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Numerical Taxonomy of 3-chlorobenzoate Degrading Bacteria isolated From Korean Coastal Waters

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한국 연안 해역에서 분리한 3-chlorobenzoate 분해 세균에 대한 수리학적 분류

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ABSTRACT : The bacteria utilizing 3-chlorobenzoate as a sole carbon and energy source were isolated by Most-Probable-Number technique and identified in Korean coastal waters. *Pseudomonas*, *Moraxella* and *Flavobacterium* were dominant genera and comprised 70.2% of total isolates. Forty-four biochemical, cultural, morphological and physiological tests were performed and average linking cluster analysis was conducted from the test results. Total 92.7% of isolates were clustered into 17 groups under the 80% similarity level. The degradation of 3-chlorobenzoate was performed by many heterogeneous bacteria and the species diversity of these bacterial group offers informations of the stability of bacterial communities in relation to carbon compound cycling in coastal environments.

KEY WORDS □ Bacterial isolates, 3-chlorobenzoate degradation, cluster analysis, heterogeneity.

The amounts of organic pollutant from industrial, municipal and domestic sources into rivers and sea are increasing rapidly in Korea. Organic halide compounds including pesticides, herbicides and polychlorinated biphenyl(PCB)s are especially problematic relative to others because of recalcitrance and toxicity to various aquatic organisms (Leisinger *et al*, 1981). Among the organic halide compounds, organic chlorine compounds are large in amounts and very serious in degradation.

Bacteria play an important role of a self-purification process in natural ecosystem and specific bacteria are able to degrade organic chlorine compounds. Therefore, informations about the distribution of natural bacteria which are able to grow on medium containg organic chlorine compounds as a sole carbon and energy source could be used as an indicator of chlorinated organic pollution and potency of self-purification processes in natural environment.

Numerical analysis is the method which has been widely used for the analysis of saprophytic bacteria (Bölter, 1977 ; Bölter and Rheinheimer, 1987 ; Choi and Kim 1987), heavy metal-tolerant bacteria (Austin *et al*, 1977a ; Kim *et al*, 1985) etc. Although numerical method can not reveal all the characteristics of identified bacterial groups, it has advantages in understanding of the system itself on that we have special interest. Thus many works have been done with these tools (cf. Appendix in Sneath and Sokal, 1973). Numerical taxonomy is the method based on statistics and useful for the analysis of multiple characters, too.

The organic chlorine compound degrading bacteria in Korean coastal waters were isolated with 3-chlorobenzoate as a model substrate and numerical analysis was performed with the isolated strains for elucidating the structural characters of communities of organic chlorine compound-degrading bacteria.

Materials and Methods

The sampling sites in coastal waters were Kori for an eastern coast, Kunsan for western and Masan for southern in Korea. They were chronically disturbed area with temperature rising, oil pollution and industrial waste, respectively. Sampling times were from Jun. 17 to Oct. 18 in 1988 and the season was from late spring to fall (Fig. 1).

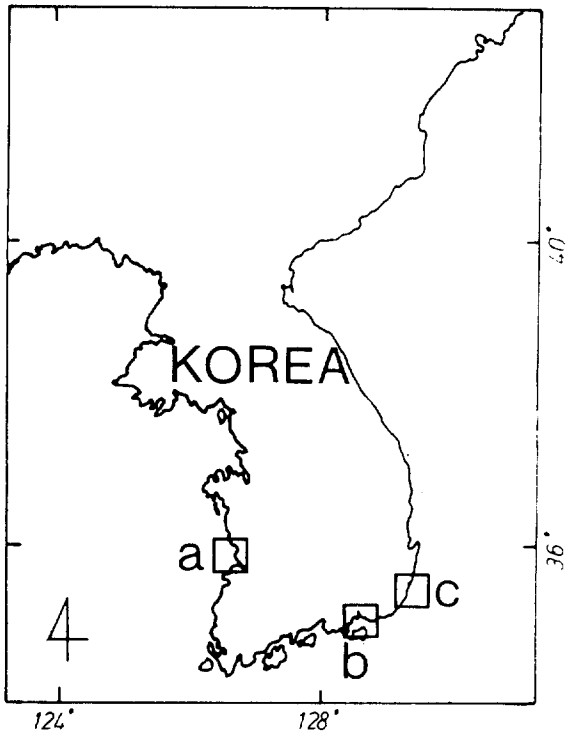

Fig. 1. *Map of Research area.* a : Kunsan, b : Masan, c : Kori.

Water samples were carried to laboratory in an ice-containg box and analyzed by following procedures. Each sample was inoculated into MPN broth medium containing 3-chlorobenzoate and resazurin as a sole carbon & energy source and a pH indicator (Table 1). The inoculated MPN media were incubated for 4 weeks at 25℃ with agitation and media occurring color-change from blue (pH 6.4 ; non-growth) into pink (below pH 3.8 ; growth) were selected for isolation. Each medium was serialy diluted with autoclaved aged-sea water. After proper dilution, 3 aliquots were inoculated onto ZoBell's 2216e agar medium by spread plate method (APHA-AWWA- WPCE, 1985) and growing colonies were isolated after 3 day incubation at 25℃. The isolated single colonies were confirmed by the degradability with inoculation into same composition of MPN broth medium and finally growing bacteria were tested 44 biochemical, cultural, morphological and physiological characteristics (Table 2). The methods and medium composition for each test were refereneced by "Manual of methods for general bacteriology" (Silvert and Krieg, 1981) and "Biochemical Tests for identification of medical bacteria"(MacFaddin, 1980).

Table 1. *Basal inorganic salt composition for MPN medium Ingradients*

$Na_2HPO_4 \cdot 12H_2O$	2.5g
K_2HPO_4	0.5g
NH_4Cl	0.5g
Aged Seawater	1L

pH 7.6

Table 2. *Tests for the characterization of bacteria*

Morphology ;	Gram Staining, Motility, Pigmentation
Growth at ;	4℃, 37℃, 45℃
	pH 5, 9, 10
	0, 1.6, 3.2, 10% NaCl
	KCl equimolar to 1.6% NaCl
Biochemical ;	Aesculine, Catalase, Citrate, Gas production,
Tests	Indole
	Methyl-Red, O/129, O/F, VP, Oxidase, TSI,
	KCN, Nitrate reduction
Degradation ;	Casein, Gelatin, Starch, Urea
Carbohydrate ;	Adonitol, Arabinose, Dulcitol, Fructose,
fermentation	Galactose,
	Inositol, Lactose, Mannose, Raffinose,
	Rhamnose, Salicin, Sucrose

The tested bacteria were identified by the scheme of Shewan *et. al* (1960) and Bergey's manual of systematic bacteriology (Krieg and Holt, 1984). Simple matching coefficients (S_{sm} ; Sneath, 1957) were calculated and unweighted average linkage cluster analysis (Sneath and Sokal, 1973) was conducted with the resulting S_{sm} values. All the numerical analyses were performed with FORTRAN program for VAX-11/780 computer system in Seoul National University.

Results

The number of isolated bacteria was 72 at Kori, 40 at Masan, 12 at Kunsan and total of 124 strains. Each bacterium was tentatively identified to genus level and 91.5% of isolated bacteria had capability

of degrading 3-chlorobenzoate as a sole carbon & energy source until final confirm-test. Among 124 identified bacteria, the dominant genera were *Pseudomonas*, *Moraxella* and *Flavobacterium* and constitute total 70.2% of all isolated strains(Table 3).

Table 3. *Identification of isolated bacteria*

Genus	Number of isolates	Percentage
Pseudomonas	39	31.5
Moraxella	36	29.0
Flavobacterium	12	9.7
Pasteurella	9	7.3
Acinetobacter	8	6.5
Acromonas	6	4.8
Aeromonas	3	2.4
Neisseria	2	1.6
Cardiobacterium	2	1.6
Corynebacterium	2	1.6
Proteus	2	1.6
Branhamella	1	0.8
Chromobacter	1	0.8
Cytophaga	1	0.8
Total	124	100

The result of identification showed that 14 different genera participate in 3-chlorobenzoate degradation reactions and might play an important role in self-purification process of environment.

The results of 44 biochemical, cultural, morphological and physiological tests were listed in Table 4. 97.6% of the tested bacteria were Gram negative and rod-shaped. Most of them were non-pigmented and motility was more active at 37℃ (22.6%) than 25℃ (6.5%) but growth was inhibited at high temperature (45℃ ; 83.1% were negative). Wide range of cultural conditions was observed for pH from 5 to 10 and salinity(NaCl & KCl results in Table 4). All bacteria were able to grow at 3.2% of NaCl concentration but thier growth was substantially inhibited in media without salt supplementation (NaCl 0%). A large group were positive in catalase and Voges-Proskauer (VP) reactions relative to other physiological tests. Most of tested bacteria were resistant to 2,4-diamino-6,7-diisoprophyl-pteridine compound (O/129 in Table 4) and none of them was gas-producer. The ability of degrading polysaccharide (starch) and carbohydrate fermentation with various substrate as a sole carbon source was substantially low with the exception of fructose, mannose and sucrose.

Table 4. *Test results for the determination of characteristics of bacteria isolated from Korean coastal waters.*

Tests	No. of ⊕ strain	%
Biochemical tests ;		
Aesculine	28	22.58
Catalase	75	60.48
Citrate	23	18.55
Gas production	0	0.00
Indole	65	52.42
Methyl Red	28	22.58
O/129	2	1.61
O/F	41	33.06
Voges-Proskauer	17	13.71
Oxidase	111	89.52
TSI	74	59.68
KCN	0	0.00
Cultural tests ;		
Growth at 37℃	28	22.58
45℃	21	16.94
pH 5	86	69.35
pH 9	123	99.19
pH 10	110	88.71
NaCl 0%	81	65.32
NaCl 1.6%	123	99.19
NaCl 3.2%	124	100.00
NaCl 10%	89	71.77
KCl 1.6%	62	50.00
Morphological tests ;		
Gram	2	1.61
Rod-shaped	121	97.58
Motility at 25℃	8	6.45
Motility at 37℃	28	22.58
Pigmentation	27	21.77
Physiological tests ;		
Casein	12	9.68
Gelatin	78	62.90
Starch	24	19.35
Urea	18	14.52
Arginine	110	88.71
Lipase	13	10.48
Adonitol	2	10.48
Arabinose	21	16.94
Dulcitol	1	0.81
Fructose	38	30.65
Galactose	11	8.87
Inositol	15	12.10
Lactose	6	4.84
Mannose	38	30.65
Raffinose	5	4.03
Rhamnose	4	3.23
Salicin	15	12.10
Sucrose	41	33.06

Cluster analysis was performed with the tested characteristics under the condition of 80% similarity- level and characters of each cluster were analyzed. 92.7% of isolated bacteria were clustered into 17 groups (Fig. 2). A cluster which contained 20 strains was the largest one and it comprised 16.1% of total isolates. Most of strains were splitted into small clusters and 60 strains (48.4% of total isolates) were pertained to 4 major clusters which contained more than 10 bacteria (Table 5). For each samplig site, strains isolated from Kunsan belonged to 3 different clusters but from other sites were belonged to most clusters. Only cluster 2 had 30% of isolates from Masan. Cluster 1 and 4 were constituted their mem-

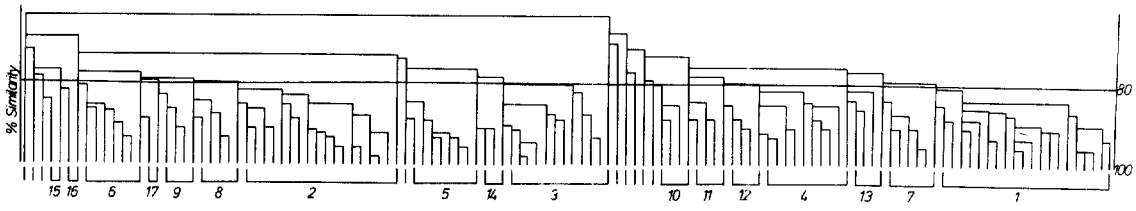

Fig. 2. *Dendrogram of 124 strains of 3-chlorobenzoate degrading bacteria.*
At the 80% similarity level, 92.7% of the isolates were clustered into 17 phenetic groups.

Table 5. *Composition of each cluster in relation to sampling sites under the 80% similarity level.*

Cluster number	Number of strain	Kori		Masan		Kunsan	
		#[1]	%[2]	#[1]	%[2]	#[1]	%[2]
1	20	13(65.0)	18.1	2(10.0)	5.0	5(25.0)	41.7
2	18	6(33.3)	8.3	12(66.7)	30.0		
3	12	3(25.0)	4.2	4(33.3)	10.0	5(41.7)	41.7
4	10	9(90.0)	12.5	1(10.0)	2.5		
5	8	2(25.0)	2.8	4(50.0)	10.0	2(50.0)	16.7
6	7	5(71.4)	6.9	2(28.6)	5.0		
7	6	5(83.3)	6.9	1(16.7)	2.5		
8	5	3(60.0)	4.2	2(40.0)	5.0		
9	4	3(75.0)	4.2	1(25.0)	2.5		
10	4	3(75.0)	4.2	1(25.0)	2.5		
11	4	2(50.0)	2.8	2(50.0)	5.0		
12	4	3(75.0)	4.2	1(25.0)	2.5		
13	4	4(100)	5.6				
14	3			3(100)	7.5		
15	2	2(100)	2.8				
16	2	1(50.0)	1.4	1(50.0)	2.5		
17	2	2(100)	2.8				
non-grouped	9	6(66.7)	8.3	3(33.3)	7.5		

1) Number of bacteria in cluster from each sampling site.
Values in parenthesis are percentage with respect to the number of bactria in each cluster.
2) Percentage of bactria with respect to the total isolates from each site.

ber mostly with isolates from Kori(65% and 90% of the member, respectively; see Table 5). Non-clustered strains were 8.3% of isolates from Kori and 7.5% from Masan.

Of the 17 clusters, those which contains more than 5 strains were selected and compositions of each genera in the clusters were calculated (Table 6). It showed that each cluster was composed with some heterogeneous bacterial genera. *Pseudomonas* was dominant group in most clusters, especially in cluster number 3 and 6. And the cluster number 7 was solely comprised with *Moraxella*.

Table 6. *Composition of tentatively identified genera in selected cluster. Values in parenthesis are percentages of each genera in cluster.*

Cluster No.	No. of strain	PSEUD	MORAX	FLAVO	PASTE	ACINE	ACROM	AEROM	CARDI	CORYN	BRANH	CHROM
1	20	7(35.0)	6(30.0)	3(15.0)		3(15.0)	1(5.0)					
2	18	4(22.2)	9(50.0)	1(5.6)			1(5.6)	1(5.6)		1(5.6)	1(5.6)	
3	12	6(50.0)	2(16.7)	1.(83)		3(25.0)						
4	10	3(30.0)	2(20.0)	1(10.0)	3(30.0)					1(10.0)		
5	8	3(37.5)	2(25.0)	1(12.5)				1(12.5)	1(12.5)			
6	7	5(71.4)		1(14.3)								1(14.3)
7	6		6(100)									
8	5	1(20.0)		2(40.0)		1(20.0)		1(20.0)				

#) PSEUD ; *Pseudomonas*, MORAX ; *Moraxella*, FLAVO ; *Flavobacterium*, PASTE ; *Pasteurella*, ACINE ; *Acinetobacter*, ACROM ; *Acromonas*, AEROM ; *Aeromonas*, CARDI ; *Cardiobacterium*, CORYN ; *Corynebacterium*, BRANH ; *Branhamella*, CHROM ; *Chromobacter*.

Discussion

The degradation for organic chlorine compounds exists in many different pathway and special interest had been pointed to the chlorinated aromatic compounds (Shamat and Maier, 1980 ; Kong and Sayler, 1983 ; Fathepure *et al*, 1988 ; Larsson *et al*, 1988) because of their toxicity (Weightman *et al*, 1985). The most common intermediate compounds of chlorinated aromatics via degradation pathway are chlorobenzoate(Furukawa *et al*, 1978 ; Masse *et al*, 1984). Various bacterial strains participate in those metabolic pathways in the aquatic habitat as well as in soil. For the isolation of 3-chlorobenzoate degrading bacteria, from the coastal waters, we have used the MPN technique which has advantages in counting and cultivation of many aquatic bacteria (DiGeronimo *et al*, 1978 ; Roubal and Atlas, 1978).

From the results of identification of the isolated bacteria (Table 3), appearance of *Pseudomonas* and *Moraxella* was remarkable. *Pseudomonas* is known as a group which has a wide capability of various compounds and it had been also reported that many *Pseudomonas* species were able to degrade chlorobenzoate componds (Hartmann *et al*, 1979). *Moraxella* is highly competent bacteria in genetic transformation(Krieg and Holt, 1984).The genetic transformation could be also important in the degradation of xenobiotics and recalcitrant compounds. Microbial degradation process is complecate one that mixed population participate in and their cometabolism is largely dependent upon specific plasmid gene (Adriaens *et al*, 1989 ; Furukawa and Chakrabarty, 1982). Thus genetic transformation between these groups might occur and it accelerates the degradation processes. The results of taxonomic diversity of the isolated bacteria suggest that the possibility of cometabolism of the organic chlorine compound in nature. It can be compared with the report of Shelton and Tiedje(1984) which isolated 7 different bacteria from methanogenic consortium able to use 3-chlorobenzoate.

The results of 44 tests gave another aspects of information on the bacterial isolates. More strains were motile at 37℃ than at 25℃ and their growth conditions had wide ranges (Table 4). Our sampling times were rather a summer season and therefore bacteria which are active at high temperature might be abundant in that environment. Bacteria which are able to degrade toxic compounds also have to be adaptive different environmental conditions. These are supported by the results of identification that 3 genera of bacteria comprised 70.2% of all isolates (Table 3).

Although high ratio of bacteria being analyzed were clustered (92%), most of clusters contained small number of bacteria. These showed that bacteria of having heterogeneous characteristics constitute total population and this well agree with above results. The different site and sampling time also explains the heterogenity of isolates. In relation with carbon flow, the taxonomic diversity of 14 genera contribute the maintainance of stable bacteial communities. From this viewpoint, coastal water near Kunsan seems to be a less stable ecosystem because of its samll diversity (see Table 5 ; Atlas, 1984).

적 요

한국 연안 해역에서 3-chlorobenzoate를 유일한 탄소원으로 이용하는 세균을 MPN 방법에 의하여 분리, 동정하였다. 분리된 전체 세균 군집중 *Pseudomonas, Moraxella, Flavobacterium*이 전체의 70.2%로 우점종을 차지하였다. 분리한 세균에 대하여 44개의 생화학적, 배양학적, 형태학적, 생리학적 특성을 조사하여 average linking cluster analysis를 수행하였다. 전체 세균중 92.7%가 80%의 유사도 수준에서 17개의 유사집단으로 나뉘어졌다. 연안 해역에서의 3-chlorobenzoate의 분해는 다양한 세균 군집에 의하여 이루어지며 이러한 종 다양성이 연안 생태계에서의 탄소 물질 순환과 연관하여 미생물 군집의 안정성에 관한 정보를 제공하여 준다.

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