COMPARISON OF TRANSPORT PATHWAYS OF CATECHOL-SUBSTITUTED CEPHALOSPORINS, BO-1236 AND BO-1341, THROUGH THE OUTER MEMBRANE OF *ESCHERICHIA COLI*

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The penetration of β-lactam antibiotics through the outer membrane is a prerequisite for exhibiting the antibacterial activity against Gram-negative bacteria. The elucidation of penetration routes has been the subject of many papers, and our understanding of the functions of a variety of outer membrane proteins has increased in recent years. It is known that major outer membrane proteins which form porin channels, OmpF and OmpC, allow small hydrophilic molecules including β-lactam antibiotics to diffuse into the periplasmic space[1]. On the other hand, catechol-substituted β-lactam antibiotics, such as BO-1236[2], BO-1341[3,4], E-0702[5], M14659[6], and pirazmonam[7] which all have potent anti-pseudomonal activity utilize a unique transport pathway, the *tonB*-dependent iron transport pathway in *Escherichia coli*[8]. The uptake of iron in *E. coli* is known to require receptor proteins and the *tonB* gene product[9] in conjunction with siderophores. Recently CURTIS *et al.*[10], and NIKAIDO and ROSENBERG[11] identified the iron-regulated outer membrane receptor proteins, Fiu and Cir, specific to the catechol-substituted cephalosporins.

In our paper, we report the diversity of the transport pathway as assessed from the characterization of spontaneous mutants of *E. coli* selected with BO-1236 and BO-1341. We also note the influence of bacterial growth under anaerobic condition and in the presence of ascorbic acid as well as iron concentration in the medium, on the antibacterial activity of these catechol-substituted cephalosporins.

BO-1236 and BO-1341, both of which were cephem antibiotics as shown in Fig. 1, were synthesized at the Okazaki Research Laboratories of Banyu Pharmaceutical Co., Ltd. (Okazaki, Japan). Ceftazidime, and cefoxitin were commercial products of Glaxo Japan Co., Ltd. (Tokyo, Japan), and Banyu Pharmaceutical Co., Ltd. (Tokyo, Japan), respectively. The strains used in this study were *E. coli* CS109, *E. coli* K-12 substrain, and *E. coli* CS197, an OmpF and OmpC deficient mutant derived from CS109[12]. The mutants resistant to BO-1236 and BO-1341 were spontaneously isolated on the antibiotic-containing agar plates. The culture media used were: Mueller-Hinton agar (Difco) for the isolation of mutants and the determination of MIC, and M9 medium[13] containing 0.2% Casamino Acids without iron chelators for the outer membrane preparation. Cells diluted to 10^6 cfu/ml were inoculated onto the antibiotic-containing agar plates for the MIC determination. The outer membranes were prepared by differential centrifugation, and treatment with sodium sarcosinate[14]. The resulting preparations were used for the analysis of outer membrane proteins with the SDS/PAGE system described by LUNDRIGAN and EARHART[15].

The spontaneous mutants resistant to BO-1236 and BO-1341 were isolated from colonies of the parent strain, CS109, grown on agar plates containing the highest concentration of BO-1236 and BO-1341 by spreading the overnight culture. Prior to selection of a mutant, approximately ten colonies randomly picked out were confirmed to have similar resistant levels against the antibiotics tested. Thus the two mutants of *E. coli* CS109, BB5936 and BB5937 were selected on the agar plates containing 16-fold-greater MIC of BO-1236 (isolation frequency: approximately 7×10^{-8}) and 64-fold greater MIC of BO-1341 (isolation frequency: approximately 4×10^{-7}), respectively. As shown in Table 1, the strain BB5936, with the 8-fold greater MIC of BO-1236 by the conventional method of

Fig. 1. Chemical structures of BO-1236 and BO-1341.

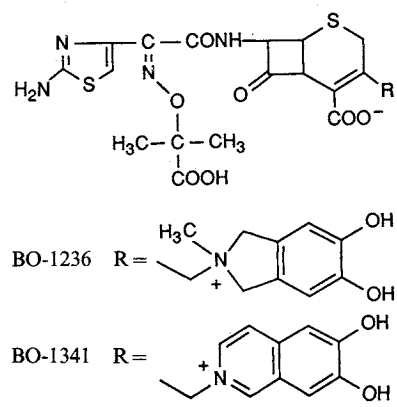


Table 1. Susceptibility of *Escherichia coli* mutants selected with BO-1236 and BO-1341.

Strain[a]	Characteristics	MIC (μg/ml)[b]			
		BO-1236	BO-1341	Ceftazidime	Cefoxitin
CS109	Wild	0.05	0.013	0.1	3.13
BB5936	*envZ* mutant	0.39	0.1	0.39	12.5
BB5937	*tonB* mutant	0.78	3.13	0.1	3.13
BB5938	*envZ tonB* double mutant	6.25	25	0.39	12.5
CS197	$OmpF^-$, $OmpC^-$	0.05	0.013	0.39	25

[a] See the text.
[b] Determined by agar dilution method with Mueller-Hinton agar (Difco).

MIC determination, exhibited relatively low resistance to ceftazidime and cefoxitin as well as BO-1236 and BO-1341, compared with the susceptibility levels of the parent strain, CS109. The strain BB5937, having the 256-fold greater MIC of BO-1341, was identified as a *tonB* mutant, as previously reported[2]. The *tonB* mutant apparently acquired the moderate resistance specific to BO-1236 and BO-1341 as indicated by the higher MICs than those for the strain BB5936. The strain BB5938, which was derived from the strain BB5937 with further exposure to 2-fold greater MIC of BO-1341 (isolation frequency: approximately 1×10^{-7}), showed much higher resistance to both BO-1236 and BO-1341 than did the *tonB* mutant, and low resistance to ceftazidime and cefoxitin. The OmpF and OmpC deficient mutant strain (CS197) showed resistance to ceftazidime and cefoxitin, but was fully susceptible to BO-1236 and BO-1341.

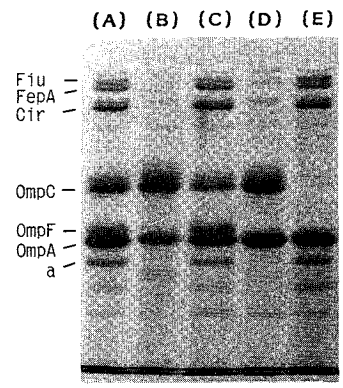
The profiles of outer membrane proteins of the mutants was shown in Fig. 2. There were no appreciable differences in the OmpF and OmpC proteins between wild-type strain, CS109, and the *tonB* mutant, BB5937, while the strain CS197 was devoid of these porin proteins. The strains of BB5936 and BB5938, having similar outer membrane profiles to each other, were deficient in OmpF protein, protein a, and the iron-regulated outer membrane proteins (Fiu, 83 kilodaltons (kDa); FepA, 81 kDa; Cir, 74 kDa), and overproduced OmpC protein. These profiles phenotypically agreed with the description of the *envZ* (*perA*) mutant reported by LUNDRIGAN and EARHART[15], and the resistance to λ phage also supported the evidence of mutation at *envZ* locus. The deficiency of the OmpF protein reduced the susceptibility to ceftazidime and cefoxitin (Table 1)[16]. Hence, the strains (BB5936 and BB5938) were assigned to be *envZ* mutant, and *envZ tonB* mutant, respectively.

In this study, we found the reduced levels of their possible receptor proteins, Fiu and Cir, as a result of *envZ* mutation (Fig. 2). However, the exclusive *fiu cir* mutant was not isolated probably due to its extremely low isolation frequency of double mutant. The *tonB* mutant (BB5937) was 8-fold and 256-fold less susceptible than the wild-type strain to BO-1236 and BO-1341, respectively. The *envZ tonB* mutant, BB5938, spontaneously derived from the *tonB* mutant, BB5937, also showed the further 8-fold increase in the resistance to both BO-1236 and BO-1341, indicating that the both compounds diffuse through the OmpF channel as well as the *tonB*-dependent iron transport pathway.

We previously reported that the *tonB* mutant was selected with BO-1341 based on analysis using relevant mutants and phages[3], so that the cephem penetrates via the *tonB*-dependent iron transport

Fig. 2. Comparison of outer membrane proteins of the strains spontaneously isolated mutants resistant to BO-1236 and BO-1341.

Strains were: (A), CS109 (wild-type); (B), BB5936; (C), BB5937; (D), BB5938 and (E), CS197 ($OmpF^-$, $OmpC^-$).



The 25 μg of each protein was run with the 8 M urea-containing SDS/PAGE described by LUNDRIGAN and EARHART[15].

Table 2. Susceptibility of *Escherichia coli* CS109 under the various conditions.

Culture condition	MIC (μg/ml)			
	BO-1236	BO-1341	Ceftazidime	Cefoxitin
Control[a]	0.05	0.013	0.1	3.13
$FeCl_3$[b]	0.39	0.2	0.1	3.13
Anaerobic[c]	0.78	6.25	0.1	3.13
Ascorbic acid[d]	0.39	0.78	0.1	3.13

[a] Determined by agar dilution method with Mueller-Hinton agar (Difco).
[b] Ferric chloride, 1 mM, was added in the medium.
[c] Cultured under anaerobic condition using GasPak Jar (BBL).
[d] Ascorbic acid, 0.1% (w/v), was added in the medium.

pathway. However, the attempt to isolate spontaneous mutants resistant to BO-1236 resulted in the *envZ* mutant. In order to confirm whether BO-1236 also utilizes the *tonB*-dependent iron transport system, the influence of bacterial growth, under the anaerobic condition and in the presence of ascorbic acid as well as ferric iron in the medium, on the antibacterial activity of BO-1236 and BO-1341 was investigated (Table 2). Although the degree of reduction in susceptibility to BO-1236 was smaller than that to BO-1341, the susceptibilities to both antibiotics were reduced under anaerobic condition and in the presence of ascorbic acid as well as ferric iron. In contrast, the susceptibilities to ceftazidime and cefoxitin were not influenced under these conditions. The result reflected the suppression of *tonB*-dependent ferric iron uptake system under these conditions[17], and BO-1236 seemed to penetrate the outer membrane through the *tonB*-dependent iron transport pathway as did BO-1341.

The *envZ* mutant selected with BO-1236 showed the reduced levels of the iron-regulated outer membrane proteins, and OmpF protein (Fig. 2) that are responsible for the resistance to catechol-substituted cephalosporins and the cephalosporins like ceftazidime and cefoxitin[15], respectively. The unaltered susceptibility of CS109 and CS197, the OmpF and OmpC deficient mutant, to BO-1236 and BO-1341 was attributed to the efficient penetration through the *tonB* pathway, even if OmpF and OmpC were not available.

The *tonB* mutant (BB5937) showed higher increase in resistance to BO-1341 than to BO-1236 (Table 1). It is likely that BO-1341 largely depends on the *tonB*-dependent iron transport pathway for the penetration route, while BO-1236 does not utilize the *tonB*-dependent iron transport pathway so much as BO-1341. Taking into consideration that the wild-type strain does not produce detectable β-lactamase, and both cephalosporins have similar affinity for PBPs 1A, 1B and 3 of *E. coli* (data not shown), it is suggested the *tonB*-dependent transport pathway is more effective than porin channels for these cephalosporins, because of the 4-fold higher activity of BO-1341 than that of BO-1236 against the strain. In contrast, BO-1236 has the merits that the susceptibility to BO-1236 was less affected by *tonB* mutation than that to BO-1341, and the frequency of emergence of spontaneous mutant resistant to BO-1236 was not only lower than that to BO-1341, but also a *tonB* mutant did not emerge.

We showed the possible development of spontaneous resistance to catechol-substituted cephalosporins in patients. The *tonB* mutant seems to be nonpathogenic due to the growth defect under iron-deficient *in vivo* environment[6,10], and we also found that the *envZ* mutant with lower resistance was even more susceptible to both cephalosporins under iron-deficient medium (data not shown). However, our results suggest that facultative anaerobic bacteria including such spontaneous mutants might survive in the infection sites where the bacteria can grow anaerobically, because the *tonB*-dependent transport pathway is mainly exploited by the catechol-substituted cephalosporins under aerobic condition.

Nevertheless, the utilization of *tonB*-dependent transport pathway besides porin channels allows the effective penetration of antibiotics to periplasm of bacteria which have low outer membrane permeability like *Pseudomonas aeruginosa*[2~4].

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References

1) NIKAIDO, H. & E. Y. ROSENBERG: Effect of solute size on diffusion rates through the transmembrane pores of the outer membrane of *E. coli*. J. Gen. Physiol. 77: 121~135, 1981
2) NAKAGAWA, S.; M. SANADA, K. MATSUDA, N. HAZUMI & N. TANAKA: Biological activity of BO-1236, a new antipseudomonal cephalosporin. Antimicrob. Agents Chemother. 31: 1100~1105, 1987
3) SANADA, M.; T. HASHIZUME, K. MATSUDA, S. NAKAGAWA & N. TANAKA: Mode of action of

BO-1341: Transport pathway through the outer membrane of *Escherichia coli*. Drugs. Exp. Clin. Res. 14: 397～402, 1988

4) NAKAGAWA, S.; M. SANADA, K. MATSUDA, T. HASHIZUME, Y. ASAHI, R. USHIJIMA, N. OHTAKE & N. TANAKA: In vitro and in vivo antibacterial activities of BO-1341, a new antipseudomonal cephalosporin. Antimicrob. Agents Chemother. 33: 1423～1427, 1989
5) WATANABE, N.; T. NAGASU, K. KATSU & K. KITOH: E-0702, a new cephalosporin, is incorporated into *Escherichia coli* cells via the *tonB*-dependent iron transport system. Antimicrob. Agents Chemother. 31: 497～504, 1987
6) MOCHIZUKI, H.; Y. OIKAWA, H. YAMADA, S. KUSAKABE, T. SHIHARA, K. MURAKAMI, K. KATO, J. ISHIGURO & H. KOSUZUME: Antibacterial and pharmacokinetic properties of M14659, a new injectable semisynthetic cephalosporin. J. Antibiotics 41: 377～391, 1988
7) BUSH, K.; S. K. TANAKA, S. OHRINGER & D. P. BONNER: Mode of action studies: Pirazmonam in *Escherichia coli* and *Pseudomonas aeruginosa*. Program and Abstracts of the 27th Intersci. Conf. on Antimicrob. Agents Chemother., No. 1218, p. 309, New York, Oct. 4～7, 1987
8) BRAUN, V.; R. E. W. HANCOCK, K. HANTKE, & A. HARTMAN: Functional organization of the outer membrane of *Escherichia coli*: Phages and colicin receptors as components of iron uptake system. J. Supramol. Struct. 5: 37～58, 1976
9) BAGG, A. & J. B. NEILANDS: Molecular mechanism of regulation of siderophore-mediated iron assimilation. Microbiol. Rev. 51: 509～518, 1987
10) CURTIS, N. A. C.; R. L. EISENSTADT, S. J. EAST, R. J. CORNFORD, L. A. WALKER & A. J. WHITE: Iron-regulated outer membrane proteins of *Escherichia coli* K-12 and mechanism of action of catechol-substituted cephalosporins. Antimicrob. Agents Chemother. 32: 1879～1886, 1988
11) NIKAIDO, H. & E. Y. ROSENBERG: Cir and Fiu proteins in the outer membrane of *Escherichia coli* catalyze transport of monomeric catechols: Study with β-lactam antibiotics containing catechol and analogous groups. J. Bacteriol. 172: 1361～1367, 1990
12) PUGSLEY, A. P. & C. A. SCHNAITMAN: Outer membrane proteins of *Escherichia coli*. VII. Evidence that bacteriophage-directed protein 2 functions as a pore. J. Bacteriol. 133: 1181～1189, 1978
13) MILLER, J. H. (*Ed.*): Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, 1972
14) SAWAI, T.; R. HIRUMA, N. KAWANA, M. KANEKO, F. TANIYASU & A. INAMI: Outer membrane permeation of β-lactam antibiotics in *Escherichia coli*, *Proteus mirabilis*, and *Enterobacter cloacae*. Antimicrob. Agents Chemother. 22: 585～592, 1982
15) LUNDRIGAN, M. & C. F. EARHART: Reduction in three iron-regulated outer membrane proteins and protein a by the *Escherichia coli* K-12 *perA* mutantion. J. Bacteriol. 146: 804～807, 1981
16) JAFFÉ, A.; Y. A. CHABBERT & E. DERLOT: Selection and characterization of β-lactam-resistant *Escherichia coli* K-12 mutants. Antimicrob. Agents Chemother. 23: 622～625, 1983
17) HANTKE, K.: Regulation of ferric iron transport in *Escherichia coli* K12: Isolation of a constitutive mutant. Mol. Gen. Genet. 182: 288～292, 1981