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Effects of Subminimal Inhibitory Concentrations of Ampicillin on Hemagglutination of *Escherichia coli*

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ABSTRACT

The hemagglutination (HA) activity of *Escherichia coli* was enhanced by subminimal inhibitory concentrations (sub-MICs) of ampicillin. One half of the MIC of ampicillin caused a bacterial filamentation and diminished bacterial piliation (as observed by light and electron microscopies) as well as an increase of HA activity. HA activity, however, decreased after separation of ampicillin-treated bacteria. These results indicate that the increase in HA activity by ampicillin is mainly due to filament formation.

Key words: *Escherichia coli*, Ampicillin, Hemagglutination

It is generally thought that the adherence of bacteria to various host cells is necessary for bacterial infections[9]. The adherence of bacteria occurs in a highly specific manner, and it frequently appears to involve interactions between lectin-like components of the bacterial cell surface and saccharide residues on the host cell surface[5]. *Escherichia coli* possesses type 1 pili which specifically bind to a mannose residue on the host cell surface, and the pili play an important role in the adherence of this bacterium.

The influence of various antibiotics on bacterial adherence has recently been investigated[9]. It has been shown that the exposure of *E. coli* to subminimal inhibitory concentrations (sub-MICs) of penicillin changes HA activity[11]. Many investigators have suggested that a change in the amount of bacterial piliation affects HA activity[9,11]. However, sub-MIC concentrations of penicillin cause filament formation as well as pili formation. In the present study, we investigated the effects of filament formation on the HA activity of *E. coli* by focusing on the morphological alterations of bacterial structure.

MATERIALS AND METHOD

Bacterial strains. Two strains of *E. coli* were used. One was *E. coli* K-12/w-13 which has type 1 surface pili, the other was *E. coli* K-12 which has no surface pili. For laboratory use, the strains were subcultured every month on brain heart infusion (BHI) agar plates (Difco Laboratories, Detroit, Mich.), and stored at 4°C.

Antibiotics. Antibiotics were provided as follows: ampicillin; penicillin G; piperacillin; mecillinam; aztreonam; cephaloridine; chloramphenicol; tetracycline; norfloxacin; gentamicin and kanamycin.

Determination of MICs. The minimum inhibitory concentration (MIC) of antibiotics was determined under conditions identical to those used for the culture of bacteria in the adhesion assays. Serial twofold dilutions of each antibiotic were prepared in BHI broth (10 ml each), and inoculated with 0.1 ml each of an early-stationary-phase broth culture of the corresponding bacteria, and incubated under shaking conditions at 37°C for 18 h. The MIC was defined as the lowest concentration of each antibiotic that resulted in complete inhibition of bacterial growth.

Treatment of bacteria with antibiotics. *E. coli* strains were subcultured 1:100 from an early-stationary-phase broth culture, grown in BHI broth in the presence of one half of the MIC of each antibiotic under shaking conditions at 37°C for 4 h. After growth, the bacteria were harvested by centrifugation, washed three times with 0.01 M phosphate buffered saline (pH 7.4, PBS), and the bacterial concentration was adjusted photometrically to an optical density of 1.0 at 660 nm (5×10^9 bacteria/ml). In each experiment drug-treated bacteria and identically handled cultures exposed to non-treated diluent were compared for HA activity, mannose sensitivity, hydrophobicity and by light and electron microscopy (Fig. 1).

HA assay. HA assay was performed as previously described by Pinchichero et al[7]. Briefly, bacteria from a fresh culture were washed and resuspended in PBS at a concentration of the optical density of 1.0 at 660 nm, then serially diluted in twofold steps in PBS. Fifty microliter of the bacterial suspension in a well of a microtitration tray was mixed with 50 μl of 1% (v/v) suspension of human erythrocytes (blood type B) which had previously been washed three times in PBS. The tray was left at

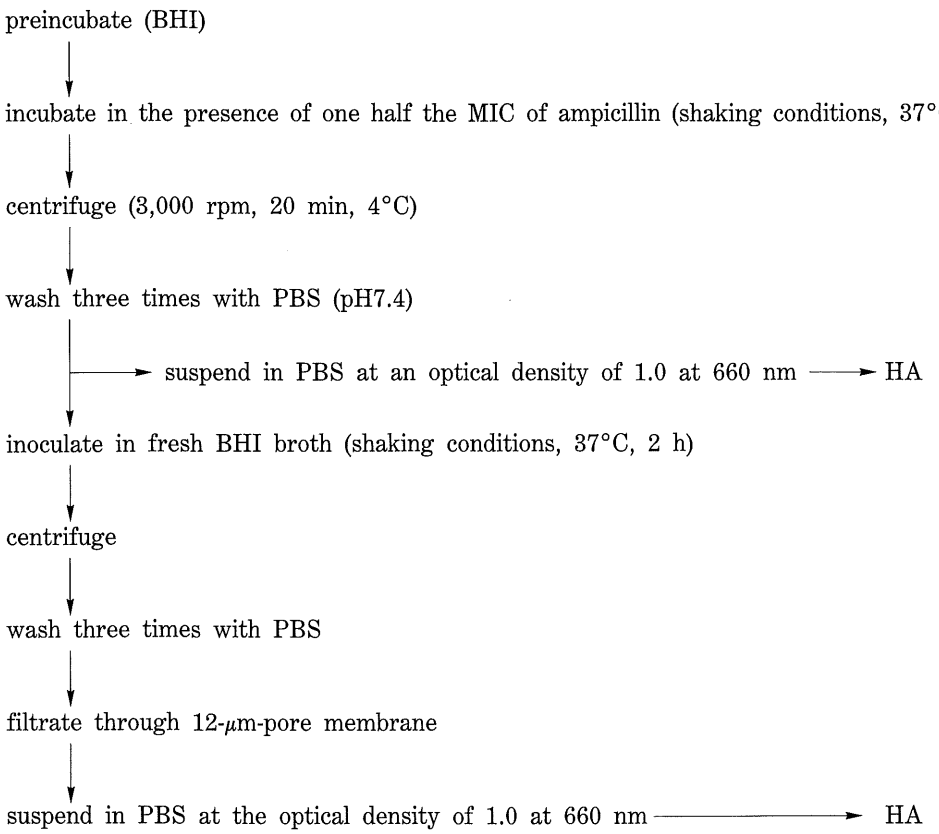


Fig. 1. Bacterial preparation for HA assay. The same sample was assayed for hydrophobicity and observed by light and electron microscopy.

room temperature for 60 min, and the highest dilution of bacteria giving visible agglutinates was recorded. The mannose sensitivity of HA was determined by mixing the bacterial suspensions with a final concentration of 2.5% solution (w/v) of α-methyl-D-mannoside on the microtitration tray when combined with 1% suspension of erythrocytes. The α-methyl-D-mannoside-treated suspension was compared to its non-treated control.

Morphological studies of bacterial piliation by electron microscopy. The samples were prepared for negative staining as described by Bamford et al[1] and observed by electron microscopy.

Adherence to hexadecane. The bacterial surface hydrophobicity was estimated using a modification of the technique for adherence to hydrocarbons described by Rosenberg et al[8]. Hexadecane (0.1 ml) was added to 2.0 ml of the bacterial suspension in a test tube (12 × 105 mm). The two phases were mixed with a mixer for 60 sec and then allowed to separate. The turbidity of the aqueous phase was measured photometrically, and hydrophobicity was expressed as the percentage reduction of initial turbidity of the aqueous suspension.

Separation of filamentous bacteria. Ampicillin-treated filamentous bacteria were inoculated into fresh BHI broth and incubated under shaking conditions at 37°C for 2 h. Non-treated bacteria were also incubated in the same medium. After the incubation, the bacteria were harvested and washed as described above. The bacteria were then suspended in PBS and filtered with gentle suction over a 12-μm-pore membrane filter (A1200A047A, Toyo Roshi Kaisha, Ltd. Tokyo) allowing passage of separated bacteria, while retaining non-separated bacteria on its surface. The filtered bacteria were resuspended in PBS at a concentration of the optical density of 1.0 at 660 nm. HA activity, mannose sensitivity and micrographs of each bacteria were examined (Fig. 1).

RESULTS

Sensitivity to antibiotics. Table 1 summarizes MICs of antibiotics for the two *E. coli* strains.

Effects of antibiotics on HA, bacterial morphology and hydrophobicity. *E. coli* K-12/w-13 was heavily piliated when grown in BHI broth, and this strain agglutinated human erythrocytes in a mannose sensitive way. *E. coli* K-12, which was devoid of pili, did not agglutinate human erythrocytes.

HA activity and the effect of α-methyl-D-mannoside on the HA of *E. coli* K-12/w-13, with and without exposure to antibiotics, are shown in Table 2. When *E. coli* K-12/w-13 was grown in the presence of one half of the MIC of antibiotics, the HA activity was changed to various extents. β-Lactams were the most effective compounds which increase HA activity. *E. coli* K-12/w-13 grown in

Table 1. MICs of antibiotics for *E. coli*

E. coli strain	MIC (μg/ml)										
	ABPC	PCG	PIPC	MPC	AZT	CER	CP	TC	NFLX	GM	KM
E. coli K-12/w-13	3.13	50	1.56	25	0.098	3.13	3.13	0.39	0.098	1.56	6.25
E. coli K-12	1.56	12.5	0.39	0.05	0.098	3.13	3.13	0.78	0.098	3.13	12.5

ABPC, Ampicillin; PCG, Penicillin G; PIPC, Piperacillin; MPC, Mecillinam; AZT, Azthreonam; CER, Cephaloridine; CP, Chloramphenicol; TC, Tetracycline; NFLX, Norfloxacin; GM, Gentamicin; KM, Kanamycin

Table 2. Effects of one half the MIC of antibiotics on hemagglutination, morphology and hydrophobicity of *E. coli* K-12/w-13

Antibiotic	HA titer[a]	mannose sensitivity	Morphology	Hydrophobicity[b]
control	2	+	rod	100
ABPC	5	+	filament	98
PCG	5	+	filament	100
PIPC	5	+	filament	98
MPC	2	+	ovoid	96
AZT	N.D.	N.D.[c]	filament	N.D.
CER	5	+	filament	100
CP	3	+	filament	100
TC	3	+	filament	98
NFLX	3	+	filament	98
GM	2	+	filament	100
KM	3	+	filament	100

Antibiotics abbreviations are given in footnote of Table 1.
[a]Log2 of the highest dilution of bacteria giving HA.
[b]Per cent turbidity remained in the aqueous phase.
[c]N.D., Not done.

Table 3. Effects of different sub-MIC concentrations of ampicillin on hemagglutination and filamentation of *E. coli* K-12/w-13

Concentration of ampicillin	filamentation	HA titer
1/2 MIC	+++	5
1/4 MIC	++	3
1/8 MIC	++	3
1/16 MIC	+	2
1/32 MIC	+	2
control	–	2

+++, long filament; ++, short filament; +, partially filamented.

the presence of one half of the MIC of each antibiotic exhibited human erythrocyte agglutination which is inhibitable by 2.5% α-methyl-D-mannoside.

It is to be noted that the exposure of *E. coli* K-12/w-13 to one half of the MICs of all antibiotics but mecillinam resulted in distinct bacterial filamentation. However, antibiotics were found not to change the surface hydrophobicity of *E. coli* K-12/w-13 (Table 2).

Effect of ampicillin on bacterial morphology. sub-MICs of ampicillin induced filament formation in cultures of *E. coli* K-12/w-13. Depending upon the increasing concentration of ampicillin, the degree of bacterial filamentation was increased (Table 3). Most bacteria in the presence of one half of the MIC of ampicillin became elongated more than a hundred-fold compared with non-treated bacteria

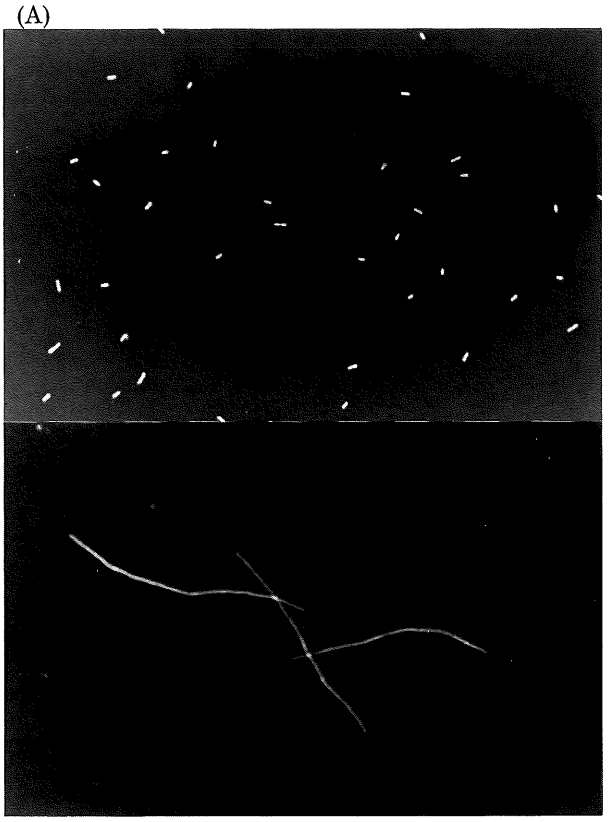
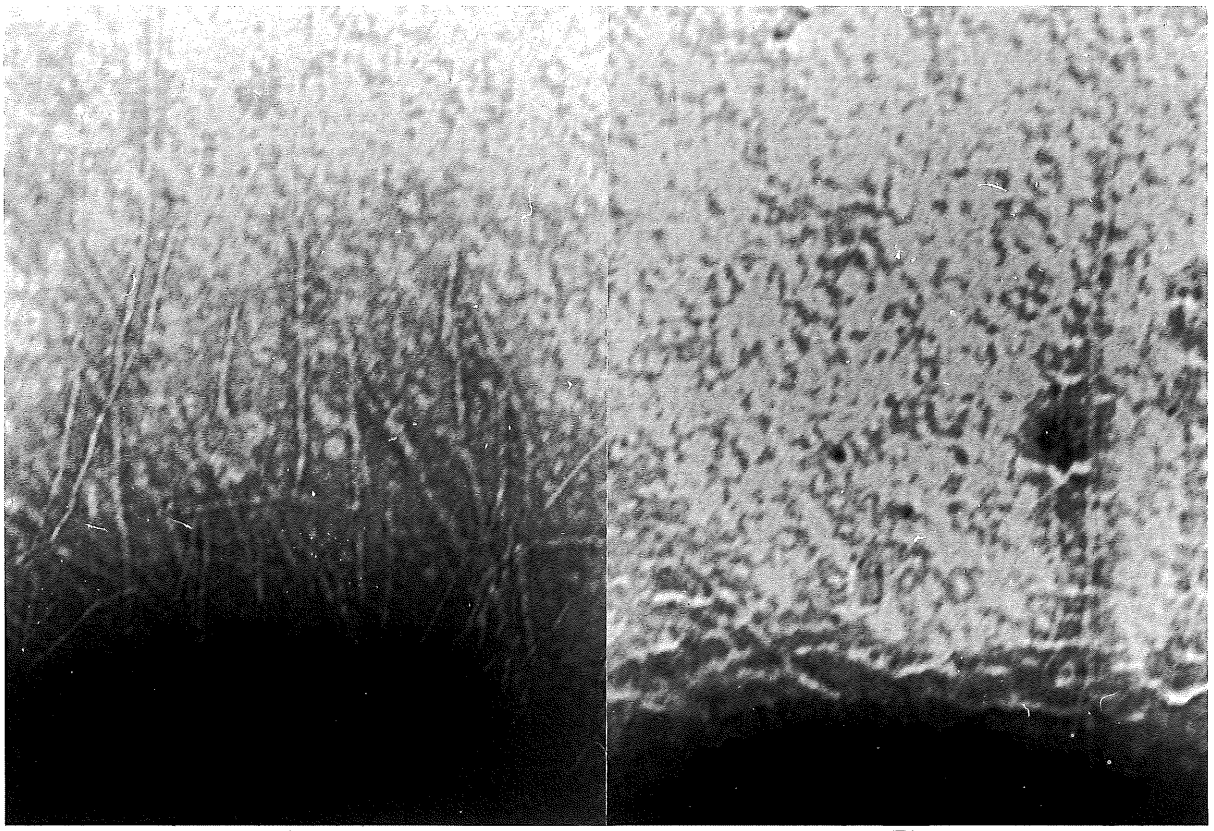


Fig. 2. Phase contrast micrographs of *E. coli* K-12/w-13 grown for 4 hours without (A) and with (B) one half of the MIC concentration of ampicillin. Drug-treated bacteria (B) are elongated compared with control (A).



(A) (B)

Fig. 3. Electron micrographs; Negatively stained with phosphotungstic acid. ×28,000. (A) *E. coli* K-12/w-13 after growth in BHI broth for 18 hours. Bacterial surface is surrounded by pili. (B) *E. coli* K-12/w-13 after treatment with one half of the MIC concentration of ampicillin. Note that only a few pili are present on the bacterial surface.

Table 4. Hemagglutination of separated cells from filaments

Bacteria	*E. coli* K-12/w-13		*E. coli* K-12	
	HA titer	mannose sensitivity	HA titer	mannose sensitivity
control	2	+	0	N.D.[a]
filamentous form	5	+	0	N.D.
separated form	0	N.D.	N.D.	N.D.

[a]N.D., Not done.

(Fig. 2). However, after the removal of ampicillin, the bacteria exhibited the formation of septum in the filament and then separated.

Effect of ampicillin on bacterial piliation. Electron microscopy shows that one half of the MIC of ampicillin decreased the amount of piliation on the surface of *E. coli* K-12/w-13 compared with non-treated bacteria (Fig. 3). After the separation of filamentous bacteria, a reduction of piliation on each separated bacterial surface was still observed. No piliation was seen on the surface of *E. coli* K-12 treated with or without ampicillin.

Effect of ampicillin on HA activity. The addition of ampicillin to *E. coli* K-12/w-13 resulted in a significant change of HA activity. Table 3 shows a good correlation between the degree of bacterial filamentation and HA activity. Especially, one half of the MIC of ampicillin had a marked effect on the HA titer, which increased from 2 to 5. However, *E. coli* K-12/w-13 completely lost HA activity after the separation of filamentous bacteria (Table 4). In contrast, the HA titer of non-treated *E. coli* K-12/w-13 always indicated the titer 2 even with experimental management. On the other hand, *E. coli* K-12 with or without exposure to ampicillin did not indicate HA activity. No morphological alterations of *E. coli* K-12, even filament formation, could make this strain HA positive.

DISCUSSION

For bacterial adhesion, the significance of pili on the *E. coli* surface has long been well known[5]. Recently, sub-MIC concentrations of antibiotics have been shown to enhance the bacterial adhesion as well as to inhibit it[3,9-11]. Many workers have shown that changes in the amount of bacterial pili-

ation is the only reason for the changes of HA activity under the influence of sub-MIC concentrations of antibiotics[9,11].

Ampicillin, the prototype of penicillin, interferes with cell wall synthesis by binding to PBPs, thereby inhibiting or distorting the terminal steps of ligand expression by preventing the anchoring of surface pili[2]. Ofek et al[6] have reported a decrease in the amount of piliation on a penicillin-treated *E. coli* surface. Our ultrastructural studies of piliation on ampicillin-treated K-12/w-13 strain surface also demonstrate a marked loss of piliation. In addition to affecting the piliation, ampicillin which specifically interferes with carboxypeptidase activity may also inhibit normal bacterial separation and thus lead to filament formation[2].

On the other hand, estimation of changes of HA activity under the influence of sub-MIC concentrations of ampicillin might be dependent on the bacterial cell number. Difficulties in the determination of the cell count because of alterations in bacterial structure are well known[4]. Consequently, one should use antibiotic-treated bacteria of the same number and/or the same size as non-treated bacteria.

We observed increased HA of ampicillin-treated K-12/w-13 when the bacterial concentration was adjusted turbidimetrically, in spite of a decrease of pili which would be the sole HA tool of this strain. There also seemed to be a correlation between HA activity and the degree of bacterial filamentation. Hence, the increased HA activity was considered to be caused by significant elongation of the bacteria. This was supported by the fact that the separated bacterial cells, whose piliation was almost the same as the elongated cells, demonstrated very low HA.

The degree of pili expression was found not to be the only factor that affected HA activity; this had already been suggested by Vosbeck et al[11] and Klein et al[3]. No precise studies have yet been performed on the alterations in the formation of bacterial structures such as filamentation under the influence of sub-MIC concentrations of ampicillin. We concluded that alterations in bacterial morphology may reflect on changes in HA activity for HA positive strain. This study indicates that changes in HA activity by ampicillin is probably mediated by both bacterial piliation and bacterial elongation.

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