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Short Communication

Locality of Genetic Determinant for the Bacteriocin Production in *Mycobacterium smegmatis*

(bacteriocin/genetic determinant/*Mycobacterium smegmatis*)

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The nature of the genetic determinant of smegmatocin, a bacteriocin produced by *Mycobacterium smegmatis* was investigated by testing the loss of bacteriocin-producing ability during treatments with various curing agents and by cultivation at an elevated temperature. As none of these treatments produced any loss of smegmatocin-producing capacity of the bacterial strain, the genetic determinant of smegmatocin is probably not of extrachromosomal DNA but rather is located on the host chromosome.

Mycobacterium smegmatis produces a bacteriocin which acts on the Runyon's group IV mycobacteria such as *M. diernhoferi*, *M. phlei*, and *M. flavescens* (1). In a previous paper, we reported the partial purification and some biological properties of a bacteriocin produced by *M. smegmatis* ATCC 14468 (smegmatocin 14468) (2). However, the genetic determinant(s) for this bacteriocin has not been identified. As the role of the bacteriocin in bacterial metabolism is unknown, the genetic features of smegmatocin should be studied in detail.

It is well known that the genetic determinants of colicins (colicinogenic factors) are located on plasmids and can be transferred from donor to recipient by cell-to-cell contact independent of the transmission of host chromosome (3–5). Concerning other types of bacteriocins, there is evidence that such are controlled by extrachromosomal DNA (bacteriocinogenic factors), particularly in cases of staphylococcin (6, 7) enterococcin (8), perfringocin and megacin (9). Therefore, it was of interest to determine whether the genetic determinant of smegmatocin is located on extrachromosomal or chromosomal DNA. In the present study, we examine the phenotypic stability of *M. smegmatis* (*butyricum*) ATCC 19979 to produce smegmatocin after treatment with some representative curing agents, acriflavine (7, 11), acridine orange (4, 6, 7, 10), ethidium bromide (6, 7, 11), and sodium dodecyl sulfate (5–7). Also given attention was the possibility the smegmatocin determinant can be eliminated after cultivation at an elevated temperature, since this treatment is known to be effective in curing certain plasmids such as the bacteriocinogenic factor for staphylococcin (6, 7) and megacin (12), and the plasmid for exfoliative toxin synthesis (13).

Curing tests for the gene of smegmatocin with chemical agents were performed essentially by the method of Jetten and Vogels (7) or in some cases by the method of Dajani and Taube (6). To a 15 hr culture at 37°C in heart infusion broth containing 1 % glycerol and 0.05 % Tween ® 80 (HIGT broth) of *M. smegmatis* ATCC 19979 was inoculated into 200 vol. of HIGT broth containing a prescribed amount of curing agents such as acridine orange, acriflavine, ethidium bromide, and sodium dodecyl sulfate (SDS). After 24 hr of incubation at 37°C with shaking (80 rpm), the bacterial cells were washed twice with phosphate buffered saline (pH 7.2) containing 1 % fetal bovine serum (FCS) (Wako Pure Chemical Indust., Osaka) and diluted to appropriate concentrations with the same buffer. The resulting diluent (0.1 ml) was then mixed with one ml of HIGT soft agar supplemented with 5 % FCS and the mixture was poured onto an HIGT agar plate. The plate was subsequently overlaid with 2.5 ml of HIGT soft agar containing 5 % FCS and cultured at 37°C for 3 days. After 1 hr UV-irradiation (15W UV-lamp, 8 cm distance) of the resultant plate, 3 ml of HIGT soft agar containing about 6×10^7 indicator cells of *M. diernhoferi* ATCC 19340 were overlaid. After a further 2-day cultivation at 37°C, colonies of *M. smegmatis* were observed for the presence of clear zones. When a colony without a growth-inhibition zone in the indicator cells was apparent, this colony was subjected to recolonization after cultivation in HIGT broth containing 3 % NaCl (This NaCl concentration suppressed the growth of *M. diernhoferi* but not of *M. smegmatis*). The presumptive non-smegmatocin producing strain was then checked for its bacteriocin-producing ability by the streaking method (1).

The loss of smegmatocin-producing ability by treatment with chemical mutagens, such as nitrosoguanidine (Wako Pure Chemical Ind.), hydroxylamine, and ethyl methane sulfonate (Sigma Chemical Co., St. Louis, Mo., U. S. A.) was studied as mentioned above.

Curing test for smegmatocin-producing gene during cultivation at 42°C was carried out as follows. A 15 hr culture of *M. smegmatis* was inoculated into 200 vol. of HIGT broth and cultivated at 42°C for 24 h. Loss of smegmatocin-production was then examined as mentioned above. Loss of the smegmatocin-producing ability by UV-irradiation (15W UV-lamp, 8 cm distance, 5—60 sec) was also studied, using a similar procedure.

As shown in Table I, none of the curing agents for bacterial plasmid DNA caused a loss of smegmatocin-producing ability of *M. smegmatis* ATCC 19979, at concentrations which are reported to be fully effective in the elimination of various bacteriocinogenic factors (6—8, 13), exfoliative toxin-plasmid (13, 14), penicillinase plasmid in *Staphylococcus aureus* (15, 16), and other plasmids (5). Cultivation of *M. smegmatis* cells at 42°C also failed to eliminate smegmatocin-gene (Table II). Many of the bacterial plasmids can be eliminated by treatment with certain curing agents such as acridine orange, acriflavine, ethidium bromide, and SDS (3—7, 9, 13, 14—16) or by cultivation at an elevated temperature (6, 7, 12, 14), at the frequency of 10 to 100 %. Therefore, the present observations strongly suggest that the genetic determinant of

TABLE I. *Effects of Some Curing Agents on Elimination of Smegmatocin-producing Gene from M. Smegmatis ATCC 19979*

Agents	Concentration (μg/ml)	No. of colonies treated	No. of weakly smegmatocin producing colonies*	No. of smegmatocin non-producing colonies
Acridine orange	0.2	708	0	0
	0.5	1247	0	0
	1.0	551	0	0
Acriflavine	0.5	1183	0	0
	1.0	464	0	0
	2.0	1219	0	0
	3.0	880	0	0
Ethidium bromide	0.5	973	0	0
	1.0	937	0	0
	2.0	870	2	0
	3.0	698	0	0
Sodium lauryl sulfate	5	501	0	0
	10	658	0	0
	50	252	0	0
	100	595	0	0
Nil.	—	3303	0	0

* The colonies, with no growth-inhibitory zone on the agar plates but producing smegmatocin in the streaking test, were regarded as to be weakly smegmatocin-producing colonies.

TABLE II. *The Failure of Cultivation at High Temperature or UV-irradiation of M. Smegmatis ATCC 19979 to Eliminate the Smegmatocin-producing Ability*

Treatment	Time for treatment	No. of colonies treated	No. of weakly smegmatocin producing colonies	No. of smegmatocin non-producing colonies
Cultivation at 42°C	24hr	1326	0	0
UV-irradiation*	5 sec	390	0	0
	10 sec	269	0	0
	30 sec	203	4	0
	60 sec	417	1	0

* Survivals after UV-irradiation were 61, 43, 1.3×10^{-1}, and 2.8×10^{-5}%, for 5, 10, 30, and 60 min-treatment, respectively.

smegmatocin is not located on extrachromosomal but rather on the host chromosomal DNA.

We also examined whether or not non-smegmatocin-producing strains can be obtained after UV-irradiation or after treatment with chemical mutagens (Tables II and III). These treatments also failed to induce a mutation giving a detectable amount of non-smegmatocin-producing mutants. This was as expected since nitrosoguanidine-treatment of *M. smegmatis* ATCC 607 produces the drug-resistant, phageresistant, and auxotrophic mutants at frequencies below 10^{-4} (17), and the detection-frequency for drug-resistant mutants after UV-irradiation of *M. smegmatis* Rabinowitchi and PM5 is as

TABLE III. *Effects of Chemical Mutagens on Smegmatocin Producing Ability of M. Smegmatis ATCC 19979*

Agents	Concentration (μg/ml)	No. of colonies treated	No. of weakly smegmatocin producing colonies	No. of smegmatocin non-producing colonies
Nitrosoguanidine	5	1656	0	0
	10	1248	0	0
	50	1200	0	0
	100	1367	1	0
	200	1321	1	0
Hydroxylamine	5	340	2	0
	10	442	1	0
	50	382	8	0
Ethyl methane sulfonate	5	510	14	0
	10	469	3	0
	50	413	1	0
	100	335	3	0
Nil.	—	2538	1	0

low as 10^{-9} to 10^{-6} (18).

It has been demonstrated that most of the genetic determinants of common bacteriocins are plasmid borne (3, 9), with the sole exception of pneumocin, a bacteriocin of *Streptococcus pneumoniae*. Pneumocin determinants were shown to be chromosomally located, by one apparently unconfirmed study (19). Taniguchi *et al.* (20) reported that *M. smegmatis* R32 and P53 had more than two plasmids with a molecular size of $2-4\times10^6$, and that these plasmids may play some roles in photochromogenicity and smooth colony phenotype but not in bacteriocinogenicity. This finding is consistent with our speculation that the smegmatocin determinant is not of extrachromosomal DNA. In a separate experiment, we also found that the addition of mitomycin C (0.2—20 μg/ml), H_2O_2 (0.0001—1 %), or ethanbutol (10—100 μg/ml) to the cultivation medium of *M. smegmatis* produced no appreciable increase in the production of free and cell-associated smegmatocin. Moreover, UV-irradiation (15W UV-lamp, 20 cm distance, 5—20 min) did not increase the production of cell-associated smegmatocin. These results indicate that smegmatocin is not an inducible bacteriocin, thereby differing from colicins, megacin, and others (4, 15). This finding may support the concept that the genetic determinant of smegmatocin is not located on the plasmid but rather on host chromosome. Studies using a recombination system of various genes of M. smegmatis, including the smegmatocin-producing gene, should provide data that will lend support to this hypothesis.

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