INHIBITION OF BACILLUS SUBTILIS TRANSFORMING SYSTEM BY ACRIFLAVIN

S. R. AYAD

Department of Biological Chemistry, The University, Manchester 13, U.K.

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Dyes of the acridine series such as proflavin (2,8 diamino acridinium), acriflavin (a mixture of 2,8 diamino acridinium and 2,8 diamino acridine) and 5-amino acridine, are mutagenic for bacteriophages but not for bacteria [1,2]. Acriflavin impairs the "dark-repair" of UV-induced prototrophy in *Escherichia coli* [3–6] and inhibits the host cell reactivation in *Haemophilus influenzae* and *E. coli* infected with irradiated phage [7–10]. Moreover, the ability to transfer the sex and the resistance factors (autonomously replicating cytoplasmic components), from male to female bacteria can be eliminated by acriflavin treatment [11–13]. The present paper reports the effect of acriflavin on the *Bacillus subtilis* transforming system.

DNA from the Marburg strain of *B. subtilis* was prepared by Marmur method [14] and stored at 0° at a concentration of 600 μg/ml in 0.15 M NaCl–0.015 M sodium citrate, pH 7.0. Cells of *B. subtilis*, 168I⁻ (indole-requiring mutant) were made competent as described previously [15]. The effect of acriflavin-HCl (AF) on the transforming system of *B. subtilis* was studied by incubating competent cells at 34° with Marburg DNA (final concentration 5 μg/ml) for 90 min, followed by treatment at 37° for 5 min with DNase (50 μg/ml) and $MgSO_4$ (0.6 mg/ml). Samples were subsequently diluted and plated to determine the frequency of cells transformed to indole-independence. Fig. 1 indicates that the relative transforming activity (i.e. the activity at a particular AF concentration to that at zero concentration) gradually decreased with increasing AF concentration. No biological activity was observed at AF concentrations greater than 2 μg/ml.

In order to determine whether AF was affecting

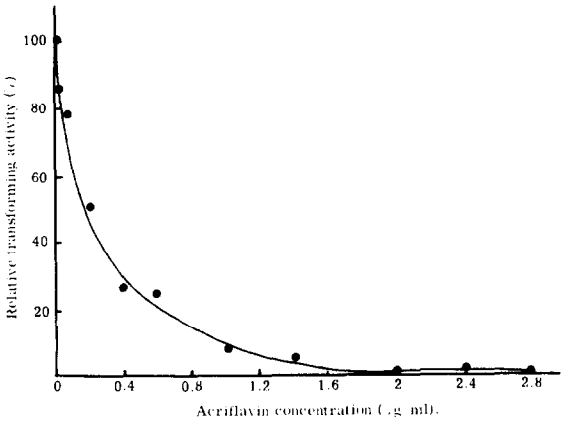

Fig. 1. Effect of AF concentration on the *B. subtilis*, 168I⁻ transforming system. Competent cells were incubated with various concentration of AF and Marburg DNA at a final concentration of 5 μg/ml at 34° for 90 min followed by DNase treatment (see text). Samples were diluted and plated to determine the frequency of cells transformed into indole-independence. The results are expressed as the % relative transforming activity (which is the activity at a particular AF concentration to that at zero concentration).

the competent cells or the donor DNA, competent cultures were incubated with (a) donor DNA (5 μg/ml) in the absence of AF, (b) donor DNA (5 μg/ml) and AF (2 μg/ml), (c) donor DNA which had been pretreated with AF (2 μg/ml) and dialysed against 0.15 M NaCl–0.015 M sodium citrate, pH 7.0, for 48 hr and (d) AF (2 μg/ml) for 5 min at 34° and the cells washed twice with Spizizen minimal medium [16]. The results (table 1) indicate that the biological activity was restored by either dialysing the AF-treated DNA, or by washing the AF-treated cells with minimal medium, and suggest that the transforming activity is reduced only when AF is present *in situ*

Table 1

Effect of AF on *B. subtilis*, 168I⁻ transforming system. Competent cultures were incubated with: 1) Marburg DNA (at a final concentration of 5 μg/ml) pretreated with AF (final concentration 2 μg/ml), and dialysed for 48 hr; and 2) AF (final concentration 2 μg/ml) washed twice with minimal medium [16], followed by incubation with Marburg DNA (final concentration 5 μg/ml). The transformation assays were terminated by the addition of DNase (see text) and samples diluted and plated to determine the frequency of transformation.

Competent cells incubated with	Number of transformants per ml X 10^{-3}
a) DNA	37
b) DNA + AF	0.003
c) DNA (pretreated with AF and dialysed for 48 hr)	29
d) AF for 5 min at 34°C and washed twice with minimal medium and then incubated with DNA	21

with the competent cells and donor DNA.

To ascertain whether DNA is taken up by competent cells in the presence of AF, competent cultures were exposed to [^{3}H] BU-labelled transforming DNA (final concentration 5 μg/ml and specific activity 2.9 X 10^5 cpm/μg) for different intervals of time in the presence and absence of AF at 34°. The cells were treated with DNase as described above and samples diluted and plated to determine the frequency of cells transformed to indole-independence. The remaining bacteria were rapidly cooled to 0°, centrifuged and washed three times with cold 0.15 M NaCl–0.05 M EDTA, pH 8.0, and the DNA extracted by Marmur method [14]. Samples (0.1 ml) of the DNA were mixed with 0.2 ml hyamine hydroxide and heated for 2 hr at 60°. 10 ml scintillator (0.4%, 2,5-diphenyloxazole and 0.04% 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene dissolved in xylene) was added and the samples counted in the Packard "Tricarb" liquid scintillation spectrometer with an efficiency of 35%. The results obtained (table 2) indicate that AF did not inhibit DNA uptake, but reduced the transforming activity to a negligible value. Moreover, [^{3}H]BU-labelled DNA samples, isolated from cells incubated for 10 min in the presence and absence of AF (table 2), were fractionated by preparative CsCl density gradient centrifugation. On attaining equilibrium drop fractions

Table 2

Effect of AF on the uptake and transforming activity of *B. subtilis* DNA.

Time of incubation (min)	cpm X 10^2/μg/ml DNA		No. of transformants X 10^{-3}	
	– AF	+ AF	– AF	+ AF
2	32	29	12	0.02
4	24	36	26	0.004
6	74	62	32	0.05
8	82	73	38	0.009
10	95	81	35	0.006

[^{3}H] BU labelled DNA was prepared by growing the marburg strain of *B. subtilis* for 16 hr at 37° in 100 ml. Spizizen minimal medium [16]. 60 ml was transferred to 2 L of the same medium to give an extinction of 0.25 at 600 mμ and incubated at 37° with vigorous aeration for 2 hr followed by the addition of 2 mc [^{3}H] 5-bromouracil (specific activity 1530 mc/mM) and the cells were grown further for 4 hr. DNA was prepared by Marmur method [14] (specific activity 2.9 X 10^5 cpm/μg DNA).

Competent cells were incubated with [^{3}H] BU-labelled DNA (final concentration 5 μg/ml) in the presence and absence of AF for different intervals of time followed by DNase treatment (see text). A sample was diluted and plated to determine the frequency of transformation. The remaining cells were washed, lysed and the DNA was extracted and counted in the Packard liquid scintillation spectrometer (see text).

were collected and the distribution of radioactivity and extinction at 260 mμ in the density gradient determined. The elution profile of the DNA extracted from the untreated competent cells (fig. 2a) consisted of three peaks representing labelled heavy donor (ρ = 1.786 g/cm^3), non-labelled light recipient (ρ = 1.703 g/cm^3) and intermediate labelled integrated DNA (ρ = 1.742 g/cm^3). (Evidence for the intermediate DNA representing an integrated molecule has been published elsewhere [17,18]. In the case of AF-treated cells (fig. 2b) no intermediate peak was observed, indicating that AF inhibited the integration of the donor DNA into the recipient genome.

The results presented above are in agreement with those of Patrick and Rupert [10], who showed that the transforming activity of UV-irradiated donor DNA was diminished in the presence of AF in the *Haemophilus influenzae* transforming system, indicating that the UV-repair mechanism was inhibited by AF. Okubo and Romig [19] also observed that the addition of AF to competent strain of *B. subtilis*

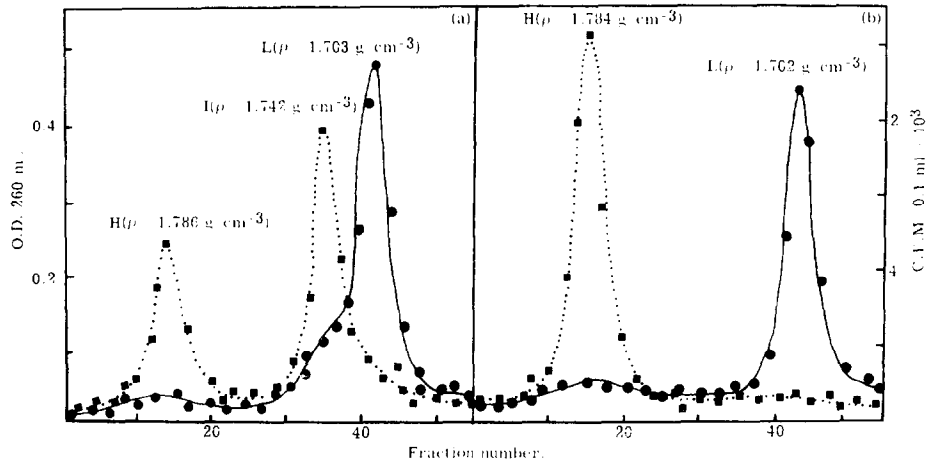

Fig. 2. Buoyant density distribution of DNA fractions extracted from competent cells of *B. subtilis* incubated with [^{3}H] BU donor DNA in the (a) absence and (b) presence of AF (see text). H, "heavy" [^{3}H] BU donor DNA; I, [^{3}H] BU integrated DNA; L, "light" recipient DNA. ●—● extinction at 260 mμ, ■·····■ cpm/0.1 ml.

decreased the number of infectious centres produced by the phage DNA, and suggested that this was due to the binding of AF to the phage DNA. Moreover, recent studies of the interaction of acridines with DNA by means of X-ray analysis and other physical techniques [20–22] strongly indicate that at low concentration, acridine molecules are inserted or intercalated between adjacent base pairs in the DNA resulting in the extension and unwinding of the phosphodiester backbone. It is therefore possible that a similar alteration to the structure of donor DNA by acriflavin could prevent its integration into the recipient genome of the competent cells of *B. subtilis*.

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