BIOCHEMICALLY ACTIVE SUBSTANCES FROM ACTINOMYCETES AND OTHER ORGANISMS. I

"MOTILOMETRY", A SIMPLE AND QUANTITATIVE ASSAY METHOD FOR MOTILITY INHIBITORS

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(Received for publication November 29, 1973)

A simple assay method (motilometry) for screening of compounds which affect the motility of *Escherichia coli* strain S-26 was devised using a semi-solid medium packed in a thin glass tube and applied to the culture filtrates of 86 Actinomycetes strains. In the presence of 0.04 M glucose, the spearhead of the migrated cell population became dense and formed a distinct band (Band I) followed by the second band (Band II) at a constant distance from the inoculated area during 14~17 hours of incubation at 37°C in either a vertical or horizontal position. The motility was easily quantified by measuring the distance from the inoculated area to Band I or II. Conditions affecting the migration in this system were studied in detail, and a standard procedure was established.

Although the brilliant history of antibiotic studies during these past 20 years led to the discovery of more than 2,000 different antibiotics from cultures of actinomycetes, almost completely disregarded were other metabolites of actinomycetes which had no or little antimicrobial activity, but which might have unique biological activity making them useful for other medical purposes. An exceptional example of the latter approach is the extensive work on enzyme inhibitors by UMEZAWA's group and others[1]. In an attempt to screen such unique substances from actinomycetes, we have been investigating some possible model systems where the function, the integrity and the structure of biomembranes were mainly involved[2]. Our purpose is to find microbial products that specifically react with certain biochemical processes occurring at the bacterial cell membrane. When a suitable system is employed, such *in vitro* reactive compounds may have unique activities never before detected among actinomycete metabolites.

This paper describes the first example of such a model, a biochemical screening system in which compounds affecting the motility of *Escherichia coli* can be detected. Based on the fact that flagella formation and hence the motility of *E. coli* are dependent upon the amount of adenosine-3′,5′-cyclic phosphate (cAMP) under catabolite repression[3,4], a simple glass tube method was devised for assaying culture filtrates of actinomycetes. Reports have accumulated to indicate that many types of compounds possess inhibitory effects on bacterial or protozoan motility. Those include neurotransmittance blockers[5], respiration inhibitors[6], microtubule depolymerizers[7] and membrane active antibiotics[8]. The primary purpose of this assay method, motilometry, is to provide a simple and quantitative model system for the screening of such compounds from culture filtrates of microorganisms which appears justified by the results

described in the subsequent paper[9]. An example will be reported elsewhere, in which a unique compound was isolated from a culture of an actinomycete strain by the use of this method and chemically characterized[10].

Materials and Methods

Bacterial Strains and Media Used

Among 5 available strains of *Escherichia coli* (K-12, K-12W3110, Q-13, NIHJ and S-26), S-26 was selected as the test organism because it showed the largest swarming area in a semi-solid nutrient agar plate (Table 1.). Further selection was made by picking up the cells from the external edge of the swarmed area. The selected clone of S-26 was maintained on 0.6% nutrient agar slants at room temperature. Strain S-26, originating from GAREN's laboratory, was kindly supplied by Prof. D. MIZUNO, University of Tokyo, and is characterized as Hfr., λ^+ and col. E_2 sensitive. Each time, preculture of S-26 grown in the preculture medium were harvested by centrifugation, washed and resuspended in an equal volume of 0.05 M sodium phosphate buffer (pH 7.5) and used for inoculation on the motility medium. The preculture medium contained (g/liter): K_2HPO_4 28.0 g, KH_2PO_4 8.0 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, $(NH_4)_2SO_4$ 1.0 g (pH 7.2) and was supplemented with 2.5 g of vitamin-free casamino acid and 0.02 M glucose. The motility medium (MM) contained the salts combined as described above and was supplemented with 0.4% casein hydrolyzate (Difco, Vitamin free), 0.2% yeast extract, 4×10^{-3} M KNO_3, and 0.05% agar. Repression MM was further supplemented with 0.04 M glucose (GMM).

Motility Measurement

After solubilizing by heating, the motility medium (MM) was packed by sucking into a sterilized thin glass tube (2 mm in diam. $\times$ 40 cm) while the medium was still warm (at 40~60°C). The solution of compounds or culture broth to be tested was placed in MM prior to the preparation of the tube. The end of the tube was covered by a Morton cap, while the other end was sealed with a plastic stopper. In usual practice, an aliquot (5 μl) of the precultured cell suspension ($1 \sim 2 \times 10^7$ cells) was carefully layered on the top of MM packed in the glass tube after cooling and then, unless otherwise stated, incubated at 37°C for 14~17 hours in a vertical position (standard assay). Turbidity change in these tubes was either recorded on a densitometer (Schoffel 2000) or judged from the thick bands formed around the spearhead of the bacterial population which migrated.

Electron Microscopic Observation

Negative-staining was consistently obtained by using a coppergrid with a carbon film. Fixation was done with 4% glutaraldehyde followed by staining with 1% phosphotungstate. Electron micrographs were taken using 50 KV at a magnification of $\times$6,900 with HS-8 Electron microscope (Hitachi).

Culture of Actinomycetes

Various actinomycetes strains isolated in our laboratory from soil samples collected in Japan were incubated at 27°C by shaking for 4 days in an Erlenmyer flask (500 ml) containing 125 ml of either A[11] or B medium[12]. The cultured broth was centrifuged at 3,000 rpm in the cold. An aliquot (0.1 ml) of the supernatant was placed in 1 ml of MM or GMM and then assayed for motility by the

Table 1. Relationship between the motility by the tube method and swarming of several *E. coli* strains

E. coli strain	Migrated distance* (mm) by the tube method		Swarming on 0.6% N-agar plate (mm $\times$ mm)
	non-repression M.M.	repression M.M.	
K-12	78	72	4.7 $\times$ 5.5
NIHJ	19	58	5.0 $\times$ 5.0
Q13	36	43	4.0 $\times$ 5.2
K12W3110	222	166	10.0 $\times$ 10.0
S-26	251	198	9.6 $\times$ 10.0
S-26**	303	266	10.0 $\times$ 10.5

* Band I.

** Selected clone from the outskirt of swarming area of strain S-26 and kept by the method described in the text.

standard method mentioned above.

Results

1. Quantitation of Motility by a Glass Tube Method

When 5 μl of cell suspension (2×10^9 cells/ml) of *E. coli* was inoculated on the top of MM packed in a thin glass tube (2 mm×40 cm) and incubated at 37°C in a vertical position, it migrated towards the tip of the glass while showing some growth. Thus, after 15-hour incubation, the spearhead of the cell population migrated approximately 30 cm from the top where the cells had been inoculated. In MM containing 0.04 M glucose (GMM), the migration was reduced by 15 % in distance (catabolite repression). A noticeable change seen in GMM is that two distinct bands of dense cell mass appeared as shown in Fig. 1.

The first and second bands, designated Band I and Band II, were reproducibly recognized to appear in a proportional position with practically all cells tested (Fig. 1), provided that the medium contained more than 5 mM glucose. It was found that the distance (mm) from the inoculated surface to Band I corresponded to the width of swarming area on a semisolid agar plate (Table 1), and was reproducibly constant at a certain glucose concentration under identical conditions. Essentially the same results were obtained when the incubation was done in a horizontal position or when cells were inoculated at the bottom (Table 2). However, for avoiding foam formation and for convenience, the vertical and downward incubation was used. That the migration is not a consequence of the growth was indicated from the fact that decrease in the glucose concentration caused an increased motility with decreased growth as judged by turbidity. Fig. 2 shows the reduction in the migration distance by increasing the concentration of glucose. A similar reduction was seen by the addition of galactose, lactose and maltose, while sucrose, glycerol and ribose showed no inhibition[9]. This repression of the motility was reversed by cyclic AMP as shown in the subsequent paper[9].

Fig. 1. Migrating profile of *E. coli* S-26 in MM along with glass tube traced by a densitometer (A420 nm), a) and appearance of distinct bands, b) Inoculation and incubation was carried out under the standard condition in the vertical (downwards) position in the presence or absence 4 mM or 40 mM of glucose.

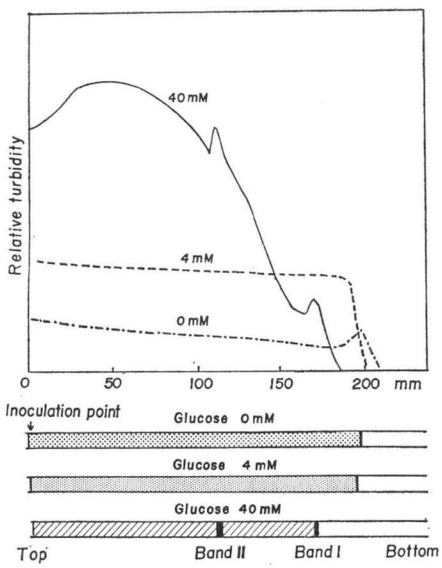


Table 2. Effect of incubation position of the migration of Bands I and II

Direction of assay tubes	without glucose		with glucose (0.04 M)	
	Band I	Band II	Band I	Band II
Vertical, downwards	299 mm*	—**	280 mm	249 mm
Vertical, upwards	289	—	266	235
Horizontal	285	—	foam formation	

* Average of two independent incubation for 15 hours under the standard condition.
** Not formed.

Fig. 2. Effect of glucose concentration in MM on the motility of S-26 strain. Average of at least 3 different run.

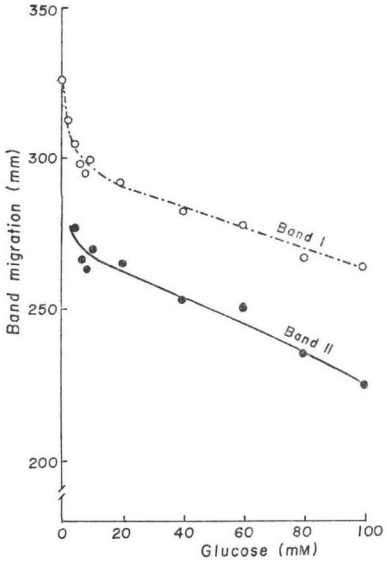
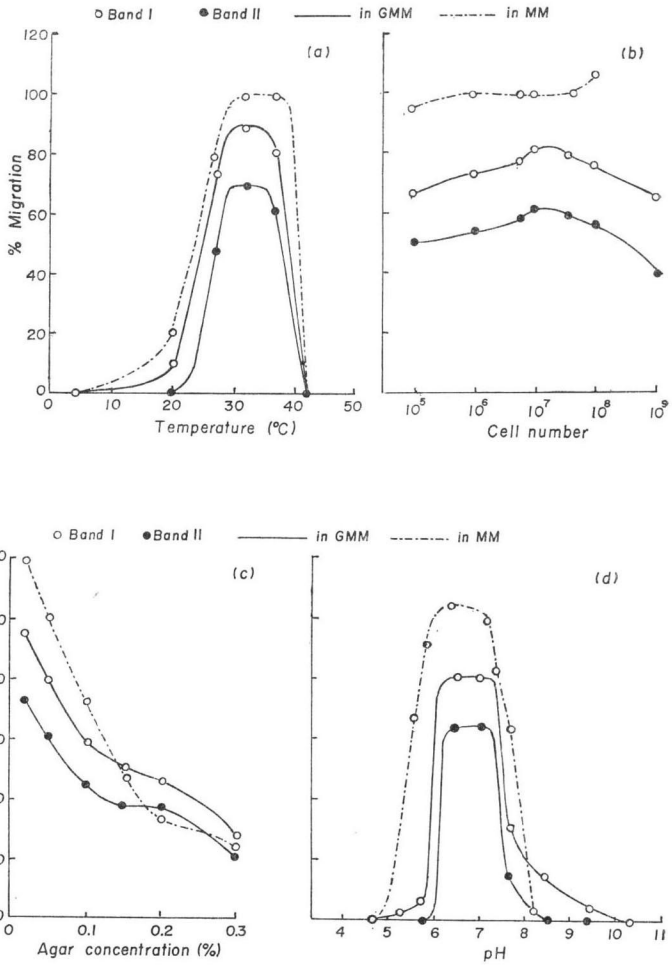


Fig. 3. Effect of temperature (a), the inoculum size (b), agar concentration (c), and pH (d) on the relative motility in the motilography. Incubation in MM or in GMM (repression MM) was done for 15 hours. Band migration was expressed as a percent of migrated distance to that incubated at 37°C for 15 hours in MM containing 0.05 % agar after 10^7 cells harvested at logarithmic phase were inoculated on the top.



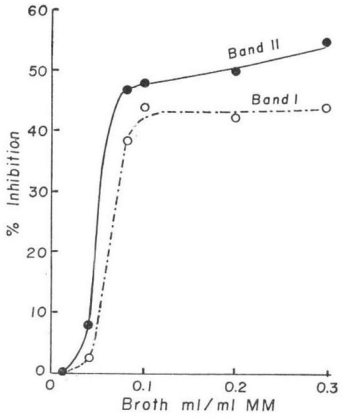
The distance of Bands I and II was affected to a great extent by the agar concentration of MM or GMM, the incubation temperature, the inoculum size, and the kind of medium, while the growth phase of the cells inoculated, the aeration and the medium at preculture had little influence on the distance of the Bands. Fig. 3(a) shows the effect of temperature on the motility. Cells were found to be almost immobile at a temperature higher than 40°C and lower than 20°C. The optimum population of cells to be inoculated was around 10^7 for the greatest migration as shown in Fig. 3(b). It is quite natural that the lower concentration of agar in MM or GMM brings about a larger migration distance (Fig. 3(c)). However, for practical reason, MM containing 0.05 % agar was used in all the experiments described hereafter. Fig. 3(d) shows that the migration is strictly dependent upon the medium pH, and high motility was seen only at around neutral pH. Different media gave a quite different pattern of the migration. Thus, incubation in nutrient broth (Eiken) containing 0.05 % agar resulted in the formation of no distinct bands although a greater migration was seen together with poorer growth.

Table 3. Culture filtrates of Actinomycete that had an inhibitory or stimulating effect on the motility by tube method

Condition of motilometry	No. of Actinomycete strains* showed			
	Inhibition		Stimulation	
	Marked**	Slight**	Marked**	Slight**
Without the addition of glucose	0	3	2	5
Under catabolite repression (0.04 M glucose)	2	6	2	1

* Total number checked=86.
** "Marked" means more than 60% effective, while "Slight" means 40~60% effective.

Fig. 4. Inhibition of the motility of a function of cultured broth of a *Streptomyces*, strain NR9GG8. Incubation was made for 15 hours under the standard condition.



From these results, a standard procedure for screening compounds affecting motility was established as follows: *E. coli* S-26 cell suspension (5 μl) with a population density of 2×10^9 cells/ml was inoculated on the top of MM or GMM containing 0.05% agar, packed (2 mm×35 cm) in a glass tube (2 mm×40 cm) equipped with a Morton cap and incubated in a vertical position at 37°C for 14~17 hours. Compounds or culture broth to be tested were contained in the MM or GMM and incubated simultaneously with a control. The distance from the top to Band I or II in the tube containing test compounds or culture broth were measured and expressed as percent distance to that of the control group.

2. Application of the Simple Assay Method for Culture Broth of Actinomycetes

Culture filtrates of actinomycetes were assayed for the effect on motility by the present tube method, motilometry. The results are summarized in Table 3. The culture filtrate of 6 strains out of 86 strains tested showed either a marked inhibition or stimulation under both catabolite repression and non-repression condition. Of these, the active principle of the culture filtrate of *Streptomyces* sp. strain NR9GG8 was isolated and characterized, the detail of which will be reported elsewhere[10]. Fig. 4 shows the inhibitory effect of NR9GG8 broth on the motility as the function of volume included in GMM. The inhibition increased linearly until 0.1 ml of the culture filtrate was added and reached a plateau thereafter. Several culture media used for actinomycetes did not affect the motility of *E. coli* tested by this method, below the level of 0.4 ml/ml of MM or GMM.

Discussion

A simple and quantitative assay method, designated here as "motilometry", for the effect on motility of *E. coli* was established and applied to the culture filtrates of actinomycetes strains. The culture filtrate of 6 strains, out of 86 strains checked, were found to contain active compounds inhibiting motility. The inhibitory or stimulating effect of a certain compound or culture filtrate was quantified as the percent migration of a distinct band formed (I or II) in the tube with the test compounds with respect to the distance in the control tube. Together

with the fact that similar migration was seen even in a horizontal position or in an upwards direction (Table 2), the strict temperature dependence of the motility in this method suggests that the migration measured here does not reflect passive migration by natural gravity but is essentially due to the cellular motility or the cell movement mediated by normal flagella, the function of which is regulated by cyclic AMP[3,4]. The rather narrow temperature range for the optimal motility was consistent with the temperature dependence of flagella formation and/or motility observed with various bacteria including *E. coli*[13], *Salmonella*[14] and *Proteus*[15], although there were some discrepancies. For instance, ADLER and TEMPLETON[13] found that *E. coli* K-12 was more motile at 24°C than at 37°C.

The meaning of the thick band formation as well as the difference in their populations remain to be answered. When cells isolated from Band I or II were inoculated into a fresh tube and incubated again under the same conditions, an almost identical migration pattern, containing both bands as the original, was obtained with a slight change in turbidity distribution, suggesting that thick band formation was not due to a double population with regard to motility. ARMSTRONG and ADLER reported that on a simi-solid plate, the motile strains formed the swarmed area in which two distinct rings were clearly seen at the outermost and the inner position of swarming[16]. Judging from the analogous conditions in the respective methods which allow cellular movement based essentially on motility, Bands I and II observed here would be closely associated with the ring formation in terms of the differential motility. Preliminary studies using electron microscopic observation revealed no apparent difference among the cells concentrated in Bands I and II, in the intermediate area and among cells which remained near the top of the tube: The number, length and wave of the flagella resembled one another.

One feature that may be associated with the motility was the shape of bacteria, since the cells in the faster migrating group generally appeared longer than those in the slower migrating group. With regard to ring formation, ARMSTRONG and ADLER[16] speculated from the experiments with the *che* mutant that cellular metabolism causes a concentration gradient of several amino acids which would make portions of cells eventually form the distinct rings on the plate. Studies are in progress relating to the biochemical characterization of flagella and cellular activity of the cells with apparently different motility.

Acknowledgement

We thank to Prof. D. MIZUNO (University of Tokyo) for the supply of Strain S-26, to Drs. K. INOUE and S. SUZUKI (National Institute of Health of Japan) for the assistance in the electron microscopic experiments and to Drs. Y. YAGI and K. TAKANO of our institute for their encouragement and critical comments throughout the experiments.

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