表４　市販鶏肉の部位別カンピロバクターの検出状況

部位	検体数	陽性数（%）			
		Campylobacter jejuni/coli	*C. jejuni*	*C. coli*	*C. jejuni+coli*
モモ	49	31(63.3)	23(46.9)	5(10.2)	3(6.1)
ムネ	36	29(80.6)	24(66.7)	1(2.8)	4(11.1)
ササミ	12	9(75.0)	9(75.0)	0	0
その他	3	2(66.7)	2(66.7)	0	0
計	100	71(71.0)	65(65.0)	13(13.0)	7(7.0)

表５　市販鶏肉の部位別検査法別ウェルシュ菌の検出状況

部位	総検体数	陽性検体数（%）	方法	ウェルシュ菌検出内訳			
			パウチ法	－	＋	－	＋
			増菌培養	－	－	＋	＋
モモ	49	18（36.7%）		31	11	3	4
ムネ	36	15（41.7%）		21	10	2	3
ササミ	12	4（33.3%）		8	4	0	0
その他	3	0（ 0.0%）		3	0	0	0
計	100	37		63	25	5	7

表６　市販鶏肉の部位別黄色ブドウ球菌の検出状況

鶏肉部位名	検体数	黄色ブドウ球菌検査結果						
		陰性（<1/g）検体数	陽性（1≦/g）検体数，陽性率		陽性検体内訳：CFU/g			SE s 陽性検体数
					増菌培養のみ陽性	直接・増菌培養陽性		
					1～ 10^2 未満	10^2～ 10^3未満	10^3～ 10^4未満	
モモ	49	26	23	46.9%	14	7	2	7
ムネ	36	15	21	58.3%	17	3	1	10
ササミ	12	10	2	16.7%	2	0	0	1
その他	3	2	1	33.3%	1	0	0	1
小計		53	47		34	10	3	19
合計	100							

表7 食品汚染調査由来サルモネラの血清型ライブラリーモデル

血清型名	平成24年度(鶏肉調査) 食肉(鶏肉)	輸入(再掲)	検出割合(%)	総計※1 全食品群	輸入(再掲)	検出割合(%)	平成23年度～21年度 合計 食肉※2(鶏肉類)	輸入(再掲)	検出割合(%)	食肉※3(その他)	輸入(再掲)	検出割合(%)	野菜	輸入(再掲)	検出割合(%)	その他	輸入(再掲)	検出割合(%)
Salmonella O4	14	-	23.3	65	1	16.3	55	-	13.8	9	1	2.3	-	-	0.0	1	-	0.3
Typhimurium	1		1.7	13	0	3.3	11	-	2.8	2	-	0.5	-	-	0.0	-	-	0.0
Schwarzengrund	12		20.0	33	0	8.3	32	-	8.0	1	-	0.3	-	-	0.0	-	-	0.0
I 4:i:-			0.0	2	0	0.5	2	-	0.5	-	-	0.0	-	-	0.0	-	-	0.0
Schwarse			0.0	1	0	0.3	1	-	0.3	-	-	0.0	-	-	0.0	-	-	0.0
Agona	1		1.7	5	0	1.3	4	-	1.0	-	-	0.0	-	-	0.0	1	-	0.3
Derby			0.0	3	0	0.8	-	-	0.0	3	-	0.8	-	-	0.0	-	-	0.0
Not typed			0.0	8	1	2.0	5	-	1.3	3	1	0.8	-	-	0.0	-	-	0.0
Salmonella O7	32	-	53.3	239	1	59.8	231	1	57.8	8	-	2.0	-	-	0.0	-	-	0.0
Infantis	32		53.3	192	1	48.0	187	1	46.8	5	-	1.3	-	-	0.0	-	-	0.0
Thompson			0.0	1	0	0.3	1	-	0.3	-	-	0.0	-	-	0.0	-	-	0.0
Bareilly			0.0	1	0	0.3	1	-	0.3	-	-	0.0	-	-	0.0	-	-	0.0
Braenderup			0.0	2	0	0.5	2	-	0.5	-	-	0.0	-	-	0.0	-	-	0.0
Virchow			0.0	1	0	0.3	1	-	0.3	-	-	0.0	-	-	0.0	-	-	0.0
Livingstone			0.0	2	0	0.5	2	-	0.5	-	-	0.0	-	-	0.0	-	-	0.0
Not typed			0.0	40	0	10.0	37	-	9.3	3	-	0.8	-	-	0.0	-	-	0.0
Salmonella O8	3	-	5.0	44	2	11.0	38	1	9.5	6	1	1.5	-	-	0.0	-	-	0.0
Manhattan			0.0	30	1	7.5	27	1	6.8	3	-	0.8	-	-	0.0	-	-	0.0
Corvallis	1		1.7	3	0	0.8	2	-	0.5	1	-	0.3	-	-	0.0	-	-	0.0
Hadar			0.0	3	1	0.8	2	-	0.5	1	1	0.3	-	-	0.0	-	-	0.0
Kottbus			0.0	1	0	0.3	-	-	0.0	1	-	0.3	-	-	0.0	-	-	0.0
Yovokome			0.0	1	0	0.3	1	-	0.3	-	-	0.0	-	-	0.0	-	-	0.0
Duesseldorf	1		1.7	0	0	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0
Virginia	1		1.7	0	0	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0
Not typed			0.0	6	0	1.5	6	-	1.5	-	-	0.0	-	-	0.0	-	-	0.0
Salmonella O9	-	-	0.0	5	0	1.3	5	-	1.3	-	-	0.0	-	-	0.0	-	-	0.0
Enteritidis			0.0	5	0	1.3	5	-	1.3	-	-	0.0	-	-	0.0	-	-	0.0
Not typed			0.0	0	0	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0
Salmonella O6,8	9	-	15.0	4	0	1.0	4	-	1.0	-	-	0.0	-	-	0.0	-	-	0.0
Manhattan	9		15.0	3	0	0.8	3	-	0.8	-	-	0.0	-	-	0.0	-	-	0.0
Muenchen			0.0	1	0	0.3	1	-	0.3	-	-	0.0	-	-	0.0	-	-	0.0
Salmonella O3,10		-	0.0	2	0	0.5	-	-	0.0	2	-	0.5	-	-	0.0	-	-	0.0
Orion			0.0	1	0	0.3	-	-	0.0	1	-	0.3	-	-	0.0	-	-	0.0
Not typed			0.0	1	0	0.3	-	-	0.0	1	-	0.3	-	-	0.0	-	-	0.0
Salmonella O11		-	0.0	1	0	0.3	-	-	0.0	-	-	0.0	1	-	0.3	-	-	0.0
Aberdeen			0.0	1	0	0.3	-	-	0.0	-	-	0.0	1	-	0.3	-	-	0.0
Salmonella O16		-	0.0	1	0	0.3	-	-	0.0	1	-	0.3	-	-	0.0	-	-	0.0
Hvittingfoss			0.0	1	0	0.3	-	-	0.0	1	-	0.3	-	-	0.0	-	-	0.0
Salmonella O18	1	-	1.7	0	0	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0
Cerro	1		1.7	0	0	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0
Salmonella O8,20		-	0.0	1	0	0.3	1	-	0.3	-	-	0.0	-	-	0.0	-	-	0.0
Yovokome			0.0	1	0	0.3	1	-	0.3	-	-	0.0	-	-	0.0	-	-	0.0
Salmonella O35		-	0.0	2	0	0.5	2	-	0.5	-	-	0.0	-	-	0.0	-	-	0.0
Alachua			0.0	2	0	0.5	2	-	0.5	-	-	0.0	-	-	0.0	-	-	0.0
Salmonella O不明	1		1.7	36	0	9.0	31	-	7.8	4	-	1.0	1	-	0.3	-	-	0.0
計	60	-	100.0	400	4	100.0	367	2	91.8	30	2	7.5	2	-	0.5	1	-	0.3

血清型名	平成23年度 食肉(鶏肉類)	輸入(再掲)	検出割合(%)	食肉(その他)	輸入(再掲)	検出割合(%)	野菜	輸入(再掲)	検出割合(%)	その他	輸入(再掲)	検出割合(%)
Salmonella O4	14	-	12.6	4	-	40.0	-	-	0.0	-	-	
Typhimurium	1		0.9	1		10.0			0.0			
Schwarzengrund	11		9.9			0.0			0.0			
I 4:i:-			0.0			0.0			0.0			
Schwarse	1		0.9			0.0			0.0			
Agona			0.0			0.0			0.0			
Derby			0.0	1		10.0			0.0			
Not typed	1		0.9	2		20.0			0.0			
Salmonella O7	63	-	56.8	2	-	20.0	-	-	0.0	-	-	
Infantis	45		40.5	2		20.0			0.0			
Thompson			0.0			0.0			0.0			
Bareilly	1		0.9			0.0			0.0			
Braenderup			0.0			0.0			0.0			
Virchow	1		0.9			0.0			0.0			
Livingstone			0.0			0.0			0.0			
Not typed	16		14.4			0.0			0.0			
Salmonella O8	15	-	13.5	1	-	10.0	-	-	0.0	-	-	
Manhattan	12		10.8	1		10.0			0.0			
Corvallis			0.0			0.0			0.0			
Hadar			0.0			0.0			0.0			
Kottbus			0.0			0.0			0.0			
Yovokome			0.0			0.0			0.0			
Duesseldorf			0.0			0.0			0.0			
Virginia			0.0			0.0			0.0			
Not typed	3		2.7			0.0			0.0			
Salmonella O9	-	-	0.0	-	-	0.0	-	-	0.0	-	-	
Enteritidis			0.0			0.0			0.0			
Not typed			0.0			0.0			0.0			
Salmonella O6,8	1	-	0.9	-	-	0.0	-	-	0.0	-	-	
Manhattan	1		0.9			0.0			0.0			
Muenchen			0.0			0.0			0.0			
Salmonella O3,10	-	-	0.0	-	-	0.0	-	-	0.0	-	-	
Orion			0.0			0.0			0.0			
Not typed			0.0			0.0			0.0			
Salmonella O11	-	-	0.0	-	-	0.0	-	-	0.0	-	-	
Aberdeen			0.0			0.0			0.0			
Salmonella O16	-	-	0.0	-	-	0.0	-	-	0.0	-	-	
Hvittingfoss			0.0			0.0			0.0			
Salmonella O18	-	-	0.0	-	-	0.0	-	-	0.0	-	-	
Cerro			0.0			0.0			0.0			
Salmonella O8,20	-	-	0.0	-	-	0.0	-	-	0.0	-	-	
Yovokome			0.0			0.0			0.0			
Salmonella O35	-	-	0.0	-	-	0.0	-	-	0.0	-	-	
Alachua			0.0			0.0			0.0			
Salmonella O不明	18		16.2	3		30.0	1		100.0			
計	111	-	100.0	10	-	100.0	1	-	100.0	-	-	

血清型名	平成22年度 食肉(鶏肉類)	輸入(再掲)	検出割合(%)	食肉(その他)	輸入(再掲)	検出割合(%)	野菜	輸入(再掲)	検出割合(%)	その他	輸入(再掲)	検出割合(%)
Salmonella O4	18	-	13.1	1	-	12.5	-	-	0.0	1	-	100.0
Typhimurium	4		2.9			0.0			0.0			0.0
Schwarzengrund	10		7.3	1		12.5			0.0			0.0
I 4:i:-	2		1.5			0.0			0.0			0.0
Schwarse			0.0			0.0			0.0			0.0
Agona	2		1.5			0.0			0.0	1		100.0
Derby			0.0			0.0			0.0			0.0
Not typed			0.0			0.0			0.0			0.0
Salmonella O7	93	1	67.9	4	-	50.0	-	-	0.0	-	-	0.0
Infantis	80	1	58.4	3		37.5			0.0			0.0
Thompson	1		0.7			0.0			0.0			0.0
Bareilly			0.0			0.0			0.0			0.0
Braenderup	2		1.5			0.0			0.0			0.0
Virchow			0.0			0.0			0.0			0.0
Livingstone	2		1.5			0.0			0.0			0.0
Not typed	8		5.8	1		12.5			0.0			0.0
Salmonella O8	15	1	10.9	3	-	37.5	-	-	0.0	-	-	0.0
Manhattan	9	1	6.6	2		25.0			0.0			0.0
Corvallis	2		1.5			0.0			0.0			0.0
Hadar			0.0			0.0			0.0			0.0
Kottbus			0.0	1		12.5			0.0			0.0
Yovokome	1		0.7			0.0			0.0			0.0
Duesseldorf			0.0			0.0			0.0			0.0
Virginia			0.0			0.0			0.0			0.0
Not typed	3		2.2			0.0			0.0			0.0
Salmonella O9	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0
Enteritidis			0.0			0.0			0.0			0.0
Not typed			0.0			0.0			0.0			0.0
Salmonella O6,8	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0
Manhattan			0.0			0.0			0.0			0.0
Muenchen			0.0			0.0			0.0			0.0
Salmonella O3,10	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0
Orion			0.0			0.0			0.0			0.0
Not typed			0.0			0.0			0.0			0.0
Salmonella O11	-	-	0.0	-	-	0.0	1	-	100.0	-	-	0.0
Aberdeen			0.0			0.0	1		100.0			0.0
Salmonella O16	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0
Hvittingfoss			0.0			0.0			0.0			0.0
Salmonella O18	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0
Cerro			0.0			0.0			0.0			0.0
Salmonella O8,20	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0
Yovokome			0.0			0.0			0.0			0.0
Salmonella O35	2	-	1.5	-	-	0.0	-	-	0.0	-	-	0.0
Alachua	2		1.5			0.0			0.0			0.0
Salmonella O不明	9		6.6			0.0			0.0			0.0
計	137	2	100.0	8	-	100.0	1	-	100.0	1	-	100.0

血清型名	平成21年度 食肉(鶏肉類)	輸入(再掲)	検出割合(%)	食肉(その他)	輸入(再掲)	検出割合(%)	野菜	輸入(再掲)	検出割合(%)	その他	輸入(再掲)	検出割合(%)
Salmonella O4	23	-	19.3	4	1	33.3	-	-		-	-	
Typhimurium	6		5.0	1		8.3						
Schwarzengrund	11		9.2			0.0						
I 4:i:-			0.0			0.0						
Schwarse			0.0			0.0						
Agona	2		1.7			0.0						
Derby			0.0	2		16.7						
Not typed	4		3.4	1	1	8.3						
Salmonella O7	75	-	63.0	2	-	16.7	-	-		-	-	
Infantis	62		52.1			0.0						
Thompson			0.0			0.0						
Bareilly			0.0			0.0						
Braenderup			0.0			0.0						
Virchow			0.0			0.0						
Livingstone			0.0			0.0						
Not typed	13		10.9	2		16.7						
Salmonella O8	8	-	6.7	2	1	16.7	-	-		-	-	
Manhattan	6		5.0			0.0						
Corvallis			0.0	1		8.3						
Hadar	2		1.7	1	1	8.3						
Kottbus			0.0			0.0						
Yovokome			0.0			0.0						
Duesseldorf			0.0			0.0						
Virginia			0.0			0.0						
Not typed			0.0			0.0						
Salmonella O9	5	-	4.2	-	-	0.0	-	-		-	-	
Enteritidis	5		4.2			0.0						
Not typed			0.0			0.0						
Salmonella O6,8	3	-	2.5	-	-	0.0	-	-		-	-	
Manhattan	2		1.7			0.0						
Muenchen	1		0.8			0.0						
Salmonella O3,10	-	-	0.0	2	-	16.7	-	-		-	-	
Orion			0.0	1		8.3						
Not typed			0.0	1		8.3						
Salmonella O11	-	-	0.0	-	-	0.0	-	-		-	-	
Aberdeen			0.0			0.0						
Salmonella O16	-	-	0.0	1	-	8.3	-	-		-	-	
Hvittingfoss			0.0	1		8.3						
Salmonella O18	-	-	0.0	-	-	0.0	-	-		-	-	
Cerro			0.0			0.0						
Salmonella O8,20	1	-	0.8	-	-	0.0	-	-		-	-	
Yovokome	1		0.8			0.0						
Salmonella O35	-	-	0.0	-	-	0.0	-	-		-	-	
Alachua			0.0			0.0						
Salmonella O不明	4		3.4	1		8.3						
計	119	-	100.0	12	2	100.0	-	-		-	-	

※1「総計」は平成23年度から21年度の各食品群の総和を表す。
※2「食品(鶏肉類)」は「鶏肉」または「鶏肉を含む食肉加工品」を表す。
※3「食品(その他)」は「鶏肉以外の食肉」または「鶏肉を含まない食肉加工品」を表す。

III. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル	発表誌名	巻号	ページ	出版年
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Rapid and Effective DNA Extraction Method with Bead Grinding for a Large Amount of Fungal DNA

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ABSTRACT

To identify a rapid method for extracting a large amount of DNA from fungi associated with food hygiene, extraction methods were compared using fungal pellets formed rapidly in liquid media. Combinations of physical and chemical methods or commercial kits were evaluated with 3 species of yeast, 10 species of ascomycetous molds, and 4 species of zygomycetous molds. Bead grinding was the physical method, followed by chemical methods involving sodium dodecyl sulfate (SDS), cetyl trimethyl ammonium bromide (CTAB), and benzyl chloride and two commercial kits. Quantity was calculated by UV absorbance at 260 nm, quality was determined by the ratio of UV absorbance at 260 and 280 nm, and gene amplifications and electrophoresis profiles of whole genomes were analyzed. Bead grinding with the SDS method was the most effective for DNA extraction for yeasts and ascomycetous molds, and bead grinding with the CTAB method was most effective with zygomycetous molds. For both groups of molds, bead grinding with the CTAB method was the best approach for DNA extraction. Because this combination also is relatively effective for yeasts, it can be used to extract a large amount of DNA from a wide range of fungi. The DNA extraction methods are useful for developing gene indexes to identify fungi with molecular techniques, such as DNA fingerprinting.

Fungi play an important role in food spoilage, although their use in food fermentation is well established. A large number of incidents of food spoilage by fungi *(24)*, contamination of food by toxigenic fungi *(1, 17, 18)*, and food poisoning associated with mycotoxins have been reported *(1)*. Therefore, easy and rapid methods to identify fungal species are required. Although morphological observation is the dominant method of identifying fungi, various culture methods are used. The subjective judgment of specific characteristics also complicates identification. Recently, molecular biology techniques have attracted interest because of their ease, rapidity, and objectivity. PCR-based assays and analyses of DNA sequences are used extensively to identify fungi in food or to detect toxin genes in fungi. Some researchers have reported that molecular methods can be used to identify strains that are difficult to identify morphologically *(16, 22)*.

Genetic experiments require nucleic acids, including genomic DNA extracted from cells. It is difficult to extract DNA from fungal cells, especially molds, because fungi contain many nucleases, polysaccharides, and pigments. Therefore, many methods of DNA extraction have been developed for fungi *(3, 5, 9, 26, 27, 29, 33)* and compared for quantity and quality *(2, 6, 8, 11, 15, 20)*. These methods have been used with many species of yeasts but only a few species of molds *(2, 5, 6, 8, 11, 20, 33)* or have been used with several species of only medically important fungi *(7, 21)*. To develop new tools for identifying fungal species, it is also necessary to search for genes or nucleotide sequences specific to species or groups and to understand the structure of the genome by conducting experiments that include sequencing of the whole genome, DNA-DNA hybridization, and DNA fingerprinting. Because these experiments require a large amount of DNA, rapid and effective DNA extraction methods are required. Other researchers have extracted DNA from cell suspensions or excised mycelial pieces from colonies on agar media *(2, 5, 6, 11, 20, 27)*. Under these conditions, significant time and effort with many agar plates are required to obtain a large amount of DNA because of the limited number of cells. Earlier and more efficient harvesting can be accomplished by inoculating a culture into liquid medium and collecting hyphae rather than spores as mold samples for DNA extraction. Guo et al. *(10)* compared two extraction methods and reported that those methods generated high DNA yields with freeze-dried mycelia of 25 species of molds; however, the DNA yields of only four species were greater than 1,000 μg/g. There is, therefore, a need for more rapid and effective extraction methods to obtain large amounts of DNA from a wide range of species, including various molds that commonly occur in foods.

The present study was conducted to determine whether the extraction methods could yield a high quantity of high-quality DNA from fungal cells by culturing in liquid medium. Species for DNA extraction were selected from a wide range of fungi based on their importance in the field of food hygiene.

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TABLE 1. *Fungal strains*

Fungus type	Division	Species	Strain[a]
Yeasts		*Cryptococcus neoformans*	NBRC 0699
		Rhodotorula rubra	NBRC 0909
		Saccharomyces cerevisiae	NBRC 0233
Molds	Ascomycota	*Alternaria alternata*	NBRC 31188
		Aspergillus niger	NBRC 6661
		Botrytis cinerea	NBRC 31831
		Byssochlamys fulva	NBRC 31877
		Emericella nidulans	NBRC 33017
		Fusarium subglutinans	IFM 50097
		Geotrichum candidum	NBRC 9541
		Penicillium expansum	NBRC 7604
		Talaromyces bacillisporus	NBRC 31150
		Trichoderma viride	NBRC 100100
	Zygomycota	*Absidia corymbifera*	NBRC 4009
		Mucor hiemalis	NBRC 9405
		Rhizopus stolonifer	NBRC 4781
		Syncephalastrum racemosum	NBRC 4828

[a] NBRC, National Institute of Technology and Evaluation Biological Resource Center; IFM, Institute of Food Microbiology (currently the Medical Mycology Research Center, Chiba University).

MATERIALS AND METHODS

Strains. The strains tested in this study are listed in Table 1: three yeasts (*Cryptococcus neoformans, Rhodotorula rubra,* and *Saccharomyces cerevisiae*), 10 ascomycetous molds (*Alternaria alternata, Aspergillus niger, Botrytis cinerea, Byssochlamys fulva, Emericella nidulans, Fusarium subglutinans, Geotrichum candidum, Penicillium expansum, Talaromyces bacillisporus,* and *Trichoderma viride*), and four zygomycetous molds (*Absidia corymbifera, Mucor hiemalis, Rhizopus stolonifer,* and *Syncephalastrum racemosum*). Strains were provided by the National Institute of Technology and Evaluation Biological Resource Center (NBRC; Chiba, Japan) and the Medical Mycology Research Center (Chiba University, Chiba, Japan). These species occur frequently in the field of food hygiene *(28)* and cover a wide range of taxonomic groups based on both morphological and molecular data *(12, 13)*.

Sample preparation. All strains were cultured on potato dextrose agar (Eiken, Tokyo, Japan) slants at 25°C for 7 days. Mycelia or conidia from the slant culture were inoculated into a 1-liter Erlenmeyer flask containing 400 ml of potato dextrose broth (Difco, Becton Dickinson, Sparks, MD). Fungi were grown at 25°C and shaken at 100 rpm. After 70 h, the cells in the planktonic cell suspension of *S. cerevisiae, C. neoformans, R. rubra,* or *G. candidum* were clumped by centrifugation at 6,000 × *g* for 10 min, and mycelial pellets of *A. alternata, A. niger, B. cinerea, E. nidulans, F. subglutinans, P. expansum, T. bacillisporus, T. viride, A. corymbifera, M. hiemalis, R. stolonifer,* or *S. racemosum* were wrapped in filter paper and squeezed to remove as much liquid as possible. Cells of both the planktonic cell suspension and mycelial pellets from *B. fulva* were pelleted using each method described above as samples. Each fungal pellet was divided into 100-mg samples, placed in 10 microtubes, and stored at −80°C until used.

DNA extraction. Combinations of physical and chemical DNA extraction methods or commercial kits were evaluated. Bead grinding was used as the physical method because it is frequently used in DNA extraction techniques for fungal spores *(2, 5, 6, 11, 20)*. Bead grinding was performed using the Multi-Beads Shocker (Yasui Kikai Co., Osaka, Japan) at 2,500 rpm for 1 min at 0°C with 0.5-mm zirconia beads. This procedure was followed by each of three chemical methods of cell lysis, the sodium dodecyl sulfate (SDS) method *(29)*, the cetyl trimethyl ammonium bromide (CTAB) method *(26)*, and the benzyl chloride method *(33)*, or two widely used commercial kits *(2, 14, 23, 25, 31)*: Dr. GenTLE (from Yeast) High Recovery (Takara Bio Inc., Otsu, Japan) and DNeasy Plant Kit (QIAGEN, Hilden, Germany).

The SDS method followed that described by Tapia-Tussell et al. *(29)* with minor modifications. SDS is an anionic surfactant. A 800-μl aliquot of lysing buffer was added to a tube containing 100 mg of the fungal pellet sample, and cells were resuspended by vortexing. After incubation at 65°C for 30 min, lysates were purified by mixing with phenol-chloroform. The suspensions were then centrifuged. The upper aqueous phase was transferred to a new tube, 15 μl of RNase A (10 mg/ml; Novagen, Darmstadt, Germany) was added, and tubes were incubated at 37°C. After 3 h, samples were purified by mixing with chloroform and then centrifuged. The upper aqueous phase contained the DNA, which was precipitated with chilled isopropanol, rinsed with 500 μl of 70% ethanol, dried under a vacuum, and resuspended in 100 μl of Tris-EDTA buffer.

The CTAB method followed that described by Saghai-Maroof et al. *(26)* with minor modifications. CTAB is a cationic surfactant. A 800-μl aliquot of CTAB buffer was added to a tube containing 100 mg of the fungal pellet sample and vortexed. Cell lysis, RNA digestion, and DNA purification were completed in the same way as for the SDS method.

The benzyl chloride method followed that described by Zhu et al *(33)*. Benzyl chloride can destroy the cell walls of fungi by reacting with polysaccharides and (similar to phenol) can extract proteins and other cell debris from the aqueous phase. A 500-μl aliquot of extraction buffer, 100 μl of 10% SDS, and 300 μl of benzyl chloride were added to a tube containing 100 mg of the fungal pellet sample and vortexed. The tube was incubated at 50°C for 30 min with shaking to mix thoroughly, 300 μl of 3 M NaOAc (pH 5.0) was added, and the tube was kept on ice for 15 min. After centrifugation, the upper aqueous phase was transferred to a new tube, 15 μl of RNase A was added, and the tube was incubated at 37°C for 3 h. DNA purification with chloroform and precipitation

with isopropanol were completed in the same way as for the SDS method.

The GenTLE kit was used according to the manufacturer's instructions with minor modifications. After salt precipitation of DNA, additional steps were performed. The precipitations were dissolved in 800 μl of distilled water, 15 μl of RNase A was added, and mixtures were incubated at 37°C for 3 h. DNA purification with chloroform and further precipitation with isopropanol were completed in the same way as for the SDS method.

The DNeasy Plant kit was used according to the manufacturer's instructions. All DNA extractions were performed in triplicate.

Quantity and quality of extracted DNA. The extracted DNA was evaluated spectrophotometrically to check quantity and quality using the NanoDrop 1000 Spectrophotometer V3.7 (Thermo Fisher Scientific, Wilmington, DE). Quantity was calculated based on the absorbance at 260 nm (A_{260}). Quality was assessed by the ratio of UV absorbance at 260 and 280 nm (A_{260}/A_{280}). A ratio of A_{260}/A_{280} greater than 1.8 is generally considered an acceptable indicator of DNA purity. To test amplification ability and the absence of PCR inhibition in all DNA extracts in this study, a PCR assay was carried out with TaKaRa Ex *Taq* (Takara Bio Inc.). For DNA from yeast cells and ascomycetous molds, forward primer FF1 (5′-GTT AAA AAG CTC GTA GTT GAA C-3′) and reverse primer FR1 (5′-CTC TCA ATC TGT CAA TCC TTA TT-3′) were used *(32)*. For DNA from zygomycetous molds, forward primer P1 (5′-ATC TGG TTG ATC CTG CCA-3′) and reverse primer Fun-R1 (5′-TTG TTA CGA CTT TTA CTT CCT CT-3′) were used *(30)*. These primer pairs were designed to amplify specific fragments (of 630 and 1,730 bp, respectively) in two regions of the 18S rRNA gene, which is one of the most widely used genes in systematics studies based on molecular biological techniques. Amplification reactions were performed in a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA). PCR products were electrophoresed with PCR markers (Novagen, Madison, WI) as the molecular maker using a 1.5% (wt/vol) agarose gel (Agarose L03 Takara, Takara Bio Inc.) in Tris-borate-EDTA buffer. To check the amount of intact and degraded DNA in each extract, all extracts were electrophoresed with lamda DNA digested with *Hin*dIII marker (COSMO BIO Co., Ltd., Tokyo, Japan) as the molecular maker using a 1.0% (wt/vol) agarose gel. DNA fragments and PCR products were visualized by staining with ethidium bromide and UV transillumination.

Statistics. Significance tests among proportions of the number of species were performed by multiple comparisons with Fisher's exact test. These comparisons were considered significant with P values controlled at an error rate ($\alpha = 0.05$) with Bonferroni correction. Pairwise comparisons between mean quantities of DNA were performed with the Mann-Whitney U test.

RESULTS

Quantity of DNA extracted from fungi. The physical extraction method was combined with each of the three chemical extraction methods and the two commercial kits for a total of 10 combinations. DNA extracted from pellets of fungal cells using each of the 10 combinations were converted to the quantity of DNA per 1 g of fungal cells. The mean quantity of DNA was determined (with the standard deviation) for triplicate samples (Table 2). The mean amount of DNA extracted from ascomycetous and zygomycetous molds ranged from 11.5 to 5,430.7 μg/g of pellet. This finding supported the possibility of extracting DNA from all mycelial pellets cultured in liquid medium in this study. The mean DNA yield for all species in this study with the GenTLE kit or DNeasy Plant kit tended to be lower than that obtained using the three chemical methods: SDS, CTAB, and benzyl chloride.

The mean amount of DNA recovered using each combination in each taxonomic group of fungi was calculated and compared. For yeasts (Table 2), DNA yields from *S. cerevisiae* with combinations that included the SDS or CTAB method with or without bead grinding were 2,078.1 to 2,883.9 μg/g, although DNA yields for *R. rubra* and *C. neoformans* with the same combinations were 98.4 to 860.4 μg/g. DNA yields for three yeasts with the combination of bead grinding and benzyl chloride were 831.7 to 1,481.0 μg/g, whereas DNA yields with benzyl chloride method were 195.2 to 453.2 μg/g. For ascomycetous molds (Table 2), DNA yields for all species obtained with the combination of bead grinding and the SDS method were 1,660.8 to 5,430.7 μg/g, higher than DNA yields obtained with the other nine combinations (12.5 to 3,695.8 μg/g). For zygomycetous molds (Table 2), DNA yields for all species, excluding *R. stolonifer,* with combinations of the three chemical extraction methods with or without bead grinding were 1,174.3 to 4,934.0 μg/g. DNA yields for *R. stolonifer* with these combinations were only 138.3 to 589.3 μg/g.

Five extraction methods were compared without bead grinding to clarify the best method of DNA extraction applicable to a wide range of species in each taxonomic group. With 1,000 μg/g defined as a large amount of DNA, the number of species from which more than 1,000 μg/g was extracted was calculated for each method. Significance tests on the results were not performed for yeasts and zygomycetous molds separately because numbers for individual species were not sufficient to give appropriate test results. The five methods were thus compared based on their ability to extract a large amount of DNA from a wide range of mold species, including Ascomycota and Zygomycota. Of the 14 mold species tested, 10 (71.4%) yielded more than 1,000 μg/g with the SDS method, 9 (64.3%) yielded this amount with the CTAB method, 8 (57.1%) yielded this amount with the benzyl chloride method, 1 (7.1%) yielded this amount with the GenTLE kit, and none yielded this amount with the DNeasy Plant kit. Multiple comparisons of these results with the Bonferroni correction provided the following result: SDS, CTAB, and benzyl chloride methods were significantly better than both the GenTLE and DNeasy Plant kits ($P < 0.01$). Results were not significantly different for the SDS, CTAB, and benzyl chloride methods.

The physical extraction method of bead grinding also was evaluated to determine whether it improved DNA extraction. Three chemical extraction methods (SDS, CTAB, and benzyl chloride methods) were used because they recovered more than 1,000 μg/g from significantly more species. Pairwise comparisons between mean quantities of DNA obtained with each of the three chemical

TABLE 2. *Yields of fungal DNA extracted by various methods*

Fungus type	Division	Species	Mean ± SD DNA extracted (μg/g of fungal cells)									
			SDS method		CTAB method		Benzyl chloride method		Dr. GenTLE (for Yeasts) High Recovery kit		DNeasy Plant kit	
			Bead grinding	No grinding	Bead grinding	No grinding	Bead grinding	No grinding	Bead grinding	No grinding	Bead grinding	No grinding
Yeasts		*Cryptococcus neoformans*	860.4 ± 358.9	412.0 ± 213.5	641.4 ± 298.3	175.2 ± 30.7	837.1 ± 247.6	195.9 ± 47.2	422.2 ± 324.7	1,34.5 ± 147.6	108.0 ± 11.5	38.0 ± 26.1
		Rhodotorula rubra	285.9 ± 89.3	98.4 ± 72.3	423.8 ± 32.8	206.7 ± 58.7	1,481.0 ± 809.0	453.2 ± 318.0	366.9 ± 360.1	406.0 ± 209.5	299.7 ± 41.3	22.1 ± 11.8
		Saccharomyces cerevisiae	2,602.7 ± 263.6	2,078.1 ± 286.0	2,883.9 ± 686.6	2,743.0 ± 381.2	1,220.7 ± 462.3	195.2 ± 41.7	810.2 ± 413.3	1,803.5 ± 366.2	318.8 ± 84.1	4.6 ± 1.4
Molds	Ascomycota	*Alternaria alternata*	2,780.7 ± 156.2	1,467.4 ± 313.0	2,554.4 ± 1,248.6	1,798.4 ± 279.5	2,100.6 ± 131.8	1,678.1 ± 595.4	809.6 ± 763.0	349.1 ± 116.4	199.8 ± 56.7	86.6 ± 18.0
		Aspergillus niger	2,572.4 ± 1,494.8	769.4 ± 459.7	1,843.1 ± 1,221.5	266.6 ± 139.1	1,927.8 ± 468.2	381.4 ± 89.0	1,664.0 ± 508.5	196.9 ± 124.4	164.0 ± 118.0	30.8 ± 21.7
		Botrytis cinerea	3,944.8 ± 1,210.9	2,742.0 ± 441.0	2,203.1 ± 606.6	1,347.4 ± 596.4	3,695.8 ± 1,198.0	2,340.2 ± 853.5	304.6 ± 34.0	280.0 ± 89.3	266.9 ± 168.5	192.2 ± 85.9
		Byssochlamys fulva	2,847.3 ± 621.4	2,119.2 ± 431.9	2,083.9 ± 586.4	1,730.1 ± 668.3	1,839.7 ± 347.4	990.4 ± 402.8	749.4 ± 35.2	438.1 ± 38.2	288.0 ± 68.7	133.4 ± 49.7
		Emericella nidulans	1,660.8 ± 203.2	2,340.4 ± 108.9	1,559.2 ± 298.8	1,935.4 ± 721.7	677.3 ± 26.3	2,355.0 ± 192.0	627.7 ± 77.4	135.2 ± 79.1	65.7 ± 22.1	105.4 ± 40.7
		Fusarium subglutinans	2,226.8 ± 100.6	773.5 ± 121.0	1,193.4 ± 460.7	711.1 ± 371.9	1,171.5 ± 401.4	267.2 ± 163.3	82.0 ± 20.7	531.5 ± 40.2	162.3 ± 38.3	106.6 ± 47.2
		Geotrichum candidum	1,660.8 ± 110.9	482.7 ± 134.6	752.5 ± 23.3	424.9 ± 117.9	745.1 ± 289.1	188.5 ± 16.3	593.8 ± 278.9	930.0 ± 60.9	37.9 ± 7.8	12.5 ± 6.3
		Penicillium expansum	2,909.0 ± 621.6	1,356.6 ± 331.8	850.9 ± 431.2	502.7 ± 169.9	1,664.0 ± 932.7	903.2 ± 386.1	472.3 ± 224.9	543.7 ± 210.2	206.0 ± 123.3	140.0 ± 93.3
		Talaromyces bacillisporus	3,426.1 ± 221.3	2,406.1 ± 485.1	3,273.9 ± 663.1	1,539.1 ± 68.1	3,451.6 ± 315.9	1,745.2 ± 586.0	730.2 ± 64.3	240.0 ± 40.5	367.4 ± 80.5	23.1 ± 9.7
		Trichoderma viride	5,430.7 ± 202.9	3,022.3 ± 541.2	2,637.6 ± 1,227.4	1,050.7 ± 496.6	3,432.9 ± 1,039.8	2,230.4 ± 650.7	1,851.1 ± 191.9	1,116.5 ± 316.1	1,768.7 ± 702.2	562.4 ± 328.7
	Zygomycota	*Absidia corymbifera*	4,065.9 ± 1,437.4	2,569.6 ± 1,141.4	3,252.2 ± 310.9	3,556.0 ± 596.9	2,048.2 ± 420.6	1,292.0 ± 601.2	1,461.6 ± 262.0	951.8 ± 61.6	128.4 ± 21.6	44.1 ± 9.5
		Mucor hiemalis	2,832.9 ± 360.1	1,562.7 ± 102.5	3,379.6 ± 256.0	2,757.6 ± 211.2	2,883.2 ± 231.7	1,174.3 ± 184.0	170.0 ± 44.1	158.4 ± 23.4	274.1 ± 63.3	42.4 ± 10.1
		Rhizopus stolonifer	248.9 ± 13.6	433.3 ± 260.5	589.3 ± 107.6	445.8 ± 103.6	281.2 ± 39.1	138.3 ± 54.8	73.3 ± 24.9	119.9 ± 18.1	26.3 ± 16.2	11.5 ± 5.5
		Syncephalastrum racemosum	4,943.0 ± 272.4	4,397.6 ± 373.7	4,493.6 ± 325.0	3,560.8 ± 498.5	3,690.5 ± 311.4	1,819.3 ± 770.8	1,190.0 ± 496.4	598.0 ± 256.8	101.3 ± 4.7	29.5 ± 13.0

TABLE 3. *Statistical comparison of DNA yields with or without bead grinding for three chemical extraction methods*

Fungus type	Division	Species	DNA yield comparison[a]		
			SDS method	CTAB method	Benzyl chloride method
Yeasts		*Cryptococcus neoformans*	−	>	>
		Rhodotorula rubra	−	>	>
		Saccharomyces cerevisiae	−	−	>
Molds	Ascomycota	*Alternaria alternata*	>	−	−
		Aspergillus niger	−	>	>
		Botrytis cinerea	−	−	−
		Byssochlamys fulva	−	−	>
		Emericella nidulans	<	−	<
		Fusarium subglutinans	>	−	>
		Geotrichum candidum	>	>	>
		Penicillium expansum	>	−	−
		Talaromyces bacillisporus	>	>	−
		Trichoderma viride	>	>	>
	Zygomycota	*Absidia corymbifera*	−	−	−
		Mucor hiemalis	>	>	>
		Rhizopus stolonifer	−	>	>
		Syncephalastrum racemosum	−	>	>
No. of species yielding significantly more DNA					
With bead grinding			7	9	11
Without bead grinding			1	0	1

[a] −, yield of DNA with bead grinding was not significantly different from that obtained without grinding; >, yield of DNA with bead grinding was significantly higher than that obtained without grinding ($P < 0.05$); <, yield of DNA with bead grinding was significantly lower than that obtained without grinding ($P < 0.05$).

extraction methods with or without bead grinding indicated that mean quantities of DNA with bead grinding were higher overall than those obtained without bead grinding (Table 3). The mean quantity of DNA was significantly higher with bead grinding than without bead grinding for 7 (41.2%) of the 17 species with the SDS method, 9 species (52.9%) with the CTAB method, and 11 species (64.7%) with the benzyl chloride method. In contrast, the mean quantity of DNA was significantly lower with bead grinding than without bead grinding for 1 (5.9%) of the 17 species, *E. nidulans,* with both the SDS method and the benzyl chloride method. This result suggests that bead grinding has an interactive effect with chemical extraction methods for most fungal species.

Quality of extracted fungal DNA. Of the three chemical extraction methods and two commercial kits, only the SDS, CTAB, and benzyl chloride methods (not the GenTLE and DNeasy Plant kits) were evaluated for DNA quality because these methods recovered more than 1,000 μg/g from significantly more species. The quality of DNA extracted was determined in six combinations of bead grinding and the three chemical extraction methods. The A_{260}/A_{280} ratios and the mean A_{260}/A_{280} ratio of three trials for each species and each of the six combinations were calculated. A mean A_{260}/A_{280} ratio lower than 1.8 was obtained for 1 (5.6%) of 18 combinations in yeasts (*R. rubra* with the SDS method), 5 (8.3%) of 60 combinations in ascomycetous molds (*A. niger, B. fulva, F. subglutinans,* and *G. candidum* with the benzyl chloride method and *B. fulva* with the benzyl chloride method with bead grinding), and 1 (4.2%) of 24 combinations in zygomycetous molds (*S. racemosum* with the SDS method). Multiple comparisons among these percentages indicated no significant differences; thus, there was no difference among the three taxonomic groups in terms of the extraction of low-purity DNA.

DNA extracted with the SDS, CTAB, benzyl chloride methods with or without bead grinding was compared by electrophoresis in agarose gel. *S. cerevisiae, A. alternata,* and *M. hiemalis* were selected as representatives of yeast, ascomycetous molds, and zygomycetous molds, respectively (Fig. 1A). The DNA extracted by the SDS, CTAB, or benzyl chloride method from all species in this study contained a large amount of intact DNA, especially when these methods were used in combination with bead grinding. The electrophoresis profiles of the DNA extracted with the DNeasy Plant kit were similar to those for the DNA obtained with the SDS, CTAB, and benzyl chloride methods. In contrast, electrophoresis profiles of DNA extracted with the GenTLE kit revealed degraded DNA (data not shown). The DNA extracted in this study also was evaluated to determine its appropriateness for use as a PCR template, because PCR assay are used extensively for

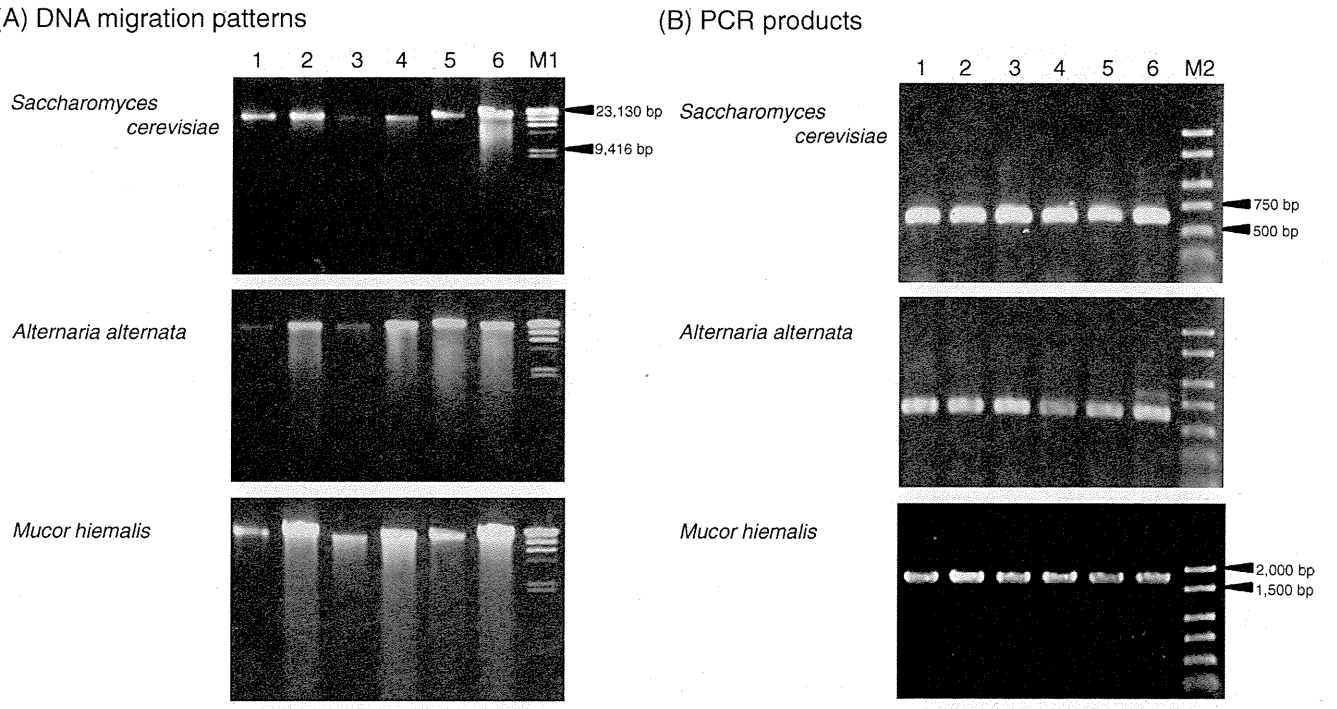

FIGURE 1. *DNA migration patterns of extracted DNA from fungi (A) and the PCR products of the 18S rRNA gene (B). Lanes 1 and 2, SDS method without or with bead grinding, respectively; lanes 3 and 4, CTAB method without or with bead grinding, respectively; lanes 5 and 6, benzyl chloride method without or with bead grinding, respectively.*

molecular biological experiments. PCR products whose template DNA was extracted by the SDS, CTAB, and benzyl chloride methods with or without bead grinding from *S. cerevisiae, A. alternata,* and *M. hiemalis* as representatives of yeast, ascomycetous molds, and zygomycetous molds, respectively, are shown in Figure 1B. The number of trials that successfully yielded PCR products from the 18S rRNA gene in triplicate were counted. In yeasts, gene amplifications were successful for all species and all trials for all combinations of DNA extraction methods. In all ascomycetous molds except *E. nidulans,* gene amplifications were successful for all species and all trials for all combinations of DNA extraction methods. The PCR for *E. nidulans* failed to amplify the gene in two extracts in triplicate obtained with the CTAB method with and without bead grinding. In zygomycetous molds, gene amplifications were successful for *M. hiemalis* and *R. stolonifer* in all trials for all combinations of DNA extraction methods. For *A. corymbifera,* the PCR failed to amplify the gene in one or two of three extracts obtained with the SDS method with or without bead grinding, in one of three extracts obtained with the CTAB method with bead grinding, and in one or all of three extracts obtained with the benzyl chloride method with or without bead grinding. In *R. stolonifer,* the PCR failed to amplify the gene in all of three extracts obtained with the SDS method with bead grinding and in all or one of three extracts obtained with the benzyl chloride method with or without bead grinding.

DISCUSSION

In developing DNA extraction methods for fungi, researchers have frequently considered rapid and effective extraction methods for a large amount of DNA targeting yeasts and molds with conidia *(2, 5, 11, 20).* The DNA extraction method for mold mycelia is still not considered sufficient. Culture in liquid medium is better for harvesting many fungal cells more rapidly than culture on agar medium. Because most molds grow mycelia but not conidia in liquid medium, it is necessary to consider rapid methods for extracting a large amount of DNA from mycelial cells. Although Fredricks et al. *(8)* and Zhu et al. *(33)* independently reported comparisons of DNA extraction methods for mycelia of molds, only single species, *Aspergillus fumigates* and *Magnaporthe grisea*, respectively, were tested. Although Tapia-Tussell et al. *(29)* developed a DNA extraction method with mycelia of five mold species, the recovery rate per weight of mycelium was not evaluated, and the developed method was not compared to other methods. Although Guo et al. *(10)* compared two extraction methods with mycelia of 25 mold species, DNA yields were not high enough for experiments that require a large amount of DNA; therefore, in the present study, DNA extraction methods were compared based on the quantity and quality of extracted DNA from mycelial pellets of a wide range of mold species after culture in liquid medium. Methods also were evaluated to determine which were effective for use in a wide range of fungi, including yeasts.

DNA extraction methods were compared for each taxonomic group: yeasts, ascomycetous molds, and zygomycetous molds. For yeasts, the combination of bead grinding and SDS extraction yielded the largest amount of DNA for *C. neoformans* and *S. cerevisiae*. For *R. rubra,* the DNA yield with the combination of bead grinding and benzyl chloride method recovered a high quantity of DNA. Gene amplification by PCR was successful for all species

and in all trials with all combinations of DNA extraction methods. These results suggest that the combination of bead grinding and the SDS method is the best DNA extraction method for yeasts, and when sufficient DNA is not obtained, the combination of bead grinding and benzyl chloride as a secondary extraction method should increase the yield. For ascomycetous molds, the combination of bead grinding and SDS extraction recovered the largest amount of DNA for all species. Gene amplification by PCR was successful for all species and all trials with the combination of bead grinding and the SDS method. These results suggest that the combination of bead grinding and the SDS method is the best DNA extraction method for ascomycetous molds. For zygomycetous molds except *R. stolonifer,* the combination of each of three chemical extraction methods with or without bead grinding recovered large amounts of DNA for all species. Gene amplification by PCR was successful for all species and almost all trials with only the combination of the CTAB method with or without bead grinding. These results suggest that the CTAB method with or without bead grinding is the best DNA extraction method for zygomycetous molds, and the use of bead grinding should increase the yield. Thus, the combination of bead grinding and the SDS method is most effective DNA extraction method for yeasts and ascomycetous molds, and the combination of bead grinding and the CTAB method is most effective DNA extraction method for zygomycetous molds. However, bead grinding tends to promote DNA degradation (Fig. 1). The bead grinding process used in this study did not produce highly degraded DNA; however, care should be taken when using bead grinding in DNA extractions.

For DNA extracted by some method combinations from *E. nidulans, A. corymbifera,* and *S. racemosum,* gene amplification was not successful in some trials, although DNA yields were high. This finding indicates that species with the target gene in the extracted DNA not amplified by PCR were limited. Some researchers have previously reported that DNA extracts of *Candida* spp. and *Stachybotrys* spp. contained PCR inhibitors *(4, 19).* Therefore, some components derived from fungal cells could cause PCR inhibition.

In this study, the best method for DNA extraction differed according to the fungal taxonomic group. However, when identifying mold isolates, it may not be possible to distinguish between ascomycetous and zygomycetous molds because isolates sometimes do not exhibit important diagnostic characteristics such as sporulation and hyphal elongation. Therefore, the efficacy of DNA extraction methods was evaluated for a wide range of fungi across taxonomic groups. Five DNA extraction methods were compared by the percentage of 14 mold species that yielded more than 1,000 μg/g. A significantly higher percentage of species yielded high amount of DNA with the SDS, CTAB, and benzyl chloride methods than with both the GenTLE and DNeasy Plant kits ($P < 0.01$). This result suggests that the SDS, CTAB, and benzyl chloride methods are more likely to recover large amounts of DNA from molds. DNA yields with bead grinding were better than those without bead grinding in all 14 species of molds except *E. nidulans* (Table 3), suggesting that bead grinding has an interactive effect with DNA extraction by chemical methods in many fungal species. The three combinations of bead grinding plus the SDS, CTAB, or benzyl chloride method were considered most likely to recover a large amount of DNA from molds; therefore, these three combinations were compared based on the DNA yield. PCR of *S. racemosum* failed to amplify the target gene in all trials with DNA extracted by both combinations of SDS or benzyl chloride method plus bead grinding. In contrast, gene amplification was successful for all mold species in at least one of three trials with the combination of bead grinding and the CTAB method. Overall, the combination of bead grinding and the CTAB method should be the first choice method for effectively extracting DNA from a wide range of molds. The combination of bead grinding and the CTAB method also yielded a relatively large amount of DNA from all species of yeasts used in this study (Table 2). Gene amplification by PCR using DNA extracted with this combination was successful in all trials for all yeasts, suggesting that bead grinding plus the CTAB method is relatively effective for DNA extraction in a wide range of fungi, including yeasts.

Our study is the first to clarify which extraction methods provide a high yield of high-quality DNA from mycelial pellets from a wide range of fungi growing rapidly in liquid medium. These methods should be very useful in genetic studies focused on identifying fungi important in the area of food hygiene, including many species of molds *(28).*

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FULL PAPER *Public Health*

Survival of *Salmonella* Strains Differing in Their Biofilm-Formation Capability upon Exposure to Hydrochloric and Acetic Acid and to High Salt

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ABSTRACT. Acidic and osmotic treatments are part of hurdle systems to control pathogens such as *Salmonella* in food. In the current study, *Salmonella enterica* isolates previously shown to differ in their ability to form biofilms were grown in diluted tryptic soy broth (TSB) (1:5 dilution in distilled water) and subsequently exposed to phosphate-buffered saline (PBS) adjusted to pH 3.0 with HCl, PBS adjusted to pH 3.9 with acetic acid or rice vinegar diluted 1:15 with distilled water (pH 3.9). Cells grown in diluted TSB were also exposed to distilled water, pH 7.6, containing 5 M NaCl. No differences in survival upon exposure to PBS adjusted to pH 3.0 with HCl or distilled water containing high salt were observed between the isolates; however, exposure to acetic acid and rice vinegar resulted in lower survival levels of isolates previously shown to be poor biofilm formers. The numbers ($\log_{10}$ cfu/m*l*) of surviving cells after exposure for 36 hr to acetic acid and rice vinegar were 4.43 ± 0.24 vs. 2.27 ± 0.87 ($P<0.05$) and 5.19 ± 0.12 vs. 2.33 ± 0.93 ($P<0.05$) for isolates with a high vs. low biofilm-forming ability. The survival data could be fitted with the Weibull model. The data suggest that the ability of *Salmonella* strains to survive in the presence of acetic acid and rice vinegar parallels their ability to form biofilms. Thus, *Salmonella* with a high biofilm-formation capability might be more difficult to kill with acetic acid found in foods or cleaning solutions.

KEY WORDS: acid, biofilm, high osmolality, *Salmonella*, tolerance.

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Bacterial survival under various stresses is a great concern to food manufacturing and processing. Frequently, acidic and/or osmotic conditions are used for food preservation and to control hazardous bacteria such as *Salmonella*. Acids are also commonly applied in washes for foods and food contact surfaces.

Many bacteria are able to grow as biofilms when in contact with surfaces or at the air/medium interface [23, 36]. The biofilms are characterized by the presence of polymeric matrices produced by the bacteria, and these matrices together with physiological changes in the biofilm bacteria are known to alter the susceptibility of these bacteria to exogenous stresses and agents [18]. For example, it is known that *Escherichia coli, Streptococcus mutans* and *Vibrio cholerae* cells embedded in biofilms differ in their acid and osmotic tolerance from free-living cells [22, 25, 27, 38, 43] and that *Salmonella* cells embedded in biofilms show reduced susceptibility to trisodium phosphate [23], desiccation [21] and chlorination [36].

It is known that isolates of *Salmonella* differ in their ability to form biofilms [21, 39]. The connection between biofilm-forming ability and risk of foodborne outbreaks has been suggested in *Salmonella* and *Listeria* [10, 28, 42]. *Salmonella* strains with high biofilm-forming abilities were also more frequently observed in fish meal and feed factories than in wild life [42]. To determine if strains differing in their ability to from biofilms also differ in their ability to cope with acidic and osmotic stress, cells were exposed to phosphate buffered saline adjusted to pH 3.0 with HCl and to pH 3.9 with acetic acid, to diluted rice vinegar and to distilled water containing 5 M NaCl, and their survival was determined.

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MATERIALS AND METHODS

Bacterial strains: Seven *Salmonella* strains were used (Table 1). These strains were previously classified as strains having high ($OD_{595}>1.0$) or low ($OD_{595}<0.03$) biofilm-formation capability based on data from a microtiter plate assay [21].

Survival experiments under acid solutions: The strains were grown in tryptic soy broth (pH 7.6) (TSB; Difco, Becton, Dickinson and Company, Sparks, MD, U.S.A.) at 37°C for 24 hr and a loop (10 μl; Sarstedt Group, Germany) of the culture was transferred into TSB (pH 7.6) diluted 1:5 with

Table 1. *Salmonella* strains used in this study

Strain	Serovar	Origin
High capability of biofilm formation		
SEC 54	Enteritidis	Patient
SEC 55	Enteritidis	Patient
SEC 105	Enteritidis	Chicken eggs
SEC 153	Mbandaka	Chicken eggs
Low capability of biofilm formation		
SEC 280	Enteritidis	Chicken eggs
SEC 282	Cerro	Chicken feed
SEC 284	Cerro	Chicken feed

distilled water (1/5 TSB) and incubated at 25°C for 24 hr in a water bath. When grown in 1/5 TSB at 25°C, *Salmonella* can efficiently form biofilm on plastic wells [21]. Under this condition, *Salmonella* cells were confirmed to reach the stationary phase and attain 10^8 cfu/m*l*. After incubation, 1 m*l* of culture was combined with 9 m*l* of phosphate buffered saline (pH 7.6) (Dulbecco's PBS(–), Nissui Pharmaceutical Co., Ltd., Japan) adjusted to pH 3.0 with 1.0 M hydrochloric acid (HCl; Wako Pure Chemical Industries, Ltd, Tokyo, Japan) or pH 3.9 with acetic acid (Wako Pure Chemical Industries, Ltd, Tokyo, Japan). Also, 1 m*l* of incubated culture was mixed with 9 m*l* of commercial rice vinegar (Japanese Consumer Co-operative Union, Tokyo, Japan) diluted 1:15 with distilled water (adjusted to pH 3.9). This rice vinegar, which contained about 0.8 M acetic acid, was purchased at a local supermarket in Tokyo. After mixing the solutions, the pH of the mixed solutions was measured and found not to be changed (HCl, pH 3.0, acetic acid, pH 3.9, 1:15 rice vinegar, pH 3.9). The samples were kept at 25°C in a water bath, and the cell numbers were counted every 6 hr. To count the cell numbers, each sample was diluted serially by 10-fold using PBS (pH 7.6). Fifty or 200 μl of the diluted samples were spread on tryptic soy agar (TSA; Difco, Becton, Dickinson and Company) using a spiral plater (EDDY JET, IUL Instruments, Barcelona, Spain). The plates were incubated at 37°C for 24 hr, and then colonies were counted. The pH value of the solution was measured after exposure to 48 hr, and it was found that pH did not change during the experiments. The experiments were performed in triplicate.

Survival experiments under high osmolar solutions: One m*l* of culture grown in 1/5 TSB culture for 24 hr at 25°C was combined with 9 m*l* of saturated saline (distilled pure water with 5 M NaCl; Wako Pure Chemical Industries, Ltd, Tokyo, Japan) (pH 7.6). The samples were kept at 25°C in a water bath, and the cell numbers were counted every 1 to 3 days by serial dilution and plating on tryptic soy agar. Colonies were counted after 24 hr of incubation at 37°C. The experiments were done in triplicate.

Survival modeling: Due to the increasing use of risk analyses aiming to control foodborne diseases, seeking the best survival model is important. To identify an appropriate survival model for the survival data, the GInaFit software was used [19]. This software is mainly used for fitting the model to the bacteria inactivation.

The Weibull model is widely used as a statistical model of the distribution of inactivation times. In this model, lethal events are considered as probabilities, and survival curves are considered a cumulative form of the distribution of lethal events [33, 41]. This model is defined as follows [11, 26].

$$\log_{10} N = \log_{10} N_0 - \left(\frac{t}{\delta}\right)^p$$

In this formula, N_0, δ and p are the initial microbial cell density (cfu/m*l*), the exposure time (hours) for the first log reduction and the shape parameter, respectively.

Albert *et al.* developed the Weibull with the tail model as follows [1].

$$\log_{10} N = \log_{10}\left[(N_0 - N_{res}) \cdot 10^{\left(-\left(\frac{t}{\delta}\right)^p\right)} + N_{res}\right]$$

N_{res}, δ and p are the residual population density (cfu/m*l*), the exposure time (hours) for the first log reduction and the shape parameter, respectively.

Performance of these models was evaluated using a prediction zone (APZ) method [31], which simultaneously assesses prediction bias and accuracy and predictive model performance. The performance factor of this method is the percentage of residuals (observed minus predicted values) that fall within an APZ from -1 (fail-safe) to 0.5 (fail-dangerous) $\log_{10}$ units. When ≥70% of the residuals are in the APZ, the model is considered to provide acceptable predictions of the test data.

Statistic analysis: The bacterial cell number observed at each time point was expressed as the mean log cfu of bacteria ± standard error of the mean (SEM) using Microsoft Office Excel. Significant differences were determined with an unpaired Student's *t*-test using the Systat 11.0 software (Cranes Software International Limited). The detection limit was set to 50 cfu/m*l*, and if the detection was under the limit, the cell number was assumed to be 25 cfu/m*l* and used in the statistic analysis.

RESULTS

Figure 1 shows survival curves in HCl solutions (pH 3.0) of strains with a high and low ability to produce biofilm. The initial average cell number ($\log_{10}$ cfu/m*l*) of the 2 groups was approximately the same (7.47 ± 0.04 (SEM) vs. 7.50 ± 0.08) at 0 hr after exposure to acid. The cell number decreased to 4.27 ± 0.30 vs. 2.90 ± 1.09 ($\log_{10}$ cfu/m*l*, P=0.22) at 48 hr of exposure for high vs. low biofilm-forming ability. At 48 hr 1 strain with low biofilm-formation capability (SEC 282) was under the detection limit. When fitted to the Weibull model, the δ values were 9.21 ± 1.45 vs. 6.39 ± 1.41 for high vs. low biofilm-forming ability, but no significant difference in the survival numbers was found during the exposure period.

Figure 2 shows survival curves of the *Salmonella* strains in acetic acid solutions (pH 3.9). Like under the HCl solutions, the initial average cell numbers of the 2 groups having high and low biofilm-forming ability were approximately the same, but significant differences were found at 24 hr (5.47 ± 0.25 vs. 2.88 ± 1.01, P<0.05) and 36 hr (4.43 ± 0.24 vs. 2.27 ± 0.87, P<0.05) for high vs. low biofilm-forming ability. At 48 hr, one strain with high capability (SEC 55) and 2 strains with low capability (SEC 280 and 282) were under the detection limit. Overall, obvious statistic differences for high vs. low biofilm-forming capability were noted from 24 hr of exposure onward. The Weibull model fitting showed this difference more clearly; the δ values were 11.42 ± 1.72 vs. 4.75 ± 3.86 for high vs. low biofilm-forming ability.

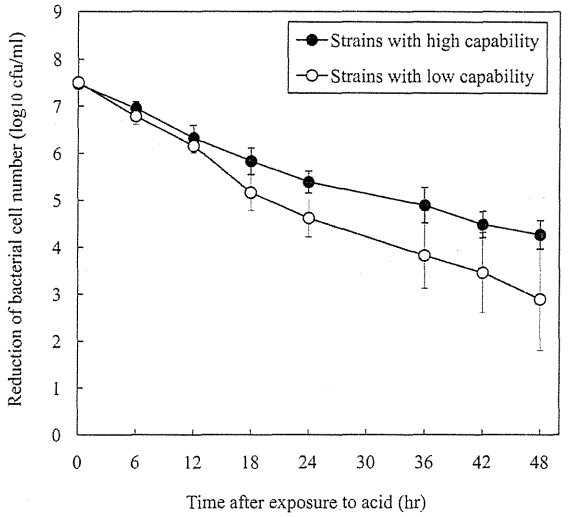

Fig. 1. Survival curves of *Salmonella* in PBS adjusted to pH 3.0 with HCl. Strains with high and low biofilm-formation capability were used. Each point on vertical bars indicates the mean±SEM (n=4 for strains with high capability, and n=3 for strains with low capability), which was calculated assuming the number below the detection limit (50 cfu/m*l*) to be 25 cfu/m*l*.

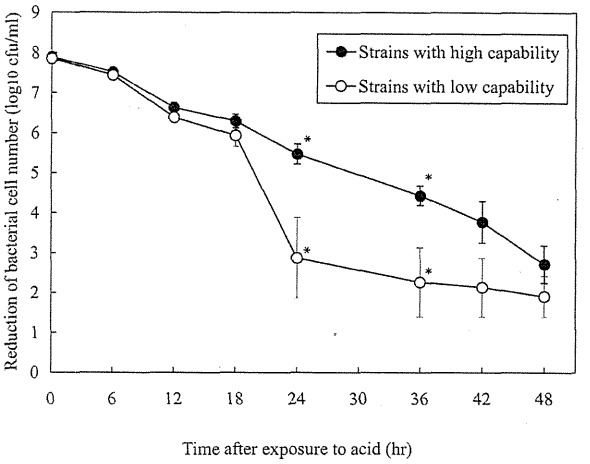

Fig. 2. Survival curves of *Salmonella* in PBS adjusted to pH 3.9 with acetic acid. Strains with high and low biofilm-formation capability were used. Each point on vertical bars indicates the mean±SEM (n=4 for strains with high capability, and n=3 for strains with low capability), which was calculated assuming the number below the detection limit (50 cfu/m*l*) to be 25 cfu/m*l*. * A significant difference was observed between the 2 groups of strains having different abilities to form biofilm at the same exposure time ($P<0.05$).

Figure 3 shows survival curves for 1:15 diluted rice vinegar (pH 3.9). The initial average cell numbers of the 2 groups having high and low biofilm-forming ability were approximately the same. Significant differences were found at 24 hr (6.03 ± 0.05 vs. 4.75 ± 0.51, $P<0.05$), 36 hr (5.19 ± 0.12 vs. 2.33 ± 0.93, $P<0.05$), 42 hr (4.92 ± 0.15 vs. 2.26 ± 0.86, $P<0.05$) and 48 hr (4.27 ± 0.22 vs. 2.03 ± 0.63, $P<0.05$) for high vs. low biofilm-forming ability. At 48 hr, 2 strains with low capability (SEC 280 and 282) were under the detection limit. The δ values were 13.19 ± 1.57 vs. 8.83 ± 3.82 for high vs. low biofilm-forming ability, but significant statistical differences in the survival numbers were not found at 42 hr and 48 hr of exposure. The reason for this seems to be that three strains (SEC 55, 280 and 282) were under the detection limit at 42 hr.

Survival curves under high hyperosmotic solutions: Figure 4 shows survival curves in saturated saline (pH 7.6) of strains with high and low biofilm-formation capability. The initial average cell numbers ($\log_{10}$ cfu/m*l*) of the 2 groups having high and low biofilm-forming ability were approximately the same (8.09 ± 0.06 vs. 8.13 ± 0.07) at 0 d. The cell numbers decreased to 1.95 ± 0.38 vs. 1.51 ± 0.11 ($\log_{10}$ cfu/m*l*, $P=0.39$) at 20 d of exposure for high vs. low biofilm-forming ability. At 20 d, 2 strains with high capability (SEC 54 and 55) and 2 strains with low capability (SEC 280 and 282) were under the detection limit. The average number for strains with high capability tended to be larger than for strains with low capability, but the δ values were not different (0.43 ± 0.12 vs. 0.12 ± 0.08 for high vs. low biofilm-forming ability), and no significant difference in survival numbers was observed.

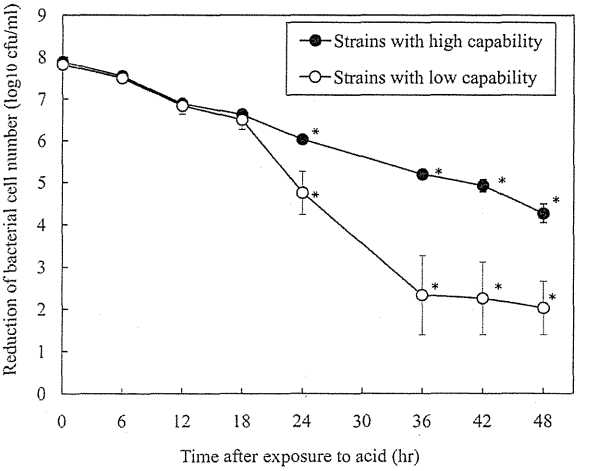

Fig. 3. Survival curves of *Salmonella* in 1:15 rice vinegar solution (pH 3.9). Strains with high and low biofilm-formation capability were used. Each point on vertical bars indicates the mean±SEM (n=4 for strains with high capability, and n=3 for strains with low capability), which was calculated assuming the number below the detection limit (50 cfu/m*l*) to be 25 cfu/m*l*. * A significant difference was observed between the two groups of strains having different abilities to form biofilm at the same exposure time ($P<0.05$).

Survival model performance: The Weibull model provided acceptable predictions (≥70% of residuals) for all data. However, the APZ value of the strains with low biofilm-forming capability in acetic acid and rice vinegar was

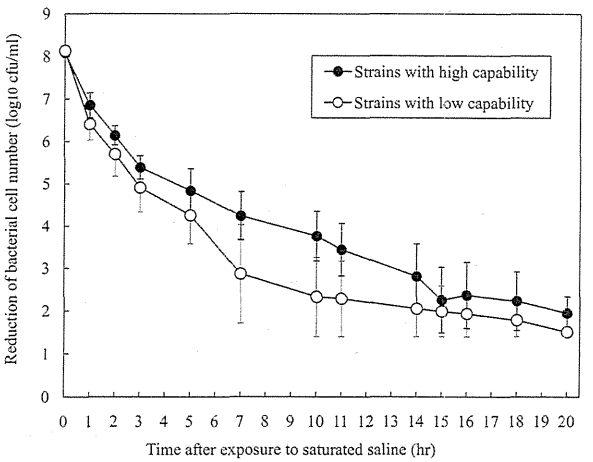

Fig. 4. Survival curves of *Salmonella* in saturated saline (pH 7.6). Strains with high and low biofilm-formation capability were used. Each point on vertical bars indicates the mean±SEM (n=4 for strains with high capability, and n=3 for strains with low capability), which was calculated assuming the number below the detection limit (50 cfu/m*l*) to be 25 cfu/m*l*.

smaller (APZ=71.4). When the Weibull with the tail model was used, the APZ values of these strains raised to 100.0, which showed more adequate fitting performance.

DISCUSSION

The ability of bacteria to form a biofilm is one of the important factors that must be considered to avoid food-borne diseases [10]. The advantage of a biofilm for bacteria is that it protects them against severe stresses such as poor nutrition and low temperature [12]. Acid and osmotic conditions are also involved in such stresses [22, 38, 43]. In this study, we used 2 groups of *Salmonella* strains, one showing a high ability to form biofilm and another showing a low ability. Previously, we have reported that biofilm formation by these strains can be facilitated on plastic wells by growing the bacteria in 1/5 TSB at 25°C for 24 hr [21]. Therefore, this growth condition was also used in this study. If the bacterial ability to form biofilm plays a key role in bacterial protection against acidic or hyperosmotic stress, such strains having high ability to form biofilm would be more tolerable to low pH and hyperosmotic conditions than strains showing a low ability.

The survival of *Salmonella* strains in HCl solutions, the pH of which was adjusted to pH 3.0 to mimic gastric acid [3], shows no significant difference in the survival curves between the 2 groups of strains having different abilities to form biofilm. However, the survival of *Salmonella* strains in acetic acid shows that the cell numbers of strains with low biofilm-formation capability decreased faster than those of strains with high capability. Acetic acid has been used as a cleanser detergent and a main material for a variety of vinegars and sources. Also, commercial rice vinegar has long been used for seasoning in Japanese and Chinese dishes. The pH value was adjusted to 3.9 for both acetic acid and rice vinegar solutions. Many studies have shown significant killing by acetic acid within minutes [9]. However, in this study, reductions of about 2 logs were observed 12 hr after acid treatment. Such a relatively slow reduction may be caused by using relatively mild acid, such as one with a pH of 3.9, and *Salmonella* cells at the stationary phase. Judging from the surviving cell numbers, acetic acid had a more severe effect on *Salmonella* survival than rice vinegar (the surviving number of cells with high capability at 36 hr was 4.43 ± 0.24 vs. 5.19 ± 0.12). Under pH 3.0 acid conditions, lactic acid has a more severe effect on cells than acetic acid and HCl [8], but Bjornsdottir, *et al.* reported that exposure to 1–20 mM of lactic acid aided the survival of *Escherichia coli* in severe acidic environments with a pH of 3.2 [7]. Since lactic acid produced by lactic acid bacteria exists at a concentration of 0.73–3.42 mM in commercial rice vinegar [17]; such low concentrations of lactic acid existing in rice vinegar solutions may also in some way have aided the survival of *Salmonella* strains in the acidified solutions.

The observed relationship between acid tolerance and biofilm-formation capability also indicates the possibility that the acid tolerance mechanism might be related to biofilm-forming mechanism. The survival curves under acidic conditions showed that the death rate decreased with incubation time in both strains, especially in strains with high biofilm-forming ability. Exposure of bacteria to low pH stress is known to induce acid shock proteins, which are involved in acid tolerance response [2, 5, 16, 29, 40]. Among these proteins, RpoS, the functional stress-responsive sigma factor, is known to be involved in the resistance of stationary phase cells to various stress-including acids [13, 24] and in the formation of biofilm by regulating CsgD expression [34, 35]. A higher level of RpoS causes a higher level of stress resistance, but the expression of this protein is widely different depending on the stress [6]. In *Listeria monocytogenes,* loss of functional stress-responsive alternative sigma factor *sigB* reduced the survival of the cells at pH 2.5 to a greater extent in the presence of organic acid (acetic acid) than in the presence of inorganic acid alone (HCl), suggesting that the protection against organic and inorganic acid may be mediated through different mechanisms [15]. Thus, *Salmonella* cells with high biofilm-formation capability might be under the high influence of acetic acid tolerance mechanisms during exposure to acetic acid, resulting in an increase in the tolerance level with incubation time. Other organic acids might also be linked to the tolerance mechanism, so further study using various organic acids will be necessary.

The results for tolerance against osmotic stress indicate that tolerance against hyperosmotic stress is independent of the biofilm-formation capability of *Salmonella*. For *Pseudomonas aeruginosa* and *Vibrio cholerae*, biofilm development was observed to be under the influence of osmotic conditions [4, 37]. Further studies using various strains and approaches will be needed to elucidate the link-

age of biofilm formation and osmotic tolerance of *Salmonella*.

In order to estimate the D-value accurately, it is necessary to seek the best survival model in the food industry. Using the Weibull model showed adequate fitting performance in each experiment. A good fit of the inactivation curve is important to obtain more valuable estimates for the controlling the survival of bacteria. The Weibull model has been shown to be a very useful model for simulating the bacteria inactivation model [20, 32]. Also, in this experiment using *Salmonella,* this model was found to be very adequate for simulation of survival under acid and high osmolar stress. The strains with low biofilm-forming capability in acetic acid, and rice vinegar showed a characteristic decrease from 24 hr of exposure onward. By using the Weibull with the tail model, these types of survival curves were found to simulate clearly.

In conclusion, the data obtained in this experiment suggest that the ability of *Salmonella* strains to survive in the presence of acetic acid and rice vinegar parallels their ability to form biofilms. The relation between biofilm-forming ability and risk of foodborne outbreaks has previously been suggested [28, 42]. Therefore, it is important to consider strain-dependent variations in biofilm-formation capability when developing strategies for prevention of *Salmonella* contamination of foods. In particular, considering that various foods with low pH have caused outbreaks of *Salmonella* infection [14, 30], this notion should be emphasized.

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