ANTRAFORMIN, A NEW INHIBITOR OF *BACILLUS SUBTILIS* TRANSFORMATION

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A convenient method was worked out which discriminates between inhibitory activity of compounds against *Bacillus subtilis* transformation and their antibacterial or growth inhibition activity. By this assay system, several drugs and antibiotics were tested and some of them were found to be more inhibitory to transformation than to bacterial growth.

This method was further applied to look for specific inhibitors among culture broths of *Streptomyces*, and during this screening program, *Streptomyces* sp. 7725-CC_1 was found to produce a specific inhibitor of *B. subtilis* transformation. The active substance was purified and named antraformin after its specific action. The molecular weight was suggested to be 279 ($C_{14}H_{21}N_3O_3$) for the compound by high resolution mass spectrometry.

Bacterial transformation consists of a complex series of interactions between transforming DNA and recipient cell components. The nature of the recipient cell is changed in such a way that transforming DNA is taken up and genes on it are expressed in recipient cells[1].

Since these phenomena are not vital to recipient cells, it is reasonable to consider that there should be specific inhibitors of transformation with no effects on becterial growth. Furthermore, such inhibitors might be useful for elucidating the mechanism of bacterial transformation. This notion tempted the author to test several chemicals and antibiotics for their inhibitory effects on the growth and transformation of *B. subtilis* by using a conventional assay method described here.

This method was further applied to search for new active substances in cultured broths of *Streptomyces* that specifically inhibit bacterial transformation.

A specific inhibitor of transformation without antibiotic activity was found to be produced by *Streptomyces* sp. 7725-CC_1 during this screening process. Isolation and some properties of the compound are reported in this paper.

Materials and Methods

Streptomyces sp. 7725-CC_1 was originally isolated from a soil sample by Dr. YONEHARA's laboratory for screening antibiotics and kept in our institute. Transforming *B. subtilis* DNA was prepared from *B. subtilis* W23 according to SAITO and MIURA[2]. Competent cells of *B. subtilis* 168 thy^- try^- were prepared according to ANAGNOSTOPOULOS and SPIZIZEN[3]. They were centrifuged, resuspended in 1/10 volume of "transformation medium" containing 10% glycerol and kept at −70°C.

"Transformation medium" consists of $(NH_4)_2SO_4$ 2 g, K_2HPO_4 14 g, KH_2PO_4 6 g, sodium citrate 1 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, glucose 5 g, casamino acids 0.1 g, thymine 10 mg, tryptophan 5 mg and arginine 5 mg in a total volume of one liter (pH 7.0).

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The drugs and antibiotics studied were obtained from the following sources: Mitomycin C and antimycin A from Kyowa Hakko Co., nalidixic acid from Daiichi Pharmaceutical Chemical Ind., bleomycin (mixture of A_2 and B group) from Nihon Kayaku Co. Ltd., cellocidin, azaserine and oligomycin from Dr. YONEHARA, colistin and penicillin G from Banyu Pharmaceutical Co. Ltd., acridine orange, acridine yellow and sodium deoxycholate from Sigma Chemical Co. Ltd., cetyltrimethylammonium bromide from Wako Pure Chemical Ind. Ltd., gramicidin S from Nikken Kagaku Co. Ltd., tetracycline from Japan Lederle Ltd., chloramphenicol from Sankyo Co. Ltd. Sterile paper discs were obtained from Toyo Roshi Co. Ltd. Avicel (microcrystalline cellulose) was from Funakoshi Yakuhin Co. Ltd.

Assay for antibacterial activity and antitransformation activity

Agar plates consisting of transformation medium (without tryptophan) and 1.5% agar were dried in an incubator overnight at 37°C. Paper discs (diameter 8 mm) impregnated with an appropriate concentration of chemicals, antibiotics or eluate from column chromatography (one disc holds approximately 0.08 ml of solution) were placed in the center of a dried plate and the plate was kept in the cold overnight to let the drug diffuse into agar medium. Five μl of DNA solution (200 μg/ml) was streaked radially 3-mm wide, 30-mm long on one side of the disc at a distance of 0.1 mm. After the DNA soaked into the agar (about 10 minutes), 10 μl of competent cell preparation was streaked on the track of DNA. Although the transformation medium contains 5 μg/ml of tryptophan, this amount is not sufficient for the try$^-$ cells to grow. Thus in this condition, only cells which became independent of tryptophan by transformation can grow on the plate. On the other side of the paper disc also at a distance of 0.1 mm, 10 μl of 100 times diluted competent cells suspended in 100 μg/ml of tryptophan solution was streaked. Dilution of the competent cells was performed so that the number of transformants on one side became equal to that of total cells on the other side of the disc. (Frequency of the transformation was 0.1%.) DNA solution and cell suspension were carefully streaked on the agar plate so that they did not touch drug-containing discs. After 20-hour incubation of the plates at 37°C, the distance between the center of the disc and the edge of any inhibited zone was measured in mms.

Localization of the activity on a silica gel thin-layer chromatogram: A piece of tissue paper was laid on agar medium in a rectangular dish and a thin-layer plate was put on it. After the minutes, the thin-layer plate and the paper were removed. After DNA solution was streaked from the beginning to the top of the track of the plate, competent cells were then streaked. Activities on the thin-layer chromatogram were detected by discontinuation of the growth of the transformed cells on agar medium.

Liquid transformation assay was conducted as follows: Forty μl of competent cell suspension were incubated with 20 μl of drug solution, 130 μl of transformation medium and 10 μl of DNA solution (200 μg/ml) at 37°C for 20 minutes. An aliquot (20 μl) was taken and streaked on a synthetic agar plate. For assaying antibacterial activity, the remaining incubation mixture was diluted 100-fold with transformation medium containing 100 μg/ml of tryptophan and 20 μl were streaked on a synthetic agar plate.

Results and Discussion

Inhibition of *Bacillus subtilis* Transformation by Known Antibiotics and Chemicals

It has been reported that in order for the bacterial transformation to occur, DNA synthesis[5] and energy metabolism[6] in the recipient cells are necessary, although the role of the former step in transformation is rather controversial. Inhibitors of DNA synthesis and energy metabolism together with some other drugs were tested for their antibacterial activity and antitransformation activity as shown in Table 1.

DNA synthesis inhibitors such as mitomycin C[7], nalidixic acid[8], bleomycin[9] and cellocidin[10]

Table 1. Effects of chemicals and antibiotics on transformation and growth of *B. subtilis*

Test compound	Concentration*	Antibacterial activity**	Antitransformation activity**
Mitomycin C	1	8.0	18.0
Nalidixic acid	100	14.0	21.0
Bleomycin	100	6.0	11.0
Cellocidin	1,000	13.5	18.0
Acridine orange	10 mg/ml	10.0	15.0
Acridine yellow	10 mg/ml	10.0	11.5
Antimycin A	1,000	4.0	4.0
Oligomycin	1,000	4.0	4.0
Sodium deoxycholate	100	4.0	15.0
Tween 20	20 mg/ml	4.0	7.0
Tween 40	20 mg/ml	4.0	4.0
Tween 60	10 mg/ml	4.0	4.0
Tween 80	10 mg/ml	4.0	10.0
Span 20	20 mg/ml	4.0	4.0
Span 80	10 mg/ml	4.0	4.0
Span 85	20 mg/ml	4.0	11.5
Cetyltrimethyl-ammonium bromide	100	10.5	10.5
Colistin	100,000 units/ml	7.0	7.5
Gramicidin S	1,000	10.0	10.0
Penicillin G	10	19.0	5.0
$NaAsO_2$	200	4.0	13.0
Azaserine	100	18.5	18.5
Rifampicin	3.3	14.5	15.0
Tetracycline	100	11.5	14.0
Chloramphenicol	100	11.5	16.5

Assays are described in Methods.
* Concentrations are expressed as μg/ml unless otherwise indicated.
** The distance between the center of a paper disc and the edge of inhibition zone is measured in millimeters. The radius of a paper disc is 4.0 mm.

had a stronger effect on *B. subtilis* transformation than on its growth. Neutral surface-active reagents such as Tween 60 and Span 85 inhibited transformation without affecting cell growth.

Deoxycholate exerted the most drastic effect on transformation at the concentration used. Azaserine (an inhibitor of *de nove* purine synthesis[7]), cationic detergent such as cetyltrimethylammonium bromide, colistin and gramicidin S (inhibitors of bacterial membrane[7]) had no specificity in their inhibitory activity against bacterial growth and transformation.

Although inhibitors of respiration such as antimycin A[7] and oligomycin[7] did not inhibit both activities (Table 1), inhibition of transformation was observed in an assay conducted in liquid medium at a concentration of 10 μg/ml (data not shown).

This limited survey indicates that the method employed is adequate to discriminate whether an agent inhibits transformation more effectively than bacterial growth or *vice versa*. From the inhibition pattern of drugs against bacterial growth and transformation, it was inferred that in nature there should be specific inhibitors of *B. subtilis* transformation without antibacterial activity. In order to test this possibility, cultured broths of *Streptomyces* were tested for antibacterial and antitransformation activity.

After examining 300 strains, one strain *Streptomyces* sp. 7725-CC_1 was found to produce a potent inhibitor of transformation that has no activity against bacterial growth.

Isolation of Antraformin

An active principle found to be produced by *Streptomyces* sp. 7725-CC_1 was named antraformin after its specific inhibitory effect on *B. subtilis* transformation.

Streptomyces sp. 7725-CC_1 was inoculated from slant culture into 10 ml of a medium in a 30 ml test tube consisting of 1.5% soybean flour, 0.2% $(NH_4)_2SO_4$, 2% soluble starch, 0.5% NaCl and 0.4% $CaCO_3$ (pH 6.4 before autoclaving) and incubated by shaking for 3 days at 30°C. One ml of the resulting cell suspension was then inoculated into 100 ml of the same medium and grown in a long neck flask (500 ml) on a reciprocal shaker at 30°C for 48 hours.

Cultured broth (1.8 liter) was centrifuged to remove cell pellets and the supernatant was extracted twice with equal volumes of butanol. The aqueous phase was saved, brough to pH 2.0 with concentrated hydrochloric acid and again extracted twice with equal volumes of butanol.

The butanol extract was concentrated *in vacuo* to 4~5 ml, 100 ml of ethyl acetate was added and the resultant precipitate was discarded. To the supernatant was added an equal volume of water, and the ethyl acetate layer was discarded. The water layer was concentrated, neutralized with conc.NH_4OH, two volumes of ethanol were added and the precipitate which formed was discarded. The ethanolic solution was concentrated to dryness and the residue was dissolved in 1 ml of butanol saturated with 0.01 N NH_4OH. The solution (0.2 ml) was charged on an Avicel (microcrystalline cellulose) column (1.8×28.5 cm) which had been equilibrated with the same solvent used for the sample. Active fractions which were eluted with the solvent described above appeared in two peaks. The first peak which eluted between 29 ml and 37 ml was contaminated with brown pigment and showed relatively less activity than the second peak (between 60 ml and 81 ml). Fractions with stronger activities of the second peak were collected and concentrated to dryness.

The resultant white material was dissolved in butanol saturated with water and purified by Sephadex LH-20 column (1.8×60 cm) chromatography using the same solvent system used for dissolving the material. Active fractions were eluted at about 0.4 column volume and each of them was chromatographed on silica gel thin-layer plates in order to examine purity (solvent system: butanol saturated with 0.01 N NH_4OH).

Substances on the thin-layer plates were detected by heating the plates on which 10% sulfuric acid had been sprayed. Fractions that showed only one spot were collected, evaporated to dryness, and further dried under reduced pressure at 50°C overnight. On silica gel thin-layer chromatography, the Rf value of the substance thus obtained coincided with that of the biologically active spot (see methods) in the following solvent systems.

1. Butanol-pyridine-H_2O (4:2:1). Rf 0.60
2. Butanol saturated with water. Rf 0.82
3. Butanol-0.01 N NH_4OH (40:7). Rf 0.2
4. Butanol-acetic acid-H_2O (4:1:1 or 5:1:5). Rf 0.88
5. Methanol-acetone (1:3). Rf 0.26
6. Methanol. Rf 0.77

7. Buthanol-formic acid-H_2O (40:15:10). Rf 0.84
8. Buthanol-1.5 N NH_4OH (1:1). Rf 0.15

Chemical Properties of Antraformin

Antraformin is a hygroscopic white powder. It is soluble in ethanol, butanol, methanol, pyridine, acetic acid, acetone, slightly soluble in water and insoluble in ethyl acetate, chloroform and benzene. The UV spectrum of antraformin, $\lambda_{max}^{CH_3OH}$ 278 nm ($E_{1cm}^{1\%}$ 59), is reproduced in Fig. 1. Its infrared spectrum measured in nujol shows characteristic absorption at 3300 cm^{-1}, 1730 cm^{-1} and 1250 cm^{-1} as shown in Fig. 2.

Fig. 1. Ultraviolet spectrum of antraformin in methanol

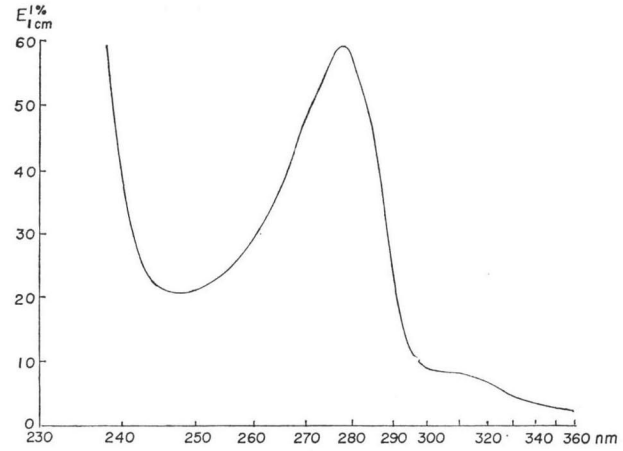
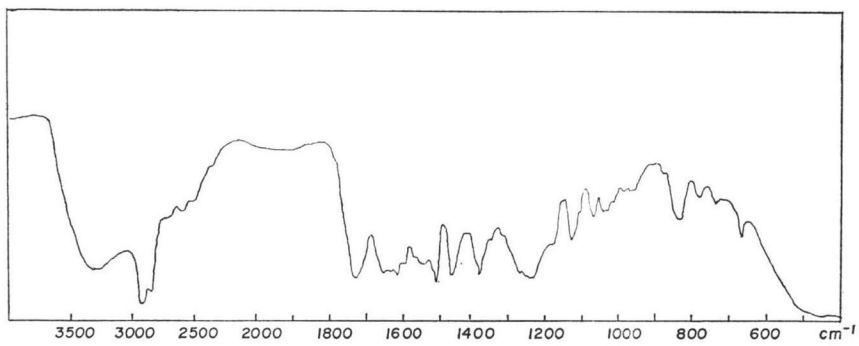


Fig. 2. Infrared spectrum of antraformin (Nujol paste).



Antraformin was subjected to mass spectroscopy analysis (electron impact method) in order to determine the molecular formula, since elementary analyses performed did not give satisfactory results. Thus a molecular weight of 279.1607 was obtained, from which a molecular formula of $C_{14}H_{21}N_3O_3$ (279.158292) was suggested for the compound. There was another possible molecular formula such as $C_{19}H_{21}N_1O_1$ (279.162314). However, since there were peaks such as $C_6H_5N_3O$ and $C_7H_5N_1O_3$ in the low field of the spectrum (data not shown), the former molecular formula was adopted.

Color reactions: positive LEMIEUX; negative ninhydrin, ferric chloride, TOLLENS, and sodium nitroprusside.

No reduction of activity was observed after heating for 5 minutes at 100°C within the range of pH 2.0 and pH 10.0.

Biological Activity

Complete inhibition of *B. subtilis* 168 transformation by antraformin was observed at a concentration of 5 μg/ml or more when the assay was conducted in liquid transformation system. On the other hand, antraformin did not show any inhibitory activity against growth of *B. subtilis* 168 in liquid assay method even at a concentration of 100 μg/ml.

On agar plates containing discs impregnated with 2 mg/ml of antraformin solution, the antibiotic gave a 25 mm radius inhibition zone against transformation of *B. subtilis* 168, but this concentration gave no growth inhibition zones against the following microorganisms:

Staphylococcus aureus FDA 209 P, *Bacillus subtilis* PCI-219, *Escherichia coli* B, *Pseudomonas aeruginosa*, *Xanthomonas oryzae*, *Piricularia oryzae*, *Pellicularia sasakii*, *Glomerella cingulata* and *Diaprothe citri*.

To the author's knowledge, a compound with the molecular formula and unique biological properties described above has not been reported so far. Although precise details of the transformation inhibition mechanism were not investigated in this study, antraformin has an attractive biological property and is expected to be a useful tool to investigate the mechanism of transformation.

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