

BULGECIN, A BACTERIAL  
METABOLITE WHICH IN CONCERT  
WITH  $\beta$ -LACTAM ANTIBIOTICS  
CAUSES BULGE FORMATION

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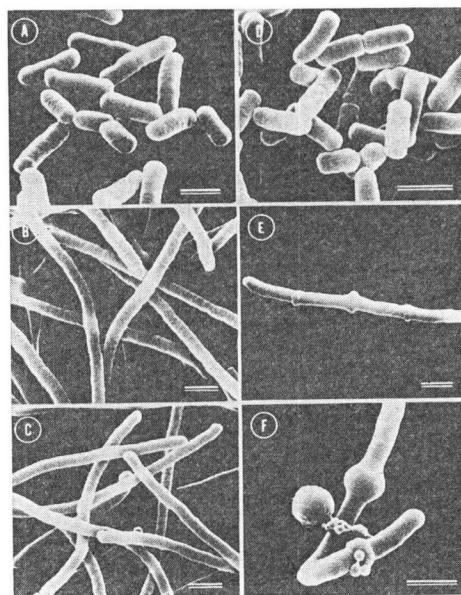
Bulge formation in Gram-negative bacteria is a morphological change caused specifically by  $\beta$ -lactam antibiotics that interact with two penicillin-binding proteins (PBPs) determining bacterial shape and septum formation<sup>1)</sup>. Here, we describe a new bacterial metabolite, bulgecin which, without irreversible binding to PBPs, induces bulge formation acting in concert with  $\beta$ -lactam antibiotics.

In a previous communication, we reported the isolation of the novel *N*-sulfonated  $\beta$ -lactam antibiotics, sulfazecin and isosulfazecin, from bacterial cultures<sup>2)</sup>. The discovery of sulfazecin was based on observations that a culture filtrate of a soil isolated bacterium had three features that indicated the presence of a  $\beta$ -lactam antibiotic: 1) Stronger activity against  $\beta$ -lactam antibiotic sensitive mutants than against their parents, 2) inactivation by  $\beta$ -lactamases, and 3) induction of bulge formation in enteric bacteria such as *Escherichia coli* and *Proteus mirabilis*<sup>2,3)</sup>. However, when the antibacterial principle, sulfazecin, was purified, it did not possess the bulge-inducing activity although it retained the other two features. This observation prompted us to investigate the factor responsible for bulge formation.

Since we were aware that sulfazecin is fairly unstable in solutions with pH above 8.5, we first treated the culture filtrate of *Pseudomonas acidophila* strain G-6302 at pH 10 for 1 hour at room temperature. The antibacterial and bulge-forming activities were destroyed by such a mild alkali-treatment. Then we added purified sulfazecin to the alkali-treated culture filtrate, and exposed *E. coli* to the mixture for 2 hours. As shown in Fig. 1E, bulges formed at sites where septation seems to take place. On the other

hand, sulfazecin alone only caused filamentation (Fig. 1B), and the alkali-treated culture filtrate produced no morphological changes (Fig. 1D). These observations clearly demonstrated that a factor cooperating with sulfazecin to produce bulge formation was present in the alkali-treated culture filtrate of strain G-6302. Similar morphological changes were induced using cefmenoxime in place of sulfazecin (Fig. 1C & 1F).

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bulge formation was then isolated from the alkali-treated culture filtrate in crystalline form. It is a glycopeptide with a molecular formula of  $C_{16}H_{28}N_8O_{14}S_2Na \cdot H_2O$  [m.w. 591.5; m.p. 208~210°C (decomp.)]. This compound showed a morphological effect identical with that of the alkali-treated culture filtrate and was given the name bulgecin. It is interesting that bulgecin was isolated not only from the culture filtrate of *P. acidophila*, which produces sulfazecin, but also from that of *Pseudomonas mesoacidophila*, which is taxonomically distant from *P. acidophila* and produces isosulfazecin, an epimer of sulfazecin<sup>2,4</sup>.

Bulgecin itself showed no antibacterial activity against any bacteria tested including  $\beta$ -lactam antibiotic hypersensitive mutants<sup>2,3</sup>. However, it showed strong synergism with  $\beta$ -lactam antibiotics. When tested by a disk diffusion assay, bulgecin greatly increased the inhibition zone formed by  $\beta$ -lactam antibiotics such as cefmenoxime and mecillinam in a dose-dependent fashion (Table 1). Cefmenoxime shows preferential affinity for PBP 3 at low concentrations and induces filamentation in *E. coli*<sup>5</sup>, and mecillinam has selective affinity for PBP 2<sup>1</sup>. In broth culture, bulgecin enhanced the lytic activity of  $\beta$ -

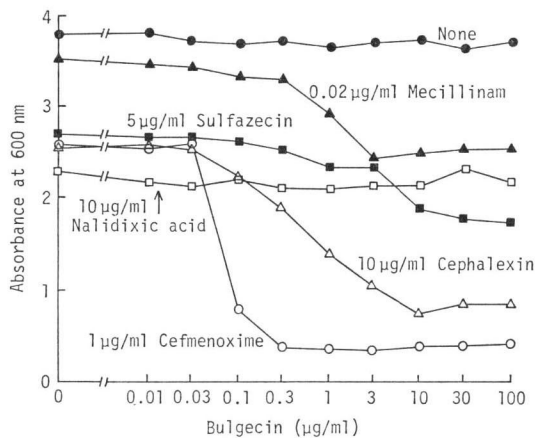
Table 1. Effect of bulgecin on antibacterial activity against *E. coli* NIHJ JC-2 of cefmenoxime and mecillinam as examined by the disk diffusion assay.

Concentration of bulgecin ( $\mu\text{g/ml}$ )	Diameter of inhibition zone (mm) in agar medium containing		
	None	Cefmenoxime 0.05 $\mu\text{g/ml}$	Mecillinam 0.01 $\mu\text{g/ml}$
0	0	0	0
1	0	0	0
3	0	13.5	11.5
10	0	23.5	16
30	0	32	21
100	0	38	25.5
300	0	42	32
1,000	0	46.5	35.5

Agar plates of nutrient medium (peptone 1.0%, meat extract 1.0%, NaCl 0.1%, pH 7.0) containing  $10^7$  colony forming unit/ml of *E. coli* NIHJ JC-2 and the antibiotics as indicated were prepared. Paper disks (diameter, 8 mm) moistened by 25  $\mu\text{l}$  of bulgecin solutions of indicated concentrations were placed on the surface of the agar plates and the plates were incubated at 37°C for 20 hours. 0 means no inhibition zone around the paper disk.

Fig. 2. Effect of bulgecin on *E. coli* NIHJ JC-2 grown in the presence of various antibiotics.

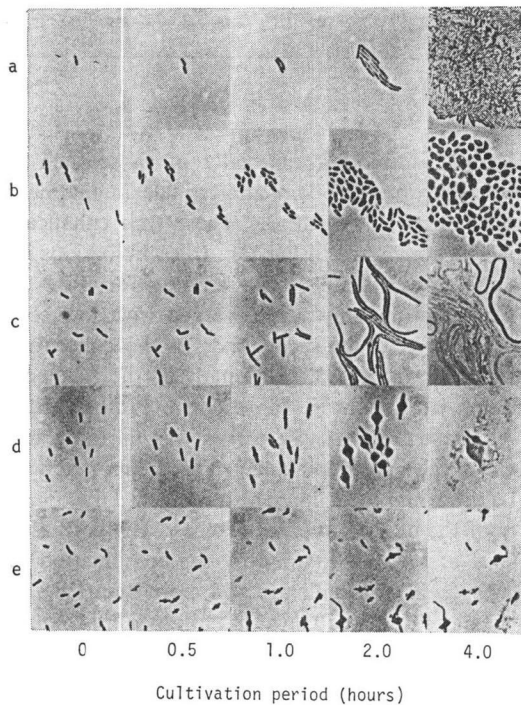
Experimental conditions were the same as those described in Fig. 1. After the 2 hours cultivation, the culture was diluted 10 times with distilled water and absorbance at 600 nm was measured in a Spectronic 20 colorimeter.



lactam antibiotics, especially of those such as cefmenoxime, cefotiam, and cephalixin, that have high affinity for PBP 3. These lysis enhancing effects are shown in Fig. 2. In combinations involving antibiotics with affinity for PBP 3, filamentation only was observed with low concentrations of bulgecin, and, as the concentration was increased, the length of the filaments decreased and bulge formation became evident. At concentrations where lysis was taking place, ghost cells predominated. The combination of bulgecin and mecillinam also decreased the opacity of the culture to some extent. The decreased level of opacity attained by the combination of 0.02  $\mu\text{g/ml}$  of mecillinam and more than 1  $\mu\text{g/ml}$  of bulgecin was equal to that attained by 1  $\mu\text{g/ml}$  of mecillinam alone. Addition of bulgecin to a culture containing 1  $\mu\text{g/ml}$  of mecillinam did not affect the absorbance further (data not shown). The combination of bulgecin and mecillinam resulted in the formation of ovoid cells, that were rounder than those induced by mecillinam alone. The combination of bulgecin and nalidixic acid is also included in Fig. 2. Nalidixic acid, although it induced filamentation in *E. coli* as did cefmenoxime, did not act synergistically with bulgecin as judged by either absorbance measurement or microscopic observations. Other non- $\beta$ -lactam antibiotics (*e.g.*, novobiocin, mitomycin C and streptozotocin) that induce filamentation

Fig. 3. Formation of bulges by mecillinam plus cefmenoxime, and bulgecin plus cefmenoxime.

a, 10  $\mu\text{g/ml}$  bulgecin. b, 0.1  $\mu\text{g/ml}$  mecillinam. c, 0.1  $\mu\text{g/ml}$  cefmenoxime. d, 0.1  $\mu\text{g/ml}$  mecillinam plus 0.1  $\mu\text{g/ml}$  cefmenoxime. e, 10  $\mu\text{g/ml}$  bulgecin plus 0.1  $\mu\text{g/ml}$  cefmenoxime. An overnight culture of *E. coli* NIHJ JC-2 in Trypticase soy broth (BBL) was inoculated into the same medium at an inoculum size of 10%. After standing for 1 hour, one loopful of the culture was inoculated on the surface of the antibiotic-containing Trypticase soy agar (BBL), which had previously been spread on a slide glass. A cover slip was placed on the top of the agar and the edges were sealed with molten paraffin. The preparation was set on a phase-contrast microscope with a temperature control unit and incubated at 37°C. Observation and photography were performed at various time intervals.



in *E. coli* or those (e.g., fosfomycin, cycloserine, vancomycin and moenomycin) that affect cell wall synthesis never acted synergistically with bulgecin in any of the test systems described above.

As far as we know, the bulge structure is generated only by  $\beta$ -lactam antibiotics. On the basis of studies on the PBP of *E. coli*, SPRATT suggested that bulges are formed by simultaneous inhibition of PBPs 2 and 3<sup>1)</sup>. This presumption was

supported by the observation that the combination of mecillinam and cephalixin gave rise to the bulge formation in *E. coli*<sup>2)</sup>. These two antibiotics had a synergistic effect on lysis. Since these observation seemed very similar to ours, we compared the process of bulge formation by mecillinam plus cefmenoxime with that by bulgecin plus cefmenoxime (Fig. 3). Cefmenoxime alone induced filamentation followed by cell lysis (c), mecillinam alone induced the formation of ovoid cell (b), and bulgecin alone had no effect (a). The morphology without antibiotics was almost identical with that shown in Fig. 3a. When mecillinam or bulgecin was added together with cefmenoxime, bulges were formed (d, e). The bulges formed by mecillinam and by bulgecin, however, were different in shape, size and rate of formation. Thus the effects of mecillinam plus and bulgecin plus cefmenoxime on the morphology of *E. coli* are similar, but the mechanisms seem different. A notable difference lay in the binding to PBPs: although mecillinam had strong affinity for PBP 2, bulgecin showed no affinity for PBPs up to 400  $\mu\text{g/ml}$  (data not shown).

The mechanism of action of  $\beta$ -lactam antibiotics has evoked great interest among those involved in antibiotic research and in basic research dealing with bacterial cell division and morphogenesis. The discovery of bulgecin, which has unique antibacterial and morphological effects as described above, offers a new approach to study the mechanism of action of  $\beta$ -lactam antibiotics.

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