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Inhibition of Microbial Growth by Ajoene, a Sulfur-Containing Compound Derived from Garlic

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Ajoene, a garlic-derived sulfur-containing compound that prevents platelet aggregation, exhibited broad-spectrum antimicrobial activity. Growth of gram-positive bacteria, such as *Bacillus cereus*, *Bacillus subtilis*, *Mycobacterium smegmatis*, and *Streptomyces griseus*, was inhibited at 5 μg of ajoene per ml. *Staphylococcus aureus* and *Lactobacillus plantarum* also were inhibited below 20 μg of ajoene per ml. For gram-negative bacteria, such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Xanthomonas maltophilia*, MICs were between 100 and 160 μg/ml. Ajoene also inhibited yeast growth at concentrations below 20 μg/ml. The microbicidal effect of ajoene on growing cells was observed at slightly higher concentrations than the corresponding MICs. *B. cereus* and *Saccharomyces cerevisiae* were killed at 30 μg of ajoene per ml after 24 h of cultivation when cultivation was started at 10^5 cells per ml. However, the minimal microbicidal concentrations for resting cells were at 10 to 100 times higher concentrations than the corresponding MICs. The disulfide bond in ajoene appears to be necessary for the antimicrobial activity of ajoene, since reduction by cysteine, which reacts with disulfide bonds, abolished its antimicrobial activity.

Garlic has been used worldwide as a spice, food, and folk medicine. It contains alliin [(+)-*S*-allyl-L-cysteine sulfoxide] as a major sulfur-containing compound. When the raw garlic clove is damaged, alliin is hydrolyzed to sulfenate, pyruvate, and ammonia by alliinase. Condensation of 2 mol of sulfenate gives allicin (diallyl thiosulfinate), a major sulfur-containing intermediate, which was isolated and identified as an antibacterial substance by Cavallito et al. (6). Additionally, antitumor effects of allicin have been reported (8, 16). These reports suggested that allicin is an important substance for the medicinal properties of garlic. Allicin, however, is rapidly converted to diallyl disulfide (DADS) and other sulfides because of its instability.

Methanolic extracts from garlic homogenate were reported by Apitz-Castro et al. (2) to inhibit platelet aggregation. From the methanol extract, Block et al. (4, 5) isolated the strongest antiplatelet compound and identified it as ajoene [(*E*,*Z*)-4,5,9-trithiadodeca-1,6,11-triene-9-oxide] (Fig. 1), a derivative of allicin. Ajoene is known as an inhibitor of platelet aggregation induced by all known agonists (2, 3) and also is expected to be a medicine for treatment of thrombosis. Various antibiotic effects of ajoene have been reported. Yoshida et al. and San-Blas et al. showed that ajoene had antifungal activity toward *Aspergillus niger* and *Candida albicans* (17) and *Paracoccidioides brasiliensis* (11, 12), respectively; Weber et al. and Tatarintsev et al. showed that ajoene had antiviral activity (13, 15); and Urbina et al. and Perez et al. showed that ajoene had antiprotozoal activity toward *Trypanosoma cruzi* (14) and *Plasmodium berghei* (10), respectively. However, antimicrobial activity of ajoene has been demonstrated only for one yeast species and two fungus species; therefore, we examined the antimicrobial activity of ajoene for bacteria and some species of yeast.

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Preparation and isolation of ajoene were carried out according to the method described by Block et al. (4). Authentic *E*- and *Z*-ajoene were separated by a high-performance liquid chromatography (HPLC) system (LC-10A, Shimadzu Co., Kyoto, Japan) with the column Supelcosil LC-Si (4.6 by 250 mm; Supelco Japan, Inc., Tokyo, Japan) (9), and their purity and identification were confirmed by HPLC and ^{1}H-nuclear magnetic resonance (Hitachi R-90H). In the serial experiments, 98% (*E*,*Z*)-ajoene, which contains more than 80% *Z*-ajoene, was used. Ajoene stock solutions were made in ethanol at 100 mg/ml and used by dilution. The microbial strains used are shown in Table 1. Nine strains of gram-positive bacteria, four strains of gram-negative bacteria, and five strains of yeast were obtained from the Institute for Fermentation, Osaka, Japan, or the Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan. Two lactic acid bacteria, *Lactobacillus plantarum* and *Streptococcus* sp., and the other strains were cultivated without and with shaking in liquid medium (Table 1). For cultivation on solid medium, 1.5% agar was added to each liquid medium. Each microbial strain was transferred from stored plates at 4°C into 5 ml of liquid medium and cultivated with shaking overnight. A preculture was prepared by transfer from this culture broth (100 μl) to fresh medium and cultivation for 18 h under the conditions shown in Table 1. To determine the MIC, fresh precultures were diluted to 10^5 cells per ml and cultivated with or without various concentrations of ajoene under the conditions shown in Table 1 for 18 h. The MIC for each microbe was evaluated by measuring the optical density at 660 nm of the medium. If the increase in the optical density at 600 nm was less than 0.1, microbial growth was considered to be inhibited. To determine the MIC on solid media, each preculture containing 10^3 cells was plated onto the solid medium with or without various concentrations of ajoene and cultivated under the conditions shown in Table 1 for 3 days. The surviving cells were detected on the plate as colonies, and the MIC on the solid medium was evaluated by the detection of no survivors.

The MICs of ajoene for each microorganism are shown in Table 1. For gram-positive bacteria, two strains of *Bacillus* spp.

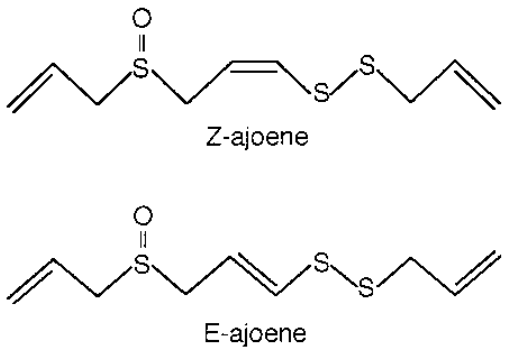

FIG. 1. Chemical structures of ajoenes.

were inhibited at less than 5 μg of ajoene per ml in the liquid medium and on the solid media. *Staphylococcus aureus*, two strains of *Mycobacterium* spp., *L. plantarum*, *Streptomyces griseus*, and five strains of yeast also were inhibited at 20 μg of ajoene per ml in the liquid medium and on the solid media. The MICs of *Micrococcus luteus* and *Streptococcus* sp. were higher than those of gram-positive bacteria described above. Gram-negative bacteria, such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Xanthomonas maltophilia*, were inhibited by 100 to 160 μg/ml. No inhibition was found even at 500 μg/ml for *Pseudomonas aeruginosa.*

The antimicrobial effects of ajoene and the other antibiotics (Wako Pure Chemical Industries Ltd., Osaka, Japan) on solid medium were compared (Fig. 2). *Bacillus cereus* spores were prepared according to the method of Amaha et al. (1). The spore suspension was used after incubation at 65°C for 45 min. Only ajoene strongly inhibited *Candida albicans*, and it was comparable to kanamycin, tetracycline, and bacitracin against *S. griseus*. Yoshida et al. (17) and San-Blas et al. (11, 12) reported the antifungal effect of ajoene, and their MICs were comparable to those for yeast strains obtained in this experiment. Although Yoshida et al. (17) stated the antibacterial effect of ajoene was only specific for *S. aureus*, our results showed strong inhibition of growth of many bacteria. Members of the genera *Bacillus*, *Mycobacterium*, and *Streptomyces* were inhibited by concentrations of less than 20 μg of ajoene per ml. These results suggested that ajoene caused strong inhibition of gram-positive bacteria and yeasts and had various degrees of inhibition of gram-negative species like *E. coli* (116 μg/ml) and *P. aeruginosa* (>500 μg/ml).

The antimicrobial activities of ajoene, diallyl sulfide (DAS; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), and DADS (Tokyo Chemical Industry Co., Ltd.) as derivatives of allicin and sorbic acid (Wako Pure Chemical Industries Ltd.) as a food preservative were compared by addition at various concentrations to the culture broth (10^5 cells per ml) of *B. cereus* and *C. albicans* (Table 2). After 24 h, the numbers of surviving cells were decreased, showing inhibition of growth by ajoene and DADS, but not by 100 μg of DAS or sorbic acid per ml. When the sorbic acid concentration was raised to 1,000 μg/ml, microbial growth was inhibited strongly for *B. cereus* and moderately for *C. albicans*. Because 100 μg of ajoene per ml decreased the number of surviving cells from 10^5 cells per ml to less than 10 cells per ml for both microorganisms, it appears to be an efficient antimicrobial agent. Although DAS, DADS, and ajoene are similar in structure, consisting of two allyl groups and sulfur(s), the inhibitory effect on microbial growth was shown only by DADS and ajoene. These results suggest that

TABLE 1. MIC and MMC of ajoene for organisms in this study

Microorganism	Strain no. (cultivation condition)[a]	MIC (μg/ml) on medium[b]:		MMC (μg/ml)
		Liquid	Solid	
Gram-positive bacteria				
Bacillus cereus	IAM 12605 (a, e)	4 ± 1.4	5 ± 0	300
Bacillus subtilis	IFO 13719 (a, e)	4 ± 1.4	5 ± 0	≥500
Staphylococcus aureus	IFO 14462 (b, e)	16 ± 2.2	20 ± 0	400
Mycobacterium smegmatis	IFO 12065 (b, e)	4 ± 1.4	13 ± 2.7	>500
Mycobacterium phlei	IFO 3158 (a, e)	14 ± 5.5	13 ± 2.7	>500
Micrococcus luteus	IFO 12708 (a, e)	136 ± 8.9	160 ± 0	≥500
Lactobacillus plantarum	IAM 1041 (a, f)	19 ± 2.2	ND[c]	ND
Streptococcus sp.	IFO 3535 (b, f)	56 ± 5.5	76 ± 8.9	>500
Streptomyces griseus	IFO 3357 (a, g)	4 ± 1.4	5 ± 0	>500
Gram-negative bacteria				
Escherichia coli	IFO 3301 (b, e)	116 ± 8.9	152 ± 8.3	400
Klebsiella pneumoniae	IAM 1063 (b, e)	152 ± 8.4	200 ± 0	>500
Pseudomonas aeruginosa	IFO 12689 (a, e)	>500	>500	>500
Xanthomonas maltophilia	IAM 12423 (a, e)	118 ± 4.5	136 ± 8.9	>500
Yeasts				
Candida albicans	IFO 1594 (c, h)	13 ± 2.7	18 ± 2.7	≥500
Hanseniaspora valbyensis	IFO 1758 (d, h)	11 ± 2.2	20 ± 0	400
Pichia anomala	IFO 0806 (d, h)	11 ± 2.2	15 ± 0	≥500
Schizosaccharomyces pombe	IFO 0347 (c, h)	5.5 ± 2.7	10 ± 0	300
Saccharomyces cerevisiae	IFO 2347 (a, h)	12 ± 2.7	17 ± 2.7	300

[a] IAM, Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan; IFO, Institute for Fermentation, Osaka, Japan. a, b, c, and d, cultivation temperatures of 30, 37, 28, and 24°C, respectively; e, cultivated in PY medium (0.5% peptone, 0.3% yeast extract, 0.3% NaCl [pH 7.0]); f, cultivated in *Lactobacillus* inoculate broth "Nissui" without shaking; g, cultivated in IFO231 medium (0.1% yeast extract, 0.1% beef extract, 0.2% NZ amine, 0.2% maltose [pH 7.3]); h, cultivated in YM medium (1% glucose, 0.5% polypeptone, 0.3% malt extract, 0.3% yeast extract [pH 6.0]).

[b] Values represent the mean of five determinations ± standard deviations.

[c] ND, not determined.

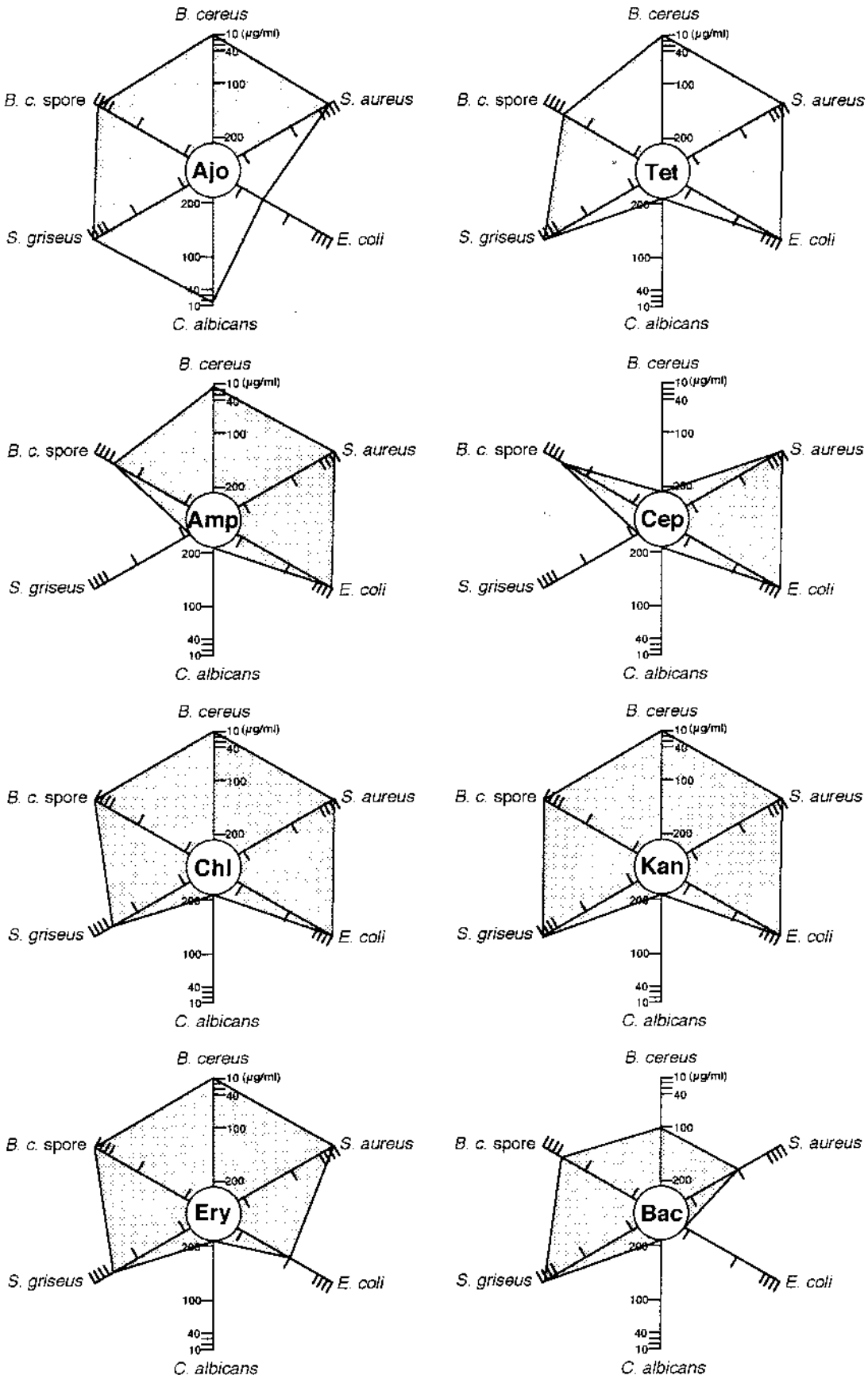

FIG. 2. Comparison of antimicrobial effects of ajoene and other antibiotics. The effects were evaluated by the MICs on the plates. The details are described in the text. Ajo, ajoene; Tet, tetracycline; Chl, chloramphenicol; Kan, kanamycin; Amp, ampicillin; Cep, cephalothin; Ery, erythromycin; Bac, bacitracin; *B. c.*, *B. cereus.*

TABLE 2. Comparison of the antimicrobial activities for *B. cereus* and *C. albicans*

Antibiotic	Concn (μg/ml)	No. of surviving cells of[a]:	
		B. cereus	*C. albicans*
Control		2.0×10^8	1.8×10^8
Ajoene	10	6.0×10^2	ND[b]
	30	ND	1.0×10^2
	100	<10	<10
DAS	100	1.8×10^8	8.3×10^7
DADS	10	2.2×10^2	ND
	30	ND	4.5×10^6
	100	<10	2.0×10^4
Sorbic acid	100	2.0×10^8	7.0×10^7
	1,000	<10	1.4×10^4

[a] Values are means of three independent experiments.
[b] ND, not determined.

the disulfide bond may be important for antimicrobial activity. Ajoene also contains a sulfinyl group, which has been reported in antimicrobial agents such as allicin (diallyl thiosulfinate). Therefore, the antimicrobial effect of ajoene is thought to result from the presence of both the disulfide bond and the sulfinyl group.

The minimum ajoene concentrations required to kill the cells (minimal microbicidal concentrations [MMCs]) were estimated by incubating resting cells and ajoene in sodium phosphate buffer to eliminate the influence of medium compounds. The precultures were prepared as described above. The cells were harvested by centrifugation (4,000 × *g*, 5 min), and then the cells were suspended in 50 mM sodium phosphate buffer (pH 7.0) at 10^5 cells per ml. Ajoene was added to 5 ml of each cell suspension and incubated with shaking for 5 h at the same temperature of cultivation. The surviving cells were detected on the plates, and MMC was evaluated by the detection of no survivors. The MMCs of ajoene for the microorganisms are shown in Table 1. The MMCs for *B. cereus*, *Schizosaccharomyces pombe*, and *Saccharomyces cerevisiae* were 300 μg/ml, and those for *S. aureus*, *E. coli*, and *Hanseniaspora valbyensis* were 400 μg/ml. The other microorganisms were not killed even at 500 μg/ml. For the tested microorganisms, the MMCs obtained were 10 to 100 times higher than the corresponding MICs. *B. cereus* and *C. albicans* were inhibited only during cultivation, suggesting that the antimicrobial action of ajoene was effective only with growing cells (Table 2).

The time courses of growth of *B. cereus* and *S. cerevisiae* in the presence of ajoene are shown in Fig. 3. The cell number of 10^5 cells per ml at the starting point was decreased or increased

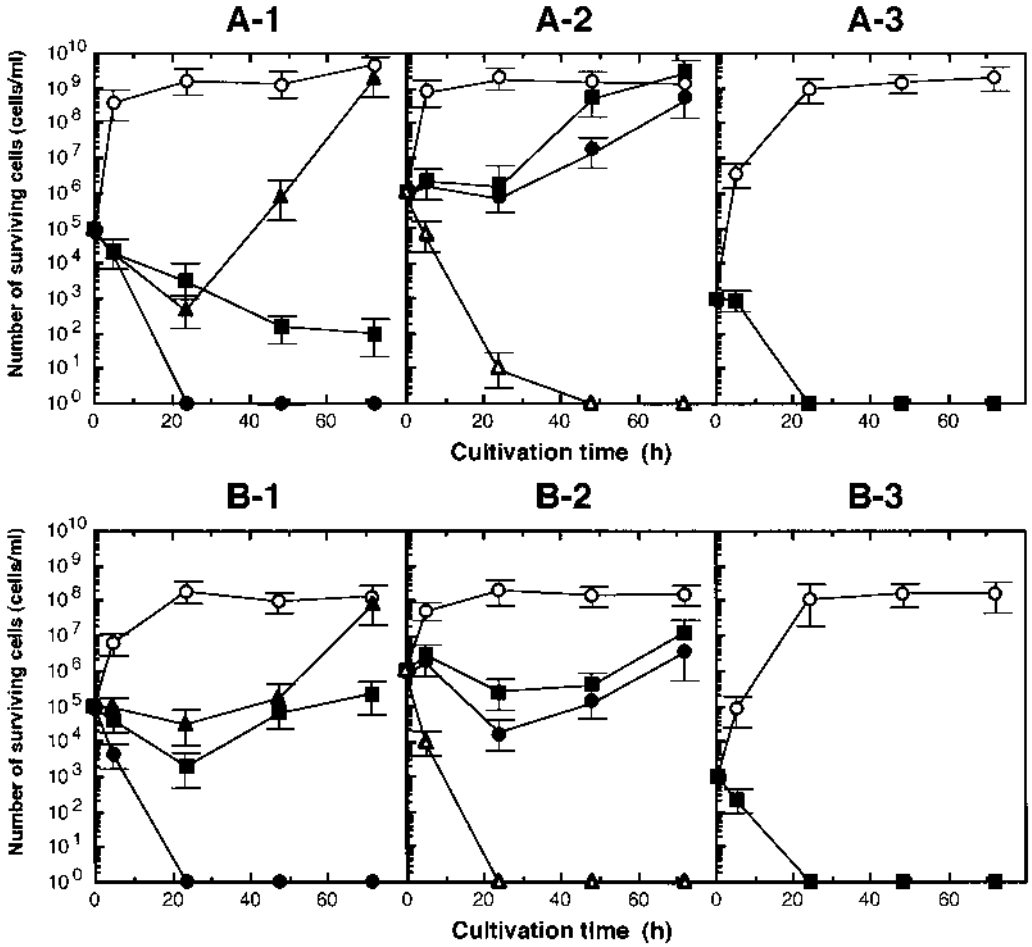

FIG. 3. Effect of ajoene on growth of *B. cereus* (A-1 to A-3) and *S. cerevisiae* (B-1 to B-3). At the starting point of cultivation, cell concentrations were adjusted to 10^5 (A-1 and B-1), 10^6 (A-2 and B-2), and 10^3 (A-3 and B-3) cells per ml in the medium shown in Table 1, and they were counted at several intervals. ○, control; ▲, 10 μg/ml; ■, 20 μg/ml; ●, 30 μg/ml; △, 50 μg/ml.

depending on the concentration of ajoene in the medium. *B. cereus* (Fig. 3A) and *S. cerevisiae* (3B) were killed at 30 μg of ajoene per ml after 24 h of cultivation. The microbicidal effect of ajoene was observed at slightly higher concentrations than the corresponding MICs. In the lower concentrations evaluated above, the surviving cell number was decreased at the beginning and then increased with time. To investigate the influence of starting cell density, *B. cereus* and *S. cerevisiae* cultivations were started at different cell densities (10^6 and 10^3 cells per ml) (Fig. 3A-2, B-2, A-3, and B-3). Although neither microorganism was killed by less than 20 μg of ajoene per ml at the starting cell density of 10^5 cells per ml, the microbicidal concentration was decreased at 10^3 cells per ml. On the other hand, the microbicidal concentrations increased to 50 μg/ml at a higher cell density (10^6 cells per ml). These results suggested that the given concentration of ajoene killed a constant percentage of growing cells.

When various concentrations of cysteine were added to ajoene-containing medium, the ajoene concentrations were decreased depending on the cysteine concentrations. For example, when twice the amount of cysteine was present with ajoene in the medium, the concentration of ajoene was immediately reduced to 20% of that without cysteine. The decreasing concentration of ajoene in the medium eliminated the antimicrobial properties of ajoene. San-Blas et al. also observed that the inhibitory activity of ajoene for the dimorphic fungus *P. brasiliensis* was reduced by addition of cysteine and dithiothreitol (11). Cysteine and dithiothreitol have a sulfhydryl group in common and are highly reactive with disulfide bonds. Cavallito et al. reported that allicin reacts with the sulfhydryl group of cysteine and forms *S*-allylmercaptocysteine (allyl-ss-cysteine) by cleaving the disulfide bond and replacing the allyl group with cysteine (7). The reduction of ajoene concentration in the medium might be caused by this reaction; consequently, the antimicrobial effects of ajoene were reduced.

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