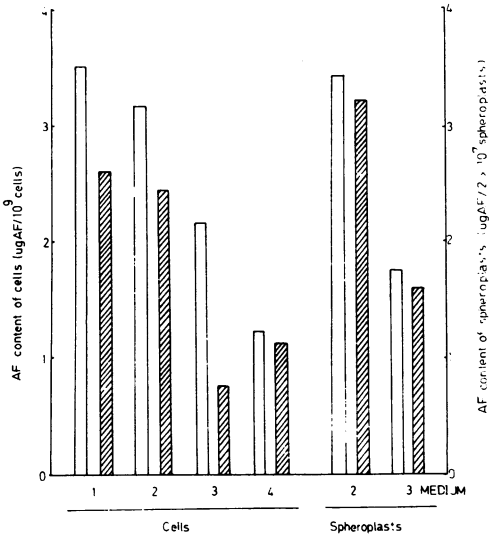

FIG. 1. *AF uptake by sensitive (unshaded) and resistant (shaded) cells and their spheroplasts in nutrient broth (1), 1.8% NaCl (2), supplemented Brain Heart Infusion broth (3), and spheroplast medium (4). Each medium contained 5 μg of AF/ml, and the values plotted are the means of three experiments.*

Temperature did not affect the uptake of AF by heat-killed cells.

Effect of pH and AF concentration. The AF binding of sensitive and resistant cells increased with increase in pH (Fig. 6). The pH effect was most pronounced in the AF binding medium since the amount of AF bound by cells grown at pH 5.7, but exposed to AF at pH 8.0, was similar to that bound by cells grown and exposed to AF at pH 8.0. Similarly, cells grown at pH 8.0, but exposed to AF at pH 5.7, bound

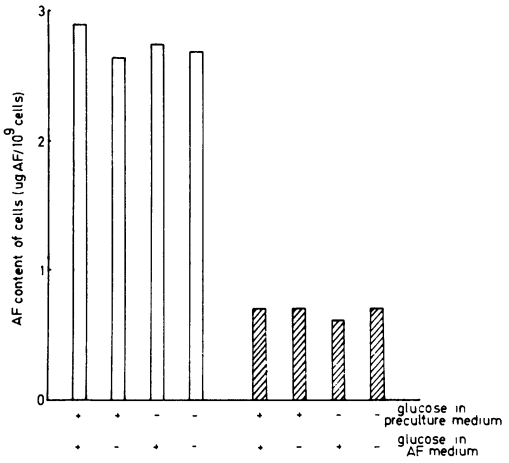

FIG. 2. *Effect of carbon starvation on AF uptake by sensitive (unshaded) and resistant (shaded) cells. Overnight, washed cultures were suspended for 3 h at 30 C in preculture media with and without glucose prior to transfer to AF (5 μg of AF per ml) binding medium with and without glucose. The AF contents are the means of three experiments.*

TABLE 2. *Effect of metabolic inhibitors and mutagens on AF binding*

Test substance[a]	AF content of cells (μg of AF/10^9 cells)	
	AF-sensitive strain	AF-resistant strain
None	3.6	2.7
Chloramphenicol	3.6	2.7
As_2O_3	3.6	2.5
Nitrosoguanidine	3.6	2.7
Bromouracil	3.6	2.7

[a] Final concentration: 80 μg/ml.

a similar amount of AF as cells grown and exposed to AF at pH 5.7. A similar pH dependency for AF uptake was shown with *E. coli* cells by Nakamura (4) and Silver et al. (5).

There was a linear correlation between the logarithm of AF uptake by both AF-sensitive and AF-resistant cells and the logarithm of the AF concentration in the medium (Fig. 7). Similar results were obtained with resistant and sensitive *E. coli* cells (3).

DISCUSSION

AF-sensitive *S. marcescens* cells bind more AF than AF-resistant mutant cells. Spheroplasts from sensitive and resistant cells are both resistant to AF and bind similar amounts of AF. Sensitive cells are resistant to AF in spheroplast medium containing 0.01 M $MgSO_4$

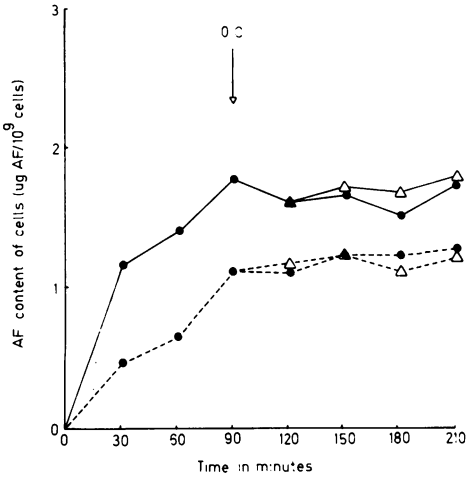

FIG. 3. *Effect of cold shock on AF uptake by sensitive cells (continuous line) and resistant cells (dotted line). Cells were incubated at 30 C (●) in broth containing 5 μg of AF/ml. After 90 min, a portion (Δ) of each culture was placed at 0 C. The values plotted are the means of four experiments.*

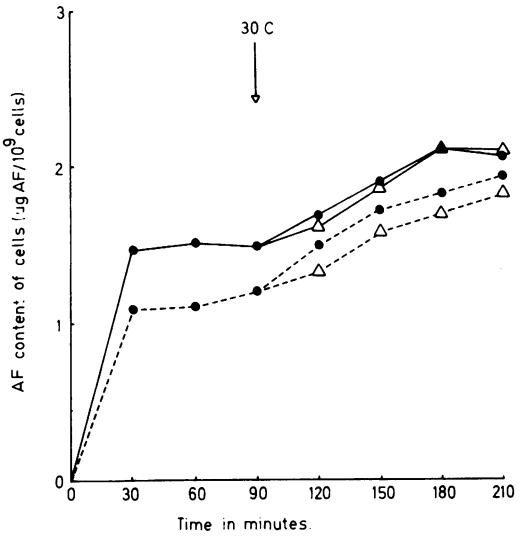

FIG. 4. *Effect of increased temperature on AF uptake by sensitive cells (continuous line) and resistant cells (dotted line). Cells were incubated at 0 C (●) in broth containing 5 μg of AF/ml. After 90 min, a portion (Δ) of each culture was placed at 30 C. The values plotted are the means of four experiments.*

and 0.5 M sucrose. Under these conditions, binding by sensitive cells was reduced to the level of binding by resistant cells. These observations suggest that AF resistance is due to a

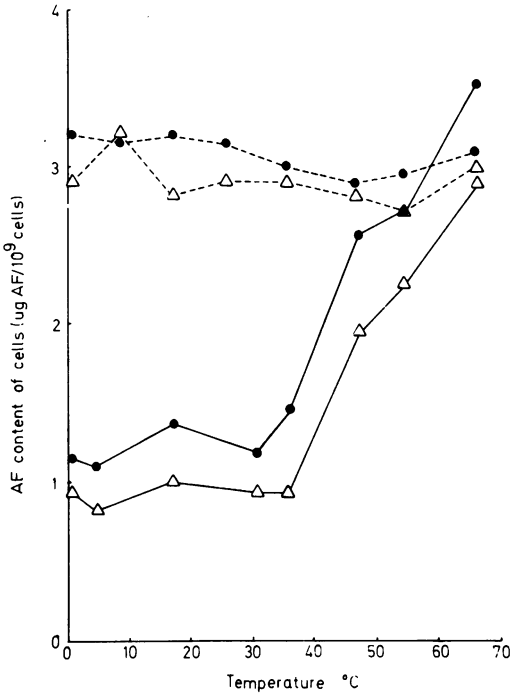

FIG. 5. *Effect of temperature on AF uptake by live (continuous line) and heat-killed (dotted line) cells. Live and heat-killed sensitive (●) and resistant (Δ) cells were incubated for 60 min at different temperatures in broth containing 5 μg of AF/ml. Each value plotted is the mean of four experiments.*

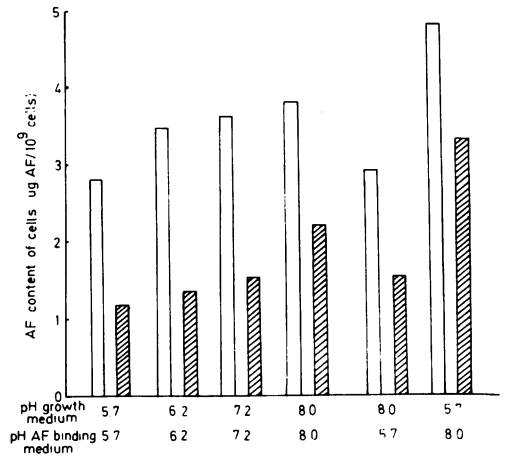

FIG. 6. *Effect of pH on AF uptake by sensitive (unshaded) and resistant (shaded) cells. Cells were incubated overnight in nutrient broth (buffered at different pH values) prior to washing and suspending for 1 h in AF (5 μg of AF/ml) binding medium buffered at the same or different pH as the growth medium.*

reduction in the AF binding capacity of the mutant cells. Nakamura (3) showed that the difference in AF sensitivity between sensitive and resistant *E. coli* cells was due to a gene controlling the AF uptake of the cells.

Active transport does not limit AF uptake in *S. marcescens* since carbon starvation, the addition of metabolic inhibitors, and temperature reduction did not decrease the AF uptake of the cells. In marked contrast to *E. coli*, these treatments did not cause an increase in AF uptake (3, 5). In *E. coli*, carbon starvation and metabolic inhibitors increase AF accumulation particularly in the resistant strain, and Nakamura (3) suggested that AF uptake in the resistant strain was prevented or interfered with by the synthesis of a specific macromolecule. AF resistance in our resistant *S. marcescens* strain is not due to a similar mechanism.

The acridine dyes are considered to associate with a component of the cell envelope during entry into the cell (6). In contrast to *E. coli*, this external binding in *S. marcescens* is not readily reversible. Since spheroplasts from AF-sensitive cells are resistant to AF and as there is no difference in the AF binding capacities of spheroplasts from sensitive and resistant cells, it is suggested that spheroplast formation resulted in a change which affected AF-specific receptor sites. The AF resistant mutant could, perhaps, be due to a mutation affecting this receptor component for AF. Woods et al. (7) reported the production of orange phenocopies from the red AF-sensitive strain by AF and the concurrent disappearance of a pigment-protein complex in red cell preparations. This complex was shown to be absent in orange AF-resistant preparations.

Kushner and Khan (1) studied proflavine uptake and release in sensitive and resistant *E. coli* cells and suggested that these cells were equally permeable to proflavine but that resistance was linked with the ability to expel the bound dye. Two different mechanisms seem to exist in *E. coli* for resistance to two closely related acridine dyes. The uptake of proflavine and other acridines by our *S. marcescens* strains will be presented in a future publication, but the characteristics of uptake of proflavine are essentially the same as those described for acriflavine.

Sucrose (0.5 M) and $MgSO_4$ (0.01 M) inhibited the action of AF; the individual effects of these substances is being determined and will be presented elsewhere. However, sucrose and Mg^{2+} separately inhibit the action of AF (unpublished results). Nakamura (4) reported the inhibition of AF action against *E. coli* by high concentrations of glucose. This effect in *E. coli* was partly due to pH as well as to some other mechanism. Massart and van der Stock (2) suggested that if the action of AF is inhibited by the presence of H^+ ions at low pH values because of competition for the negative charges on the cell surface, a similar effect could be occurring with several other mono-, di-, and trivalent cations. Mg^{2+} may compete with AF for the negatively charged groups including the specific receptor sites at the cell surface.

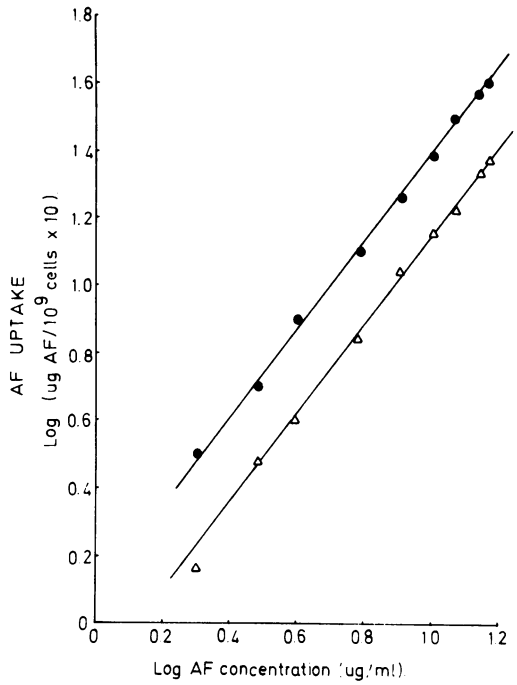

FIG. 7. *Relationship between AF concentration in the medium and AF uptake by cells. AF-sensitive (●) and -resistant (Δ) cells were incubated for 10 min in broth containing different concentrations of AF. The values plotted are the means of four experiments.*

ACKNOWLEDGMENTS

We acknowledge a Council for Scientific and Industrial Research (C.S.I.R.) Research Grant, V.R.S. acknowledges a C.S.I.R. Post-Graduate Research Bursary and P.B.W. a Barclay's Bank Scholarship.

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