Shimane J. Med. Sci. Vol. 4, pp. 36—45, 1980

Suppressive Effect of *Lactobacillus casei* on the Drug Resistance Transfer in *Escherichia coli*

(*Lactobacillus casei*/drug resistance transfer/*Escherichia coli*)

HAJIME SAITO, HARUAKI TOMIOKA, KYOKO ASANO, and TAKASHI WATANABE

Department of Microbiology and Immunology, Shimane Medical University, Izumo 693, Japan

(Received January 29, 1980)

In the mixed culture of *Escherichia coli* S-58 and S-42 strains, CP^r gene is thought to be transferred from the former to the latter by conjugation. The cultured supernatant of *Lactobacillus casei* YIT 0003 exhibited a considerable suppressive effect on the drug resistance transfer between the two *E. coli* strains. However, it failed to inhibit the R-factor transfer between *E. coli* W 3630 and ML 1410. This factor in the supernatant was heat stable, ethanol-precipitable, and the molecular weight was estimated to be approximately 6,000. This factor probably constitutes a non-dialyzable complex with some other substances in the culture fluid.

It is well known that microbes belonging to the genus *Lactobacillus* produce a large amount of organic acid such as lactate, and show a marked antimicrobial activity. This nature of *Lactobacillus* is thought to cause a stabilization of the intestinal microflora (1, 2). It remains unclear what role this organism plays on the transfer of drug resistance among various bacteria in the intestinal microflora.

In the present study, the effects of *Lactobacillus casei* on drug-resistance transmission between two different strains of *Escherichia coli* in the *in vitro* system were investigated.

We found that the cultured supernatant of *L. casei* exhibited an inhibitory action against drug-resistance transfer. This factor was heat stable, precipitated with ethanol at 80% concentration, and the molecular weight was about 6,000 as estimated by Sephadex G-75 gel filtration. Some mechanisms of drug-resistance transfer between the test *E. coli* strains were also investigated.

MATERIALS AND METHODS

Bacteria

Lactobacillus casei YIT 0003 (IAM 1045) was obtained from Dr. S. Kodaira, Yakult Institute, Tokyo, and maintained in Rogosa's medium. *Escherichia coli* W 3630 (R^+_{100-1}. mal^-) and *E. coli* ML 1410 (R^-. met^-. nal^r) were provided by Dr. H. Hashimoto, Department of Microbiology, School of

Medicine, Gunma University, Maebashi, Japan, and maintained in heart infusion (HI) agar supplemented with 25 μg/ml of chloramphenicol (CP) or 50 μg/ml of nalidixic acid (NA), respectively. *E. coli* S-42 (KM^r. CP^s. SM^r) and *E. coli* S-58 (KM^s. CP^r. SM^r) were clinical isolates, and maintained in HI agar containing 25 μg/ml of kanamycin (KM) for S-42 and 25 μg/ml of CP for S-58. The drug resistance patterns of four *E. coli* strains mentioned above are shown in Table I.

TABLE I. *Drug-resistance Pattern of E. coli Strains*

Strain	MIC (μg/ml)				
	KM	SM	CP	TC	NA
W 3630	N. T.	25	>200	>200	6.3
ML 1410	N. T.	6.3	3.2	3.2	>200
S-42	>200	>200	12.5	25	N. T.
S-58	6.3	>200	200	1.6	N. T.

N. T. : not tested.

Cultivation of L. casei

L. casei was cultured in Rogosa's medium at 37°C for 24 hr. The cultured supernatant was obtained by centrifugation at 2,000×g for 15 min, and the pH adjusted to 7.5 with 2.5N NaOH, and then passed through a Millipore filter with 0.45 μ pore size. The cell pellet was thoroughly washed with phosphate buffered saline (PBS, pH 7.2), irradiated with 15W UV lamp for 25 min at 20 cm hight, centrifuged at 2,000×g for 15 min, and finally resuspended in brain heart infusion (BHI) broth.

Determination of Antibiotic Resistance of E. coli Strains

Minimum growth inhibitory concentrations of various antibiotics against *E. coli* strains were determined by the standard method of the Japan Society of Chemotherapy (3).

Assay for the Transfer of Drug Resistance between E. coli Strains S-42 and S-58

Each of overnight cultured broth of S-42 and S-58 was diluted 100-fold with fresh BHI broth and incubated at 37°C with gentle shaking (80 cycles/min). After 3.5 to 4 hr of incubation, the bacterial cells of both strains at the exponential phase were harvested, washed with BHI broth, and then suspended in BHI broth (pH 7.5) at an equal concentration.

Equal amounts of the bacterial suspension were mixed, and added to the test solution, usually at a dose of 50 % (v/v). The initial bacterial concentration is shown in each figure. After the incubation at 37°C for up to 3 hr with shaking at 80 cycles/min, the mixed culture was cooled in ice to

stop the transfer reaction, homogenized by vigorous pipetting, and the number of total and KM-CP resistant bacterial cells on HI agar plates counted. To enumerate the number of KM-CP resistant cells, HI agar supplemented with 50 μg/ml of KM and 25 μg/ml of CP was used as a selection medium. The frequency of the transmission of drug resistance between the two bacterial strains is indicated using the parameter "KM-CP resistant cells per 10^{10} of the total cells produced in the mixed culture".

Assay for R-factor Transfer

The frequency of the R-factor transmission was measured using *E. coli* W 3630 (R^{+}_{100-1}. mal^{-}) and ML 1410 (R^{-}. met^{-}. nal^{r}) strains, according to the method of Mitsuhashi (4), with slight modifications. Both strains of donor and recipient were separately cultured in BHI broth overnight, and diluted 100-fold with fresh BHI borth. After 3.5 hr incubation with shaking, cultures were mixed in equal parts and the test solution added at a dose of 50 % (v/v). The mixed cultures were incubated for 6 hr and the R^{+} conjugants counted using HI agar plates containing 50 μg/ml of NA and 25 μg/ml of CP.

RESULTS

Polarity and Mechanism of Drug-resistance Transfer between E.coli S-42 and S-58 strains

As shown in Table II, the transmission of drug resistance between the two

Table II. *Evidence for Conjugal Transfer of the Drug-resistance between E. coli S-42 and S-58*

Addition to the culture				Colony forming units/ml			
Bacteria		Cultured fluid		0 hr		3 hr	
S-42	S-58	S-42	S-58	Total cells	KM^{r} . CP^{r} cells	Total cells	KM^{r} . CP^{r} cells
+	+	−	−	2.4×10^{9}	0	3.5×10^{9}	1.4×10^{4}
+	−	−	−	1.2×10^{9}	0	3.1×10^{9}	0
+	−	−	+	1.2×10^{9}	0	1.5×10^{9}	0
−	+	−	−	1.1×10^{9}	0	2.9×10^{9}	0
−	+	+	−	1.1×10^{9}	0	2.1×10^{9}	0

Indicated bacteria were cultured in BHI broth at 37°C for 3 hr with or without addition of cultured fluid of counterpart strain at a dose of 70% (v/v).

strains occurred only when both strains were present in the incubation mixture, thus indicating that the production of KM-CP resistant cells in the mixed culture was not due to the spontaneous mutation of either of the two strains. Moreover, the drug-resistance transfer did not occur when either strain was cultured in the presence of the culture fluid of the counterpart strain. These results indicate that the transmission of the drug-resistance gene between S-42

TABLE III. *Drug-resistance Patterns of the Recombinants Obtained from Various Mating Systems E. coli S-42 or S-58 with Other E. coli Strains*

Combination	Selective antibiotics	No. of recombinants	Resistance pattern of the recombinants
S-42(KM^r . SM^r) ×S-16(CP^r . TC^r)	KM. TC	2	KM^r . SM^r . TC^r
×S-28(SM^r . TC^r)	KM. TC	74	KM^r . SM^r . TC^r
×S-44(SM^r . CP^r . TC^r)	KM. TC	5	KM^r . SM^r . CP^r . TC^r
×S-49(CP^r . TC^r)	KM. TC	5	KM^r . SM^r . TC^r
×S-59(TC^r)	KM. TC	3	KM^r . SM^r . TC^r
×S-58(SM^r . CP^r)	KM. CP	69	KM^r . SM^r . CP^r
S-58(SM^r . CP^r) ×S-25(TC^r)	SM. TC	1	SM^r . CP^r . TC^r
×S-34(TC^r)	SM. TC	18	SM^r . CP^r . TC^r
		2	SM^r . TC^r
×S-28(TC^r)	CP. TC	9	SM^r . CP^r . TC^r

E. coli S-42 or S-58 was mated with the indicated *E. coli* strains by overnight mixed cultivation. The cultured broth was diluted with 100-fold fresh tryptosoy broth and cultured for additional 20 hr. One μl of the resulting cultured broth was spread on HI agar plates containing 50 μg/ml of the indicated antibiotics to select the recombinants. Then, the recombinants obtained were tested for the drug-resistance patterns.

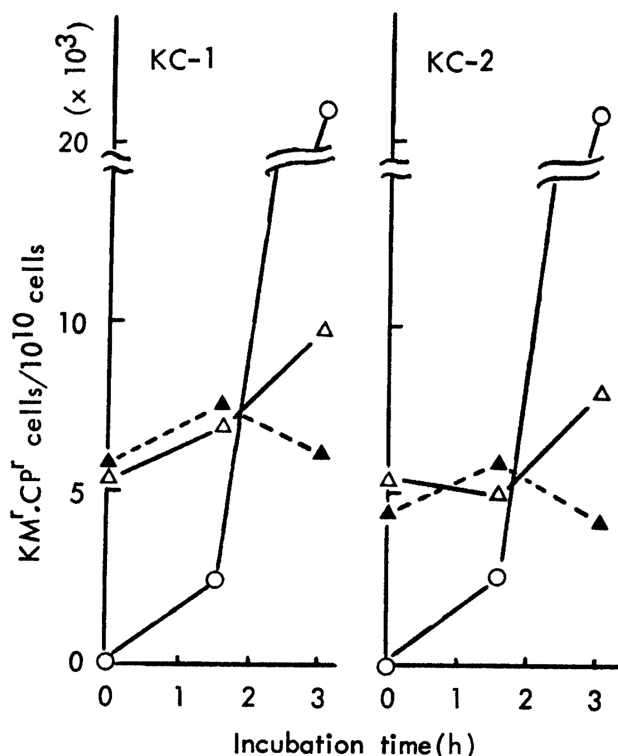

Fig. 1. Time-course of increase in KM^r . CP^r cells in the mixed cultures of *E. coli* KC-1 or KC-2 (KM^r . SM^r . CP^r) with *E. coli* S-42 and S-58. KC strains were selected from the mating culture of S-42 and S-58. Mixed culture of S-42 (1.6×10^9/ml) and KC strain (5×10^3/ml) (–△–) ; mixed culture of S-58 (1.6×10^9/ml) and KC strain (5×10^3/ml) (--▲--) ; mixed culture of S-42 (8×10^8/ml) and S-58 (8×10^8/ml) (–○–).

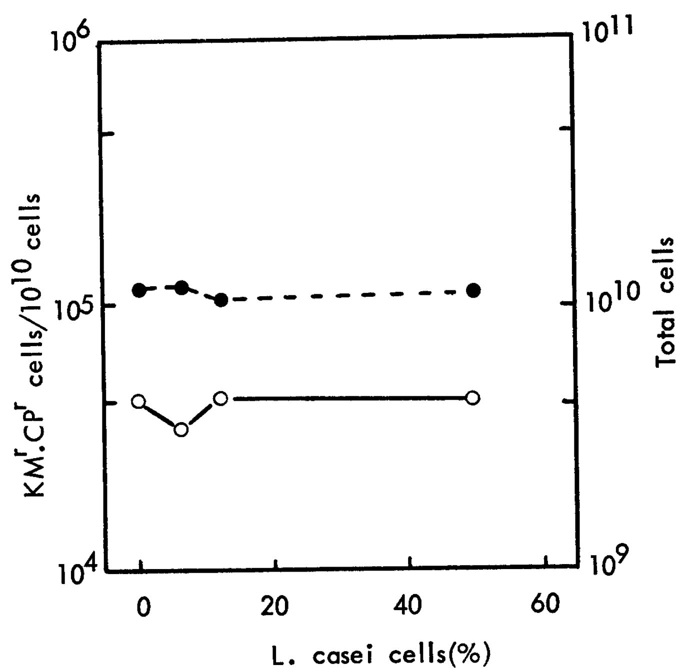

Fig. 2. Effect of *L. casei* cells on the drug-resistance transfer between *E. coli* S-42 and S-58. *L. casei* cells were suspended in BHI broth at a concentration of an optical density of 3 at 620 nm in a Spectronic 20, and added to the mixed culture of S-42 and S-58 at indicated doses. KM^r . CP^r cells produced in the mating system during 3 hr incubation (–○–) ; number of total cells after 3 hr incubation (--●--).

and S-58 strains was not caused by transformation or transduction. The transmission of drug-resistance so observed is probably due to the conjugation.

To determine the polarity of the drug-resistance transfer between the two strains, the following experiments were carried out. As shown in Table III, S-42 and S-58 strains were examined to determine potential ability as a donor or a recipient in the drug-resistance transfer, using other *E.coli* strains having various drug-resistance spectra. After an overnight mixed cultivation, the desired recombinants were selected on the HI agar plates containing various antibiotics indicated in the Table. All the recombinants derived from the mixed culture of S-42 (KM^r. SM^r) and S-16 (CP^r. TC^r) or S-42 and S-49 (CP^r. TC^r), using the selection medium containing KM and TC, were resistant to KM, SM and TC but not to CP. In this case, it is thought that S-16 and S-49 acted as donors and S-42 as a recipient and that only TC^r gene was transferable from the donor to the recipient. When S-58 (SM^r. CP^r) was mated with S-34 (TC^r) and the resulting recombinants were selected on the selection medium containing SM and TC, 18 of 20 strains were resistant to SM, CP and TC, whereas the remainder (2 strains) were resistant only to SM and TC but not to CP. This suggests that SM^r and/or CP^r genes were transferred from S-58 (donor) to S-34 (recipient). Thus, S-42 and S-58 function as a recipient and a donor, respectively, in the drug-resistance transfer.

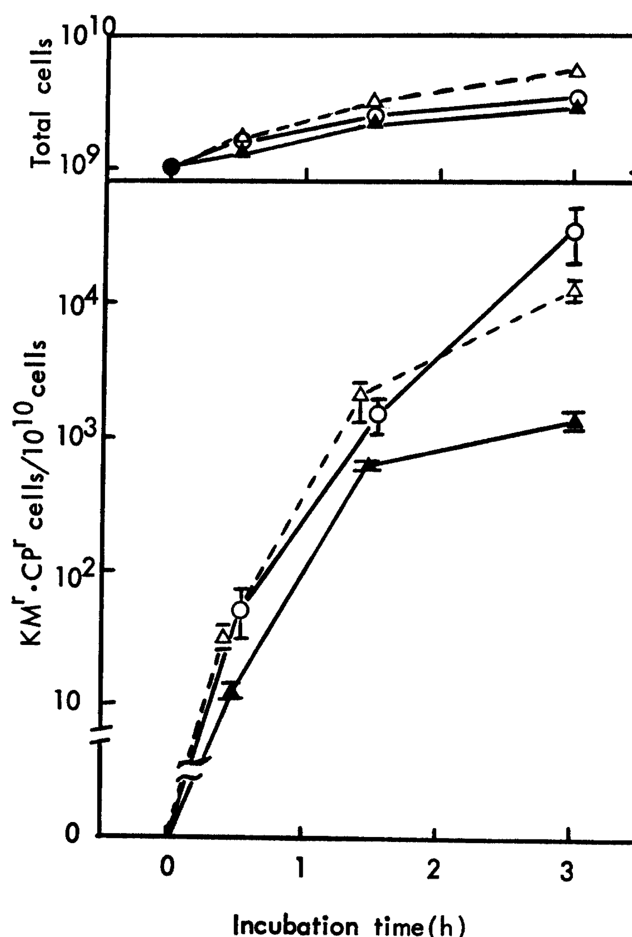

Fig. 3. Time-course of increase in KM^r . CP^r cells in the mixed culture of *E. coli* S-42 and S-58 in the presence of L. casei-supernatant fluid. The supernatant fluid was added to the mixed culture at the dose of 50% (v/v). Rogosa's medium and non-added control were also used for the test. *L. casei*-supernatant fluid (—▲—) ; Rogosa's medium(--△--) ; non added (—○—).

The above conclusion may be supported by the following observations : KM^r. CP^r recombinants (KC strain) were selected from the mixed culture of S-42 and S-58, and the kinetics of the increase in KM^r. CP^r cells in the mating system of KC and S-42 or KC and S-58 were investigated. As indicated in Fig. 1, the increase of KM^r. CP^r cells during 3 hr in the mixed culture was observed only in the mating of KC with S-42 but not with S-58. These findings indicate that S-42 functions as a recipient, and in the mating of S-42 with S-58, CP^r gene was transferred from the former to the latter by conjugation.

Effect of L. casei Cells on the Drug-resistance Transfer between E.coli S-42 and S-58 Strains

The effect of addition of UV-irradiated cells of *L. casei* (1.5 at OD_{620}) to the mixed culture of S-42 and S-58 on the drug-resistance transmission was studied. As shown in Fig. 2, the frequency of transfer was not affected

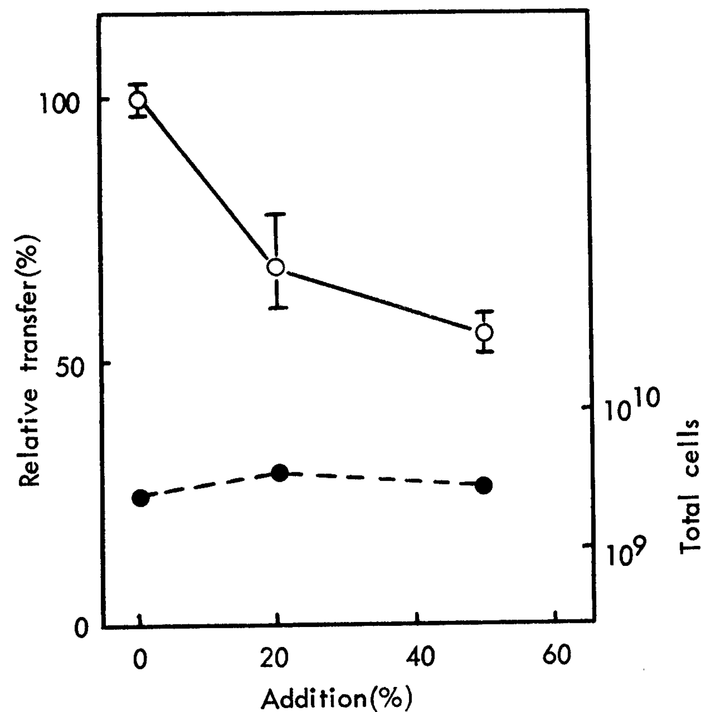

Fig. 4. Inhibition of drug-resistance transfer between *E. coli* S-42 and S-58 by addition of dialyzed cultured fluid of *L. casei*. The supernatant fluid of *L. casei* was dialyzed against Rogosa's medium (pH 7.5) and added to the mixed culture of S-42 and S-58 at indicated doses. Rogosa's medium was added to the mating system to give a final concentration of 50% (v/v) in the incubation mixture. The value obtained when Rogosa's medium alone was added at 50% (v/v) to the mating system was fitted to 100%. Relative transfer of drug-resistance(—○—) ; number of total cells after 2 hr incubation(--●--).

when the *L. casei* cells were added to this system.

Effect of the Cultured Supernatant of L. casei on the Drug-resistance Transfer between E.coli S-42 and S-58 Strains

When the supernatant of *L. casei* (pH 7.5) was added at a dose of 50 % (v/v) to the mating system between S-42 and S-58, the inhibition of the drug-resistance transfer was seen without any lag phase (Fig. 3). It may be noted that the number of viable cells of the mixed culture was not so markedly affected by addition of *L. casei* supernatant, indicating that the cultured supernatant did not exhibit any bactericidal effects on the two strains of *E.coli*. When the supernatant was thoroughly dialyzed against 50 volumes of Rogosa's medium at 4°C, the dialyzed fraction showed a suppressive action against the drug-resistance transfer (Fig. 4). The heat-treated (100°C, 5 min) supernatant produced a 95 % inhibition of the drug-resistance transfer at a dose of 50 % (v/v), while the intact supernatant showed a 93 % inhibition at the same dose. Moreover, most of the inhibitory principle was precipitated with ethanol at a concentration of 85 %. These observations clearly indicate that the cultured supernatant of *L. casei* contained heat-stable,

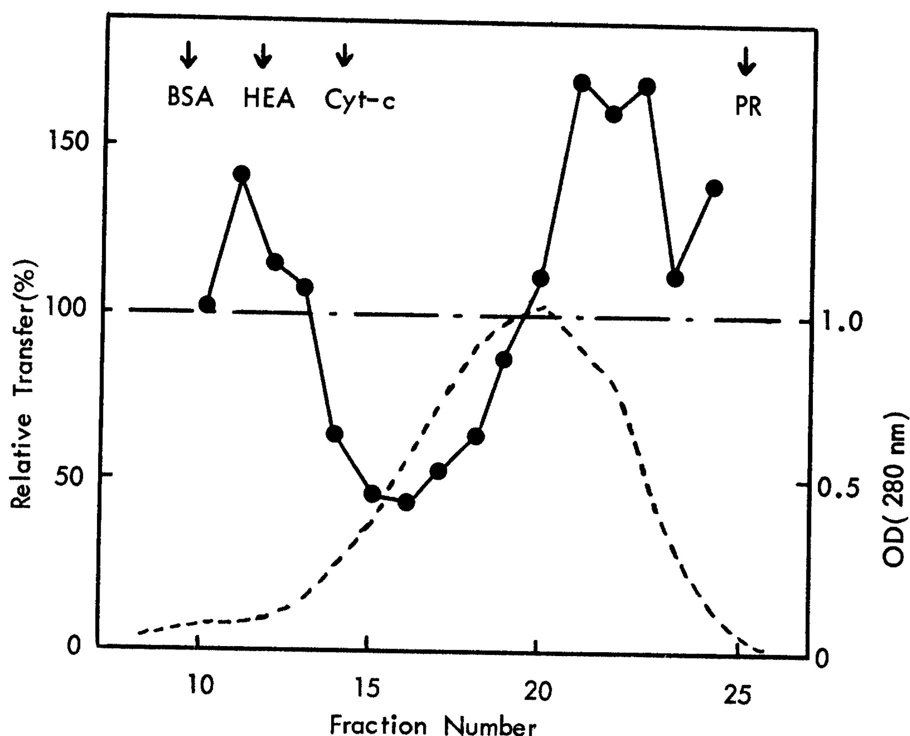

Fig. 5. Gel filtration on Sephadex G-75 of the inhibitory factor for the drug-resistance transfer produced by *L. casei*. Solid line indicates the relative transfer (%) of drug-resistance between *E. coli* S-42 and S-58 by adding the fraction at 50% (v/v). The value obtained when eluting buffer (PBS) was added to the mating system was fitted to 100%. Molecular weight of the inhibitory factor was determined from the elution profile with bovine serum albumin (BSA), hen egg albumin (HEA), cytochrome-c (Cyt-c), and phenol red PR as standard.

non-dialyzable and ethanol-precipitable inhibitory substance (s) against drug-resistance transfer between S-42 and S-58.

Partial Purification of the Inhibitory Factor against Drug-resistance Transfer from the Cultured Supernatant of L. casei

The supernatant (40 ml) obtained from *L. casei* cultured broth was dialyzed against distilled water, treated at 100°C for 5 min, and centrifuged at 15,000 ×g for 30 min. The resulting supernatant was lyophilized and the dried powder (160 μg) was redissolved in 8 ml of PBS. Then, ethanol was added to the solution to give a final concentration of 80 %. After 2 hr incubation at 0°C, the mixture was centrifuged at 2,000×g for 15 min, and the precipitate was removed and redissolved in a small amount of distilled water. The fraction thus obtained was subjected to Sephadex G-75 gel filtration (0.9×90cm) using PBS as an eluting buffer. The elution pattern is shown in Fig. 5. The inhibitory factor against the drug-resistance transfer was to some extent eluted preceding the main peak of $OD_{280\ nm}$. The molecular weight of this factor was estimated to be approximately 6,000, from the data of its elution volumes compared to those of standard (bovine serum albumin, hen egg albumin, cytochrome C, and phenol red). It may be of interest to note

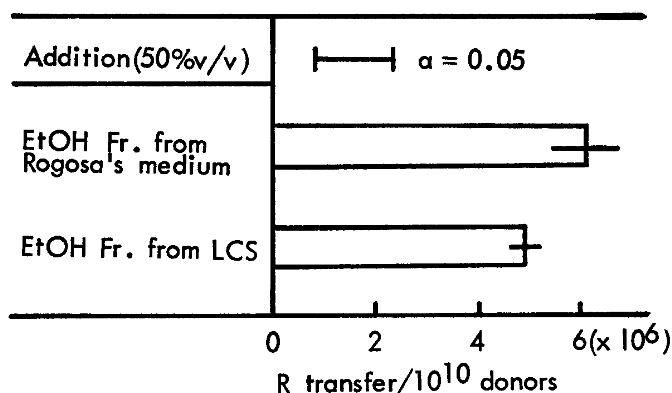

Fig. 6. Effect of the *L. casei*-originating factor on the R-factor transfer between *E. coli* W 3630 and ML 1410. The precipitate obtained from the supernatant fluid of *L. casei* (LCS) at 80% ethanol was dissolved in distilled water (1/20 vol of the supernatant fluid), and examined for the effect on R-factor transfer. Ethanol fraction of Rogosa's medium was used as the control. I ; Statistically significant at 95 % confidence limit.

that the cultured supernatant of *L. casei* contained small molecular substance (s) which shows some enhancing activity on the drug-resistance transfer between S-42 and S-58.

Effect of Cultured Supernatant of L. casei on the Transmission of R-factor

The cultured supernatant of *L. casei* (200 ml) was dialyzed against distilled water and precipitated with 75 % ethanol. The precipitate was redissolved in 10 ml of distilled water and the pH was adjusted to 7.5. This factor was examined for the effect on the R^{100-1}-factor transfer between *E. coli* W 3630 and ML 1410 strains. As shown in Fig. 6, this fraction exhibited only slight inhibition against R-factor transfer compared to that from Rogosa's medium (control).

DISCUSSION

In the present study, we investigated the effect of *L. casei* on the drug-resistance transfer between *E. coli* S-42 and S-58 strains, possessing different antibiotic-resistance spectra each other. In the mixed culture of these two strains, CP^r gene is thought to be transferred from S-58 to S-42 by conjugation. *L. casei* cells, *per se*, did not affect the drug-resistance transmission, whereas the cultured supernatant of *L. casei* exhibited considerable suppressive effect on the drug-resistance transfer between the two *E. coli* strains. This factor in the supernatant was heat-stable, and ethanol-precipitable, and the molecular weight was estimated to be approximately 6,000 from the elution profile on Sephadex G-75 chromatography.

This factor probably constitutes a non-dialyzable complex with some other substances and the molecular weight is over 10,000.

This factor failed to inhibit the R-factor transfer between *E. coli* W 3630

and ML 1410. Therefore, there is the possibility that the inhibitory factor against the drug-resistance transfer can depress the transmission of chromosome, but not of R factor, as an episome. If this assumption is valid, the drug-resistance transfer observed in the case of the mating between S-42 and S-58 may be due to the chromosomal recombination between the two. The transmission of bacterial chromosomal gene or episome has been known strictly to be dependent upon the formation of sex-pilli, such as F- and I-pilli, or the process of DNA replication (4−7).

The present inhibitory factor of *L. casei* is thought to be mucopeptide or polysaccharide from its physical properties, such as heat-stability, ethanol precipitability, and molecular weight (abuot 6,000). On the basis of these properties, this factor can be distinguished from the male-specific phage having a suppressive activity against the F-factor transfer (8) or gall and cholic acid derivative which are known to inhibit the R-factor transmission (9). Regarding detailed mechanisms of the inhibitory action of *L. casei* producing factor against the drug-resistance transfer between S-42 and S-58, further investigation is currently under way.

REFERENCES

1) Yamagishi, T., Serikawa, T., Morita, R., Takahashi, K., and Nishida, S. (1974) Effect of *Lactobacillus* product administration on the anaerobic intestinal flora of aged adults. *Japan. J. Microbiol.* **18**, 211−216
2) Watanabe, T., Morotomi, M., Kawai, Y., and Mutai, M. (1977) Reduction of population levels of some indigenous bacteria by lactobacilli in the gastrointestinal tract of gnotbiotic rats. *Microbiol. Immunol.* **21**, 495−503
3) Kosakai, N., Goshima, S., Nakazawa, S., Konno, M., Mitsuhashi, S., Matsumoto, F., Nakayama, K., Shimizu, K., and Okamoto, A. (1974) On the improvement of the assay method for minimum growth inhibitory concentration (MIC). *Chemotherapy* **22**, 1126−1128
4) Mitsuhashi, S. (1965) Transmissible drug resistance factor R. *Gunma J. Med. Sci.* **14**, 169−209
5) Brington, C. C., Gemski, P., and Carnahan, J. (1964) A new type of bacterial pillus genetically controlled by the fertility factor of *E. coli* K 12 and its role in chromosomal transfer. *Proc. Natl. Acad. Sci. USA* **52**, 776−783
6) Loeb, T. and Zinder, N. D. (1961) A bacteriophage containing RNA. *Proc. Natl. Acad. Sci. USA* **47**, 282−289
7) Hollom, S. and Pritchard, R. H. (1965) Effect of inhibition of DNA synthesis on mating in Escherichia coli K 12. *Genet. Res. Camb.* **6**, 479−483
8) Novotny, C., Knight, W. S., and Brinton, C. C. (1968) Inhibition of bacterial conjugation by ribonucleic acid and deoxyribonucleic acid male-specific bacteriophages. *J. Bacteriol.* **95**, 314−325
9) Akiba, T. and Koyama, K. (1961) Studies on the mechanism of transfer of drug-resistance in bacteria. XII. Inhibitory effect on the resistance transfer of gall and cholic acid derivatives. *Igaku To Seibutsugaku* **61**, 35−38