Correlation of Susceptibility and Resistance of Twenty-five Bacterial Strains by Analysis of MIC Database of Cephalosporins and Oxacephalosporins

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MIC database of 1,407 cephalosporins and oxacephalosporins was utilized to characterize 25 bacterial strains including Gram-positive and Gram-negative bacteria. MIC values were converted to activity rank indices, and distribution patterns of these indices were compared among the bacterial strains. *Salmonella enteritidis* No. 11 was the most susceptible bacterium, and the activity against this strain was ascribed to the binding of β-lactams to the PBPs without any significant barrier to the approach of β-lactams. Twenty-one strains were assumed to have similar types of PBPs to those of *S. enteritidis* No. 11 in the binding profile to β-lactams. Comparison of the scatter diagrams of the activity rank indices revealed two different types of resistances arising from β-lactamases (9 strains) and outer membrane permeability and/or active efflux (15 strains). The resistance factor arising from β-lactamase was affected by the nature of the C-3 substituents of β-lactams.

Minimum inhibitory concentrations (MICs) are employed nowadays as the most reliable parameter in assessing *in vitro* antibacterial activity of antibiotics. So far, MIC values have been used to characterize the antimicrobial profile of antibiotics, and no systematic attempt has been made to characterize the bacterial strains susceptible or resistant to antibiotics by the use of MIC data.

In general, the MIC values of the β-lactam antibiotics are considered to be determined mainly by the following three factors; namely binding capability of antibiotic to the penicillin binding proteins (PBPs), stability of antibiotic to β-lactamases and permeability of antibiotic through outer membrane of Gram-negative bacteria[1] and/or active efflux of antibiotic by the outer membrane components[2]. However, PBPs, β-lactamases and outer membranes of bacteria are so diverse that systematic correlation of the MIC data with these factors may be difficult as the number of bacteria are increased.

The present work was undertaken to characterize 25 bacterial strains with regards to PBP's, β-lactamases and outer membrane barriers based on the MIC analysis of 1,407 cephalosporins and oxacephalosporins. The results uncovered that many of the strains resembled in three common factors as revealed by susceptibility and resistance patterns to β-lactams. That is, 22 strains have analogous PBP's, 9 strains have common β-lactamase, and 15 strains have analogous outer membrane barrier. These results are described in this paper.

Experimental

The β-lactam antibiotics examined were 1,166 cephalosporins and 241 oxacephalosporins which were synthesized in this laboratory. About 95% cephalosporins contained aminothiazoyl group as a common C-7 substituent. The bacteria of 25 strains examined were laboratory strains kept in this laboratory including 5 Gram-positive and 20 Gram-negative bacteria. The MIC values were determined by the conventional agar dilution method using Mueller-Hinton medium (Difco) and 10^6 inoculum according to the standard method of the Japan Society of Chemotherapy.

For the analysis of MIC database, MIC values were converted to activity rank indices expressed by $[A]_i$ for a strain where i is the strain number as listed in Table 1. The [A] value is ranged from 1 to 13 corresponding to MIC value from $>100\,\mu g/ml$ to $\leq 0.025\,\mu g/ml$. Thus, higher value of [A] means that a strain was more susceptible to an antibiotic, and lower value *vice versa*.

For the computational analysis, program "QMIC" written by Microsoft Visual Basic (v.3.0) was developed on a IBM PC/AT compatible computer. Statistics, scatter diagrams and their outputs were calculated by the program.

Results and Discussion

Table 1 shows a list of 25 bacterial strains with the distribution of [A] value and the weighted average of [A] values. Among 25 strains, the most susceptible strain to the β-lactams so far examined was *Salmonella enteritidis* No. 11, in which the weighted average of [A]

Table 1. Activity distribution and averages of activity of cephalosporins and oxacephalosporins (1,407 at maximum) against 25 bacterial strains.

Strain No.	Test strain	Number of compounds to activity rank (1 to 13)													Ave. of activity ⟨A⟩	Total comps.	Correlation[a]		
		1	2	3	4	5	6	7	8	9	10	11	12	13			1	2	3
1	*Staphylococcus aureus* FDA 209P JC-1	100	65	86	117	133	178	195	171	161	111	64	17	7	(6.4)	1,405			
2	*S. aureus* Smith	103	75	99	129	152	202	193	168	130	101	42	10	3	(6.1)	1,407			
3	*Enterococcus faecalis* W-75	228	99	74	108	108	157	133	154	141	64	33	16	8	(5.5)	1,323	**		
4	*E. faecalis* W-73	862	301	69	48	29	22	9	4	2	1	1	0	0	(1.7)	1,348	**		**
5	*Bacillus subtilis* ATCC 6633	83	57	81	129	173	176	187	181	166	96	30	13	23	(6.4)	1,395			
6	*Escherichia coli* No. 29	74	46	46	45	66	93	113	139	196	180	203	145	61	(8.2)	1,407	**		**
7	*E. coli* 255	315	144	99	125	114	113	105	125	122	66	53	18	8	(5.0)	1,407	**	**	**
8	*Shigella dysenteriae shigae*	42	23	31	35	44	52	62	98	95	178	191	210	346	(9.8)	1,407	**		
9	*Klebsiella pneumoniae* PCI1602	80	51	44	52	55	95	110	119	164	170	163	168	134	(8.4)	1,405	**		**
10	*K. oxytoca* F-0100	172	71	68	95	81	91	105	61	73	56	32	20	8	(5.3)	933	**		**
11	*Proteus vulgaris* GN76/C-1	98	63	49	82	81	95	96	79	67	55	74	60	22	(6.5)	921	**		
12	*P. mirabilis* GN-310	133	53	40	60	50	72	85	107	111	121	159	170	237	(8.4)	1,398	**		
13	*Morganella morganii* 1510	320	134	121	143	152	151	158	83	50	32	26	14	11	(4.5)	1,395	**	**	**
14	*Providencia rettgeri* GN624	184	58	49	83	84	147	140	161	151	141	100	62	47	(6.8)	1,407	**		
15	*Salmonella enteritidis* No. 11	41	15	16	21	41	46	52	57	70	98	146	212	592	(10.6)	1,407	**		
16	*Citrobacter freundii* GN346	541	204	158	162	120	80	77	29	19	2	6	3	6	(3.1)	1,407	**	**	**
17	*Enterobacter cloacae* G-0005	209	75	65	74	102	129	191	184	151	105	69	36	6	(6.2)	1,396	**		**
18	*E. cloacae* GN7471	152	98	76	89	117	106	111	86	50	17	11	12	8	(4.9)	933	**	**	**
19	*Serratia marcescens* GN629	206	83	58	86	94	128	137	166	161	137	102	37	0	(6.3)	1,395	**		**
20	*S. marcescens* GN10857	250	142	119	110	85	122	49	37	12	4	3	0	0	(3.5)	933	**	**	**
21	*Pseudomonas aeruginosa* MB-3833	481	174	125	148	110	85	58	34	36	48	55	24	8	(3.8)	1,386	**	**	**
22	*P. aeruginosa* GN10362	292	118	83	95	63	69	54	41	44	55	14	4	1	(4.0)	933	**	**	**
23	*P. aeruginosa* IAM 1007	594	199	122	98	86	63	47	22	23	35	43	34	28	(3.5)	1,394	**	**	**
24	*Burkholderia cepacia* M-0527	188	83	105	187	209	187	150	35	15	10	24	38	154	(5.7)	1,385	**		
25	*Stenotrophomonas maltophilia* M-0627	855	303	66	41	47	34	33	13	5	1	3	1	3	(1.9)	1,405	**	*	**

[a] 1: Correlation of PBPs, 2: correlation of β-lactamase, 3: correlation of outer membrane barriers.
** Strong correlation, * weak correlation.

value was 10.8 corresponding to MIC = 0.1 μg/ml. The 43% β-lactams (656 compounds) showed [A] = 13 (MIC ≤0.025 μg/ml) against this strain. On the other hand, the most resistant strain was *Enterococcus faecalis* W-73, in which the weighted average was 1.7 corresponding to MIC = 100 μg/ml, and 66% β-lactams (946 compounds) showed the lowest activity [A] = 1 (MIC > 100 μg/ml).

Activity Distribution between *S. enteritidis* No. 11 and Other Strains

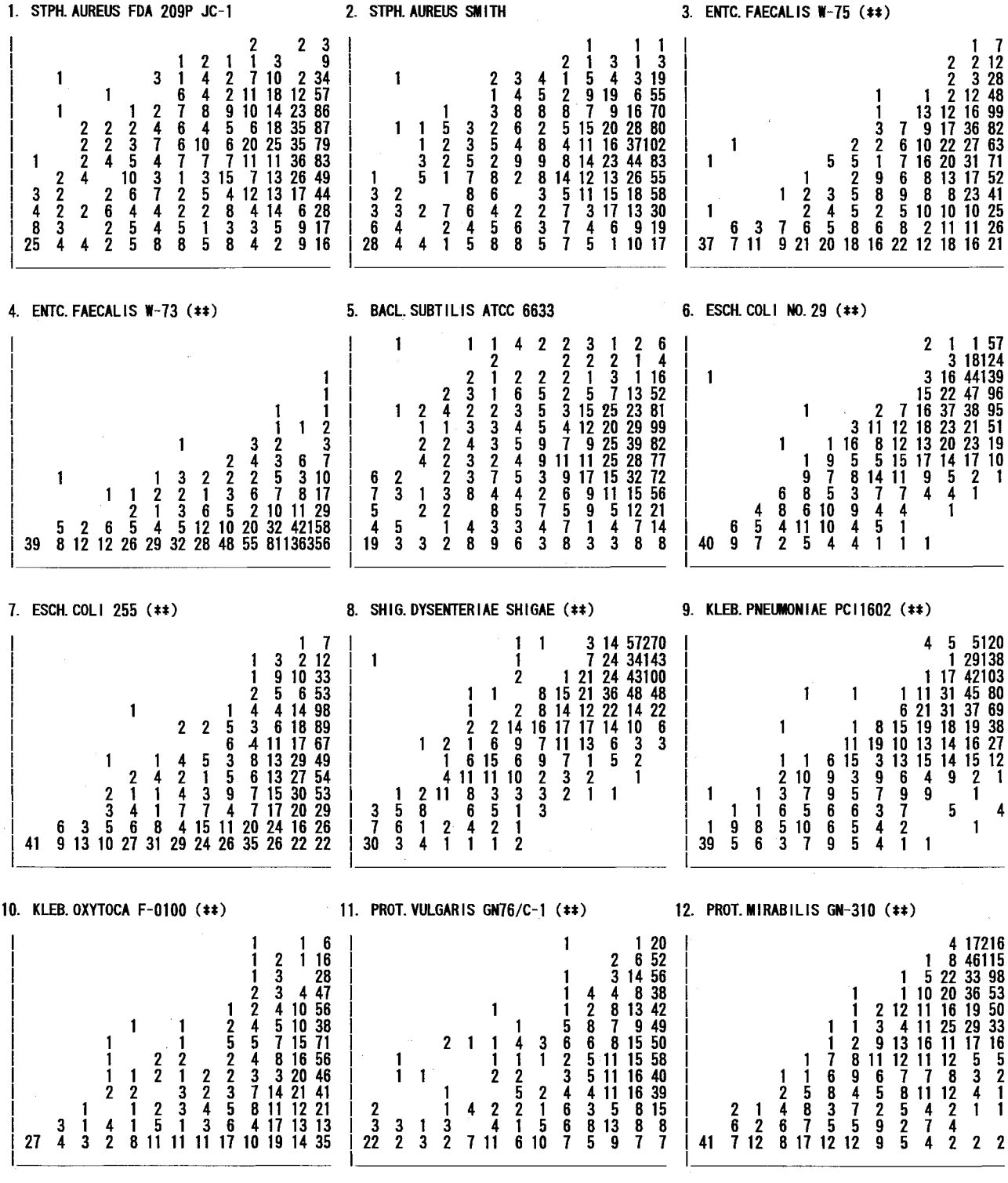
Since *S. enteritidis* No. 11 was highly susceptible to many β-lactams, an easy access of the antibiotics to the final target, PBPs was suggested with little barrier, and the MIC values may be directly related to binding strength to PBPs.

Next, we examined scatter diagrams, in which [A] values of β-lactams were distributed two dimensionally in the activity space set up with a pair of bacterial strains i and j as expressed by $[A]_i/[A]_j$. In total, 300 scatter diagrams $[A]_i/[A]_j$ were obtained from the cross combination of 25 strains. In the diagrams, we could find many similar patterns which were categorized into two types as shown in Figs. 1, 2 and 3.

Fig. 1 shows scatter diagrams between *S. enteritidis* No. 11 (strain no. 15) and all other strains $[A]_{15}/[A]_i$. When $[A]_{15}$ values were scattered against [A] of two Gram-positive *Enterococcus* strains and all Gram-negative strains, most of [A] were located one-sidedly below the diagonal line, close to *S. enteritidis* side. We named this type of distribution pattern as R type (resistant pattern) and was obtained most frequently when one partner possessed extra resistant factor out of closely related pair bacteria. If the PBPs of bacteria showed different reactivity to β-lactams, the scatter diagram of [A] should show random distribution pattern, as indicated in combination of *S. enteritidis* No. 11 with two staphylococci and one *Bacillus* sp. Another R-type pattern is shown in Fig. 2a, in which a pair bacteria were *E. coli* 255 and *E. coli* No. 29.

There was observed an another different type of distribution pattern. Fig. 3 shows $[A]_1/[A]_2$ which is activity distribution of β-lactams against *Staphylococcus*

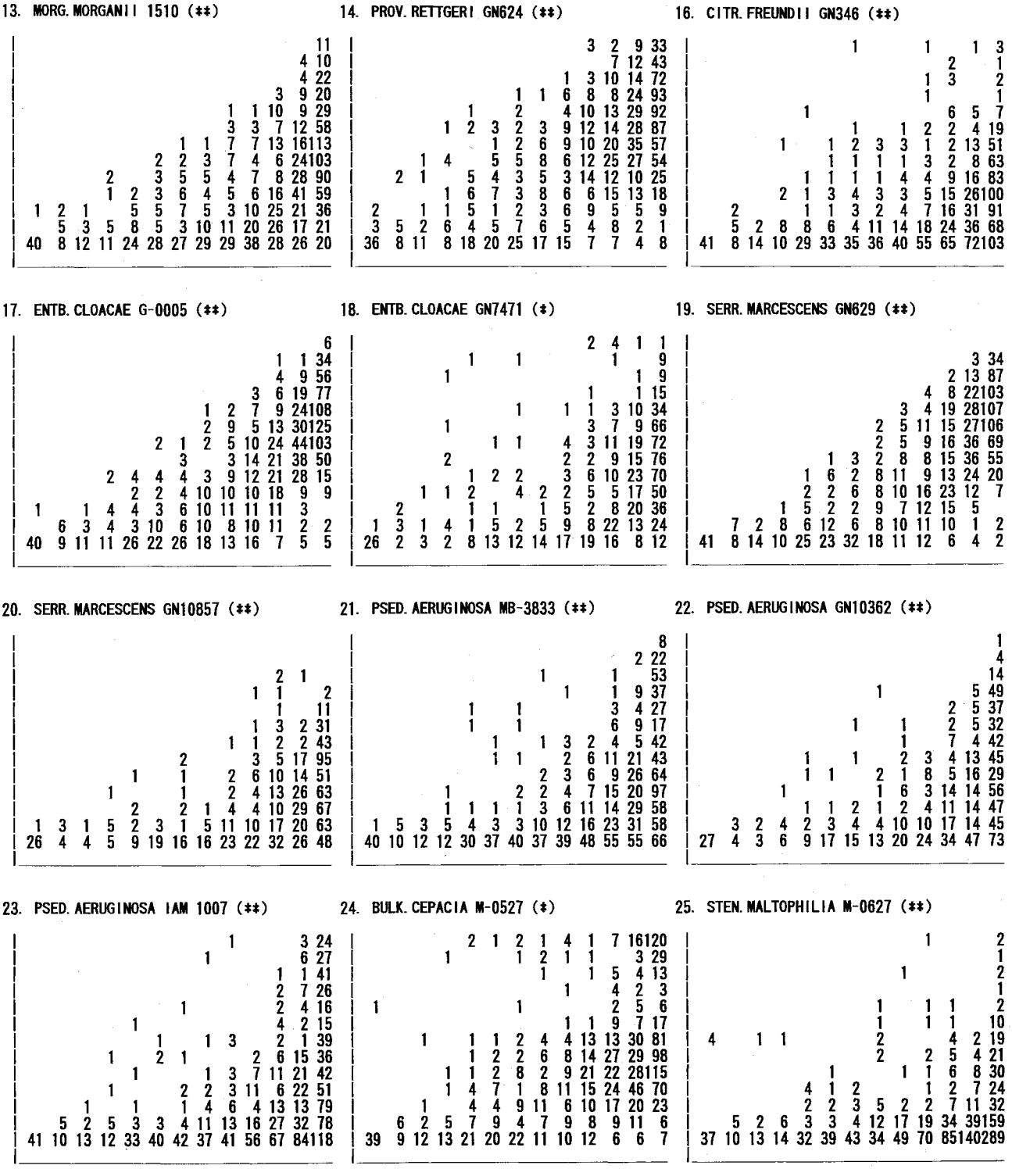
Fig. 1-1. Scatter diagrams of $[A]_{15}/[A]_i$ where transverse axis is $[A]_{15}$ of *S. enteritidis* No. 11.



** Strong correlation, * weak correlation.

aureus FDA209P JC (strain no. 1) and *S. aureus* Smith (strain no. 2) (Fig. 3a) or $[A]_1/[A]_5$ with *S. aureus* FDA209p JC and *Bacillus subtilis* ATCC6633 (strain no. 5) (Fig. 3b). In Fig. 3, almost all of β-lactams were distributed along the diagonal line in the diagram, indicating close activity response against a pair bacteria compared. The correlation coefficient of $[A]_i/[A]_j$, that is an index for similarity of susceptibility of two strains to β-lactams was R=97% in 3a and R=87% in 3b. We named this type of distribution pattern as S-type (similar pattern).

Fig. 1-2. Scatter diagrams of $[A]_{15}/[A]_i$ where transverse axis is $[A]_{15}$ of *S. enteritidis* No. 11.



** Strong correlation, * weak correlation.

It was important to recognize that a pair of two bacteria exhibiting S- or R-type distribution pattern must have at least similar final targets, which are PBPs for β-lactams. This did not always mean the morphological and structural similarity of PBPs of compaired strains, but did indicate reaction similarity to cephalosporins and oxacephalosporins. Since *S. enteritidis* No. 11 showed most frequently R- or S-type distribution with the other strains, this *Salmonella* strain was suggested to share similar PBPs with those of the compared strains

Fig. 2. An example of R-type pattern of $[A]_6/[A]_7$ (a), and scatter diagram of $[DA]_{6\text{-}7}/[A]_8$ (b).

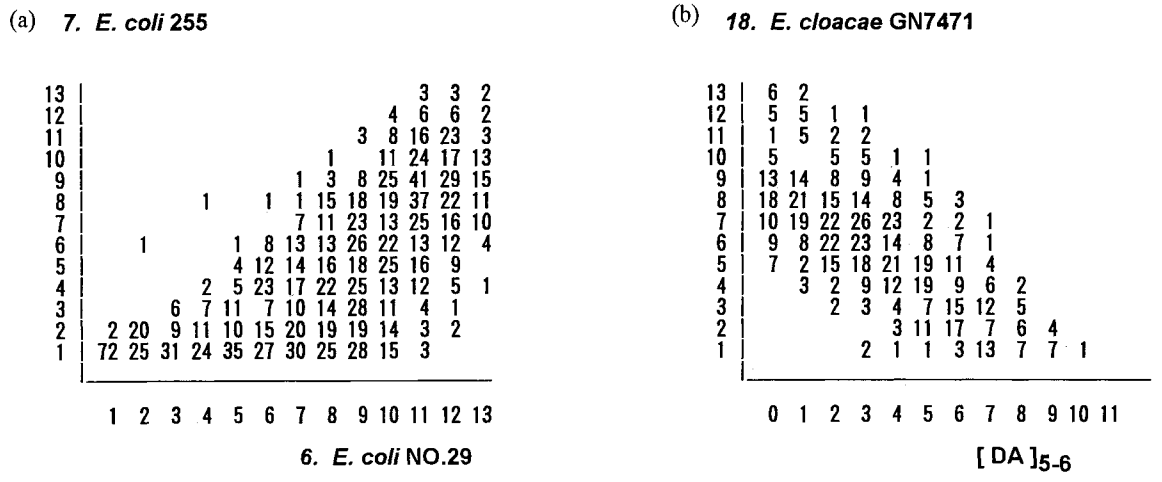
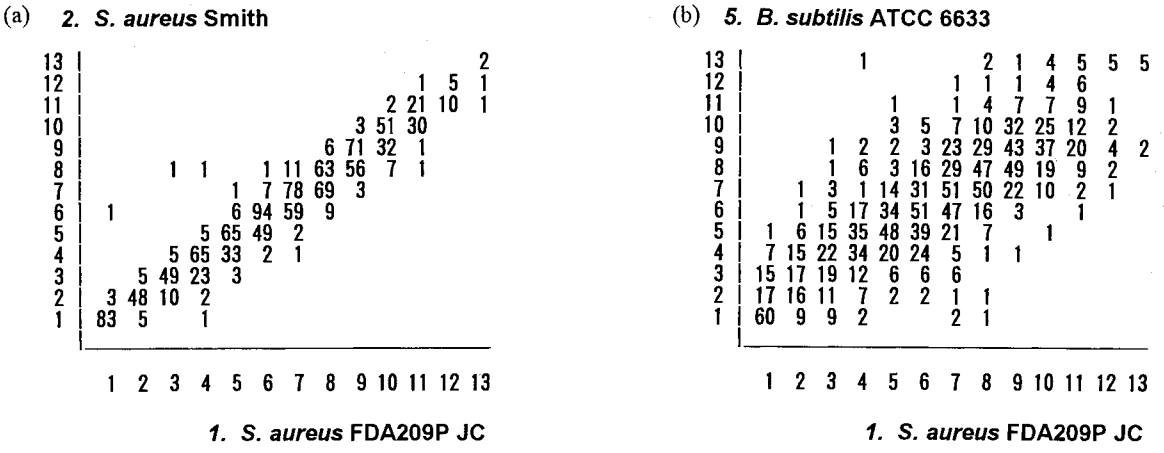


Fig. 3. Two examples of S-type patterns of $[A]_i/[A]_j$.

(a): R=97% and (b): R=87%.



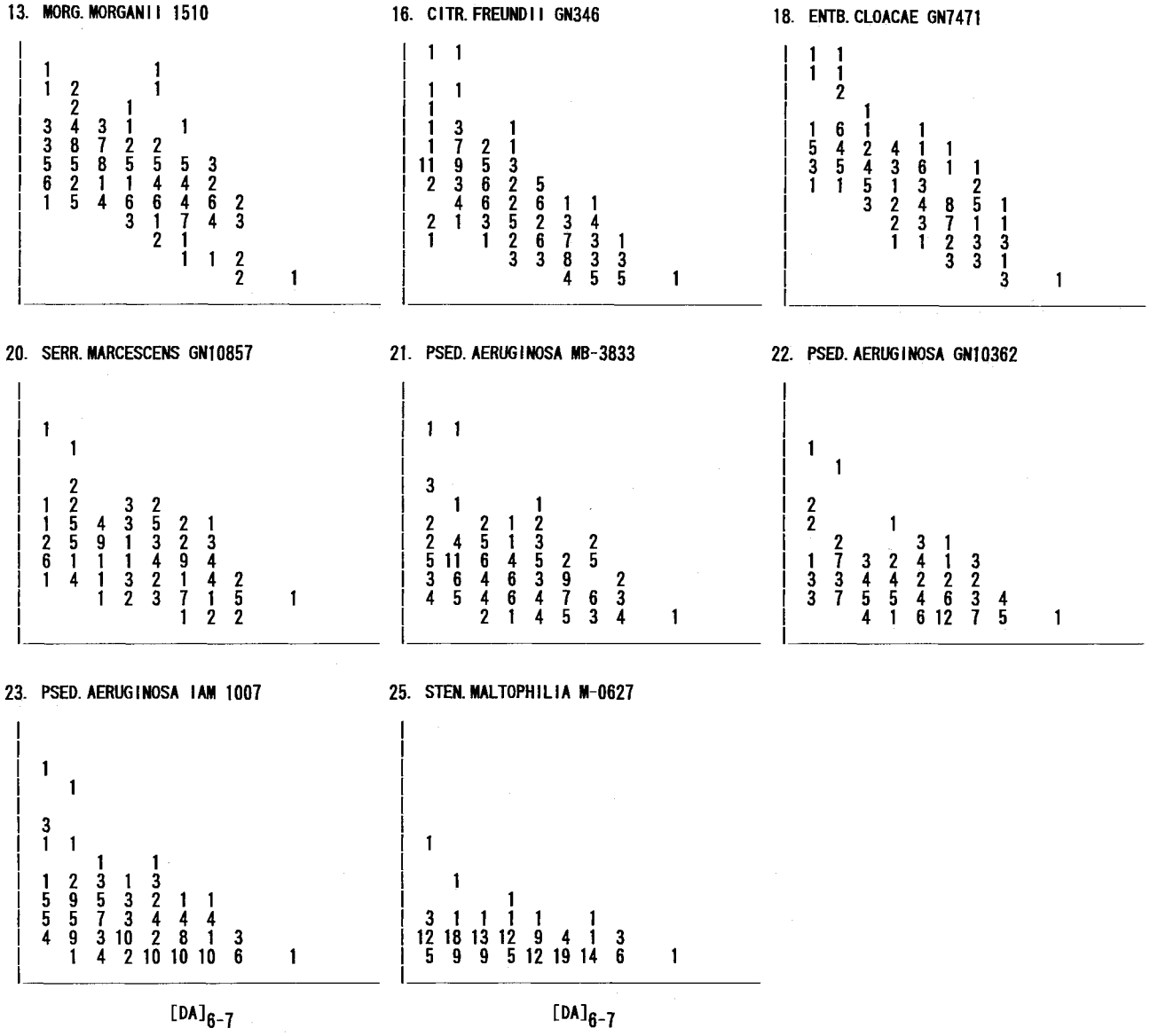
with respects to binding affinity to β-lactams examined.

Distribution of Resistance I

As mentioned before, Fig. 2a shows a scatter diagram of [A] values against *E. coli* No. 29 and *E. coli* 255, displaying a resistant pattern. The resistant factor of *E. coli* 255 was most probably β-lactamase which has been reported to be present in this strain[3]. On the contrary, the susceptible strain *E. coli* No. 29 was non-producer of β-lactamase. The scatter diagram showed downward distribution of most β-lactams towards a side of *E. coli* No. 29. In other words, the activity of β-lactam compounds against *E. coli* 255 was decreased in anti-parallel to the increasing sensitivity against β-lactamase, and the degree of shift of [A] was therefore depended on the deactivated amounts of β-lactams.

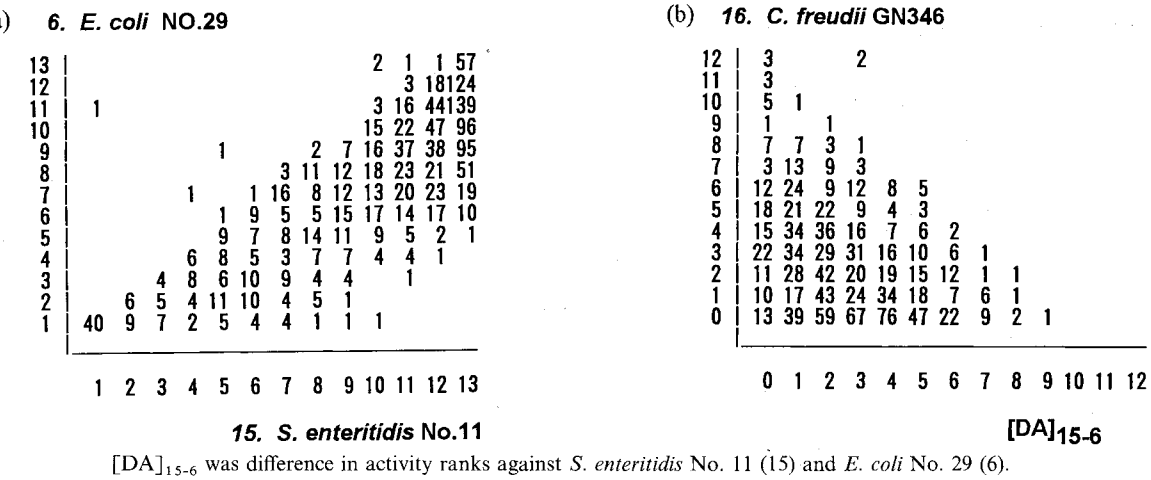
Next we introduced a new parameter of difference of activity rank indices against strain i and j as symbolized by $[DA]_{i\text{-}j}$ meaning that $[A]_i$ minus $[A]_j$. Then, $[DA]_{6\text{-}7}$ was activity difference of $[A]_6$ against *E. coli* No. 29 and $[A]_7$ against *E. coli* 255, and corresponded to the hydrolyzed amount of β-lactams by β-lactamase of *E. coli* 255. Fig. 2b shows the scatter diagram $[DA]_{6\text{-}7}/[A]_{18}$, where strain number 18 was *Enterobacter cloacae* GN7471. The characteristic feature of this diagram was that the activity of β-lactams against *E. cloacae* GN7471 was decreased with increasing $[DA]_{6\text{-}7}$. This indicated that *E. cloacae* GN7471 had a β-lactamase similar to that of *E. coli* 255 in substrate profile of β-lactams. It was further found that when the strain 18 was replaced with other strains, especially with *Pseudomonas* sp., similar scatter diagrams as that of $[DA]_{6\text{-}7}/[A]_{18}$ were

Fig. 4. Scatter diagrams of $[DA]_{6\text{-}7}/[A]_i$ where transverse axis is $[DA]_{6\text{-}7}$.



Activity data were limited to those of cephalosporins in which the C-7 substituent was fixed with methoxy-aminothiazol group. $[DA]_{6\text{-}7}$ was difference in activity ranks against *E. coli* No. 29 (6) and *E. coli* 255 (7).

Fig. 5. Scatter diagrams of $[A]_{15}/[A]_6$ (a) and $[DA]_{15\text{-}6}/[A]_{16}$ (b).

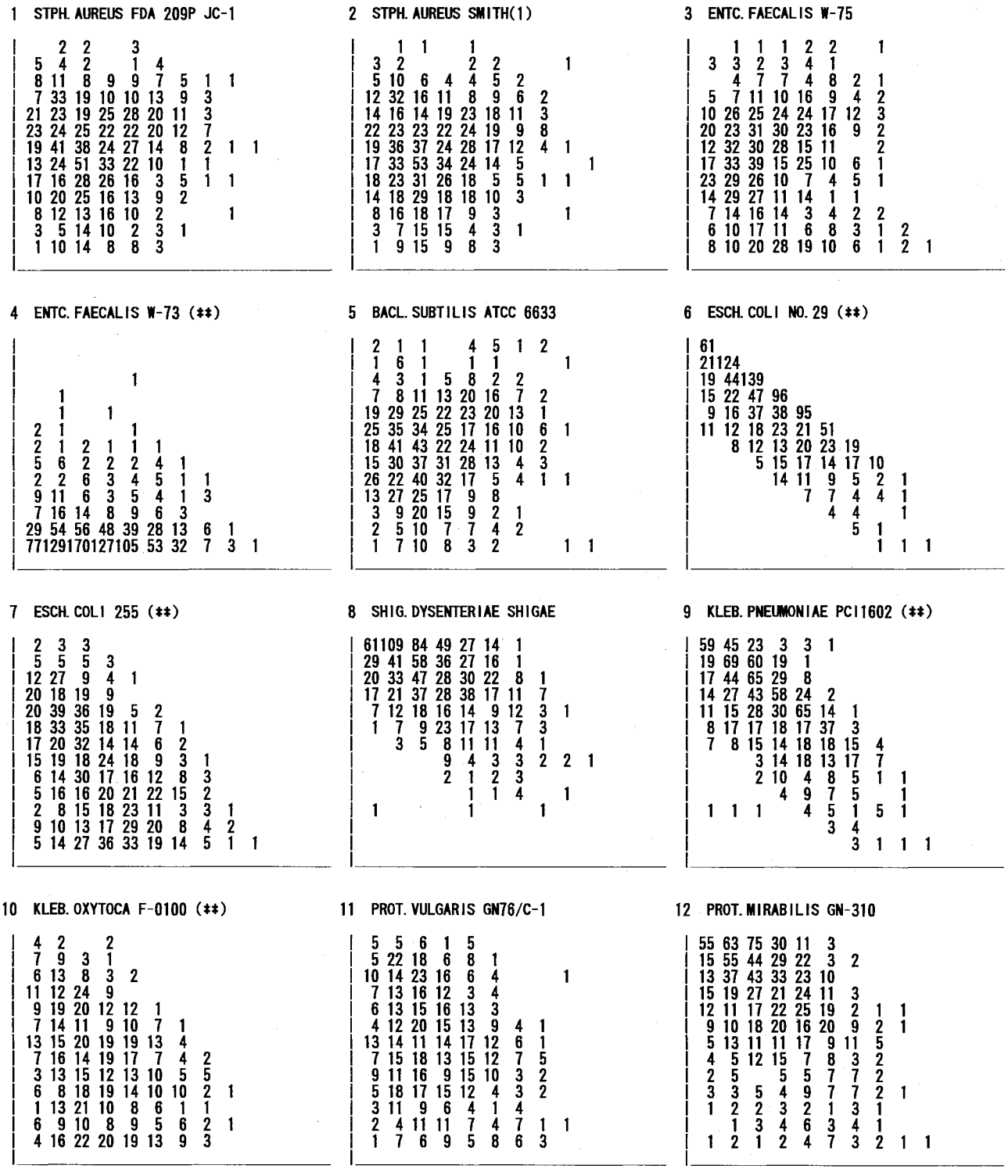


$[DA]_{15\text{-}6}$ was difference in activity ranks against *S. enteritidis* No. 11 (15) and *E. coli* No. 29 (6).

observed for other 7 strains as the β-lactams were limited to C-7 methoxy-aminothiazoyl-cephalosporins (Fig. 4). This implied that these strains had β-lactamase of similar substrate profile against cephalosporins with various C-3 substituents. Although six of 9 strains were shown to be a carrier of class-C β-lactamase by the enzymatic method[3], the presence of β-lactamase was not repoted for the remaining 3 strains, *E. cloacae* G-0005, *Pseudomonas aeruginosa* MB-3833 and *Stenotrophomonas maltophilia* M-0627.

Accordingly, when maximum [A] values against a strain at vertical axis in the scatter diagram were

Fig. 6-1. Scatter diagrams of $[DA]_{15\text{-}6}/[A]_i$, where transverse axis is $[DA]_{15\text{-}6}$.



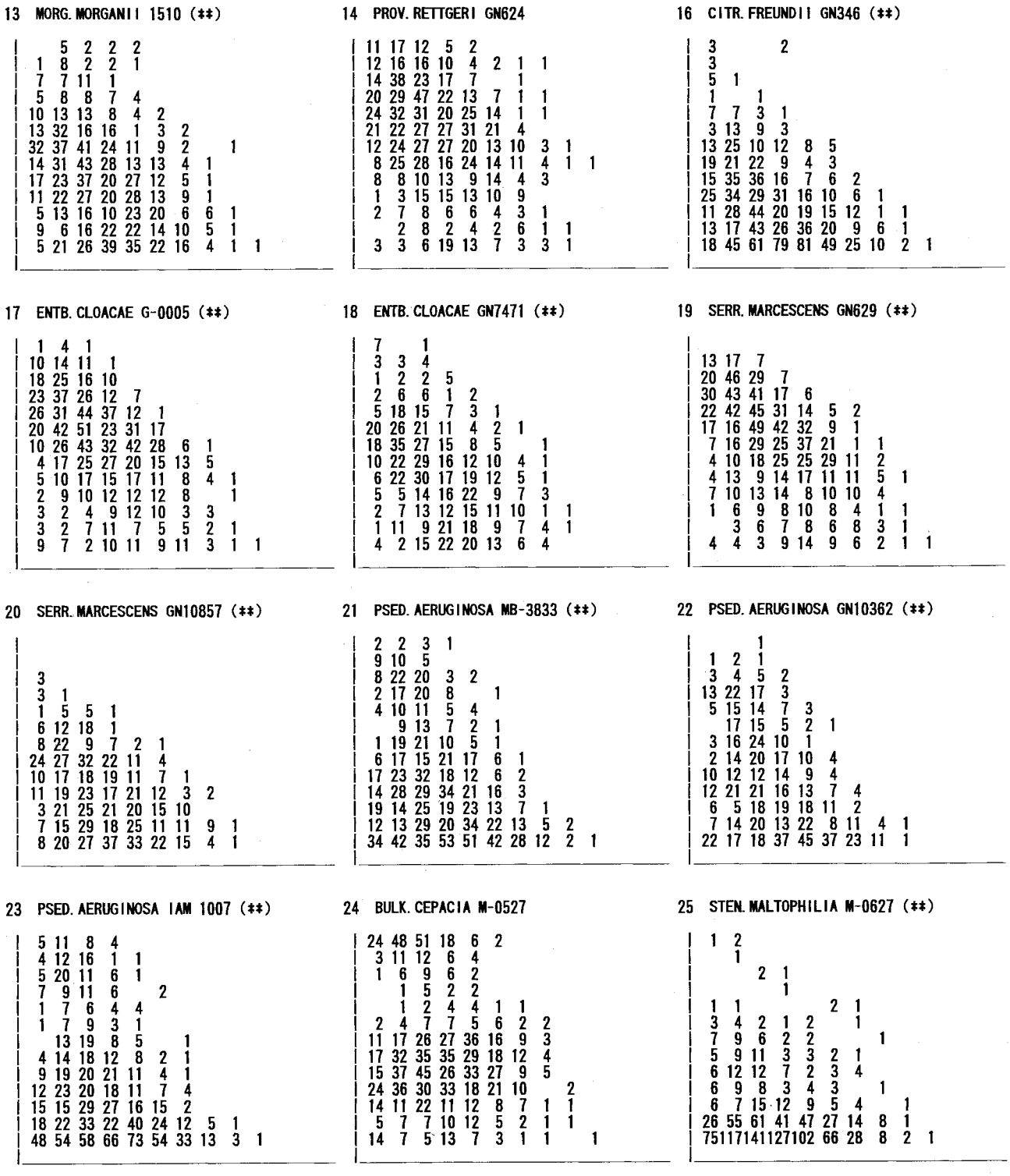
Anti-parallel correlation of $[A]_i$ and $[DA]_{15\text{-}6}$ was shown by double asterisks (strong correlation) and single asterisk (weak correlation).

gradually decreased in anti-parallel to $[DA]_{6-7}$, a strain at vertical axis can be considered to possess a resistant factor expressed by $[DA]_{6-7}$.

Distribution of resistance II

Fig. 5a shows scatter diagram of $[A]_{15}/[A]_6$, in which bacterial strains were *S. enteritidis* No. 11 (strain no. 15) and *E. coli* No. 29 (strain no. 6). This is a typical R-type pattern. In a comparable manner to that for β-lactamase, we set up an another parameter expressed by $[DA]_{15-6}$ instead of $[DA]_{6-7}$. $[DA]_{15-6}$ is activity difference between $[A]_{15}$ of *S. enteritidis* No. 11 and $[A]_6$ of *E.*

Fig. 6-2. Scatter diagrams of $[DA]_{15-6}/[A]_i$, where transverse axis is $[DA]_{15-6}$.



Anti-parallel correlation of $[A]_i$ and $[DA]_{15-6}$ was shown by double asterisks (strong correlation) and single asterisk (weak correlation).

coli No. 29. We examined all the scatter diagram using $[A]_i$ of all the strains plotted to $[DA]_{15\text{-}6}$, as shown in Fig. 6. When strain i was *Citrobacter freundii* GN346 (strain no. 16), the diagram (Fig. 5b) showed a similar distribution to that as seen in the case of β-lactamase shown in Fig. 2b. Therefore, *C. freundii* GN346 should have a resistant factor expressed by $[DA]_{15\text{-}6}$. Among the scatter diagrams examined, 15 strains exhibited R-type pattern as that for *C. freundii* GN346, and we assumed that they have a resistant factor expressed by $[DA]_{15\text{-}6}$.

Both *S. enteritidis* No. 11 and *E. coli* No. 29 are non producer of β-lactamase, and PBPs of *S. enteritidis* No. 11 were shown to be mostly exposed to β-lactams with little barrier. Therefore, $[DA]_{15\text{-}6}$ may be an another resistant factor different from β-lactamases, and most probably permeability and/or efflux factor of outer membrane. To support this, no Gram-positive bacteria that have no outer membrane did not show the R-type pattern with an exception of *Enterococcus faecalis* W-73. When $[DA]_{6\text{-}7}$ and $[DA]_{15\text{-}6}$ were plotted, no regular distribution pattern was obtained, indicating that two parameters were independent and indifferent from each other. In order to see the effect of C-3 substitution on the resistant factor of $[DA]_{15\text{-}6}$, the C-7 substituent was fixed to methoxy-aminothiazoyl group, and C-3 substituent was varied. The result is shown in Table 2, in which $[DA]_{15\text{-}6}$ of β-lactams containing neutral and cationic substituents at C-3 were plotted. The cationic substituents were correlated with lower $[DA]_{15\text{-}6}$ and neutral ones with higher $[DA]_{15\text{-}6}$. Since lower $[DA]_{15\text{-}6}$ values indicated more easy passage and/or smaller efflux of outer membrane, our data indicated that the resistant factor $[DA]_{15\text{-}6}$ could be overcome more efficiently with cationic substituents at C-3 of cephalosporins rather than neutral ones. Most of polar β-lactam antibiotics permeate outer membrane through proteineous holes, porins[4,5] which recognize polarity of passing molecules. In this respect, it was of interest that *S. enteritidis* possessed a new porin, Omp E[6], though a detailed permeability was not elucidated. Differentiation of permeability from efflux system which existed frequently in *Pseudomonas* strains[2] was difficult by this analysis.

E. faecalis W-73 which possessed analogous PBPs to those of Gram-negative bacteria, showed R-type pattern similar to that of Gram-negative bacteria when plotted with $[DA]_{15\text{-}6}$. This suggested that an unkown resistant factor different from β-lactamase and outer membrane barrier may exist for this strain.

Table 2. Effect of C-3 substituents on distribution to $[DA]_{15\text{-}6}$ in cephalosporins possessing C-7 methoxy-aminothiazol substituent.

$[DA]_{15\text{-}6}$	β-Lactams with C-3 substituent	
	Neutral	Cationic
0	2	19
1	4	31
2	12	19
3	13	15
4	29	10
5	21	5
6	13	1
7	5	0

Conclusion

The results obtained in this investigation are summarized in the last column of Table 1. First, *S. enteritidis* No. 11 was most susceptible to cephalosporins and oxacephalosporins examined, implying an easy access of β-lactams to its PBPs. Nineteen strains of Gram-negative and two Gram-positive bacteria out of 24 strains were assumed to possess PBPs similar to those of *S. enteritidis* No. 11 in binding response to β-lactams examined. Three Gram-positive bacteria showed different PBPs.

Secondly, the presence of β-lactamase was suggested for 9 strains from scatter diagrams using a resistant factor of $[DA]_{6\text{-}7}$. At least three strains have not been reported so far to have β-lactamase.

Thirdly, an another resistant factor expressed by $[DA]_{15\text{-}6}$ was detected in 15 strains, and was ascribed to the barrier of outer membrane permeability and/or active efflux except for a *E. faecalis* strain.

As shown in this paper, analysis of the MIC database from the point of view of bacteria can lead to disclosure of the common factors throughout a number of bacteria. As far as 1,407 cephalosporins and oxacephalosporins were concerned, these test strains, *S. enteritidis* No. 11, *E. coli* No. 29 and *E. coli* 255 were key strains to analyze the susceptibility and resistance of bacterial strains. For the analysis of other types of antibiotics, another set of bacteria should be selected. New drugs may be designed more efficiently by understanding the character of test strains, especially to overcome the resistant factors by using small number of test bacteria.

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References

1) SYKES, R. B. & N. H. GEORGOPAPADAKOU: Bacterial resistance to β-lactam antibiotics: An overview, *Ed.* M. R. J. SALTON & G. D. SHOCKMAN. *In* β-Lactam Anti-

biotics, pp. 199～214, Academic Press, New York, 1981
2) LI, X.-Z.; H. NIKAIDO & K. POOLE: Role of MexA-MexB-OprM in antibiotic efflux in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 1944～1953, 1995
3) SAWAI, T.; T. YOSHIDA, K. TSUKAMOTO & S. YAMAGISHI: A set of bacterial strains for evaluation of β-lactamase-stability of β-lactam antibiotics. J. Antibiotics 34: 1318～1325, 1981
4) SAWAI, T.; R. HIRUMA, N. KAWANA, M. KANEKO, F. TANIYASU & A. INAMI: Outer membrane permeability of β-lactam antibiotics in *Escherichia coli*, *Proteus mirabilis*, and *Enterobacter cloacae*. Antimicrob. Agents Chemother. 22: 585～592, 1982
5) NIKAIDO, H.; K. NIKAIDO & S. HARAYAMA: Identification and Characterization of Porins in *Pseudomonas aeruginosa*. J. Biol. Chem. 266: 770～779, 1991
6) CHART, H.; J. A. FROST & B. ROWE: Expression of a porin 'OmpE' by strains of *Salmonella enteritidis*. FEMS Microbiol. Lett. 109: 185～188, 1993