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Genetic and Functional Diversity of Agricultural Microorganisms

In conjunction with the 10th International Congress for Culture Collections

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Genetic and Functional Diversity of Agricultural Microorganisms

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Introduction to the workshop

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The National Institute of Agrobiological Sciences (NIAS) holds an International Workshop on Genetic Resources annually. The objectives of these workshops are to exchange research information with experts from around the world and to promote international collaboration for the development of technology related to the evaluation, conservation and use of plant, animal and microorganism genetic resources These workshops have helped to stimulate activities of our genebank system in Japan and help Japanese genetic resources scientists to forge collaborative linkages with scientists in other countries

This year we are holding the 12th NIAS International Workshop on Genetic Resources This workshop consisted of two themes

1 Current research on the genetic and functional diversity of microorganisms related to agriculture;
2. Ways to improve the quality and use of culture collections in the light of developments in microbial genomics and trends related to the Convention on Biological Diversity.

This workshop was held in conjunction with the 10th International Congress for Culture Collections (ICCC-10) in Tsukuba during October 2004. The theme of ICCC-10 was "Innovative roles of biological resources centers"

This book contains the proceedings of the 12th NIAS International Workshop on Genetic Resources and consists of four parts The first two parts are the papers presented at the two workshop symposia organized under the titles "Genetic and functional diversity of agricultural microorganisms" and "New paradigms of biological resources centers" In addition, the papers based on the poster presentations are included. At the time of the workshop, a training course was held for Southeast Asian microbiologists in order to help improving their culture collection techniques A report on this training course is provided in this book.

We greatly appreciate the inputs of scientists from around the world and Japan that helped to make this multi-objective workshop a success We hope that this workshop will stimulate further international developments and research cooperation on culture collections for agricultural microorganisms

The 12th NIAS International Workshop on Genetic Resources

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1. GENETIC AND FUNCTIONAL DIVERSITY OF AGRICULTURAL MICROORGANISMS

Preface

Molecular genetic tools have paved the way to significant advances in virtually all aspects of microbiological research. Remarkably, the complete genomes of over thirty agriculturally important microorganisms have been completed just within the past half-decade. Public accessibility to this wealth of genetic data via gene banks such as the DNA Data Bank of Japan and GenBank has spurred groundbreaking experimental functional analyses and descriptive phylogenetic and systematic studies in molecular plant pathology. Included among these are four phytopathogenic *Xanthomonas* species whose genomes either are being or have been sequenced. Comparative phylogenomic analyses of these data should provide new insights into the ecology and evolution of plant-microbe and animal-microbe interactions, just to mention a few.

These advances are also reflected in phylogenetic-based systematic and functional analysis of fungi, even though the genomes of relatively few agronomically important fungi have been sequenced to date. Phylogenetic analysis of plant pathogenic fungi has become essential for elucidating their genetic diversity, systematics and for providing molecular markers for their rapid detection and surveillance associated with the globalization of world trade. Phylogenetic studies are also providing a framework for understanding the evolution of virulence-associated factors such as mycotoxins

This symposium, which is held as a part of NIAS International Workshop on Genetic Resources, highlights recent research advances and future prospects in the genomics, genetic diversity, taxonomy and functional analysis of agronomically important microorganisms Special focus is directed at phytopathogenic bacteria (i.e., xanthomonads and *Ralstonia*) and phytopathogenic fungi (*i e.*, fusaria and *Pyricularia*)

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Mobile genetic elements contribution to the differentiation of closely related *Xanthomonas* genomes

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Whole-genome sequencing and comparison of *X. axonopodis* pv. *citri* (*Xac*), strain 306, and *X. campestris* pv. *campestris* (*Xcc*), ATCC33913, disclose a high degree of identity and colinearity of the chromosome backbone. Analyses of strain-specific genes preferentially located at discrete genomic islands suggest different strategies for adaptation to host and disease development *Xcc* chromosome contains a nitrate assimilation operon, higher number of avirulence genes and plant cell wall degrading enzymes probably facilitating its systemic dissemination. *Xac* has two plasmids that contain four copies of *avrBs3/pthA* gene, which has been implicated in the induction of plant cell proliferation associated with canker disease. Differences in their type III secretion systems, *rpf*, type IV fimbriae and LPS O-antigen operons were detected and may be associated with host-specific adaptation. No antibiotic synthesis was detected as part of the TE units, but some of the insertions are in close association with genes coding for toxins, drug resistance. And other genes known to be associated with pathogenicity are in close association with genetic mobile elements, mainly IS elements Both genomes were invaded by different families of IS elements and in each genome one particular IS proliferated. In addition, it is notable that these genetic mobile elements are generally associated the strain specific genes suggesting that they could be drivers of the genome evolution of this important group of plant pathogens

Introduction

Xanthomonas axonopodis pv. *citri* and *Xanthomonas campestris* pv. *campestris* are important necrogenic plant pathogens responsible for citrus canker and black rot of crucifers, respectively. In an attempt to identify the genes responsible for disease development and to unveil the strategies of the two pathogens to colonize plant tissues, the ONSA/FAPESP consortium undertook genome sequencing and comparative analyses of both bacteria. Results suggest that disease phenotype is brought by the presence of particular gene sets in each *Xanthomonas*, most of them in close association with specific rearrangements on both chromosomes Two types of genomic rearrangements were characterized in these genomes that are either changes in gene order (colinearity) and major insertion/deletions (INDEL) Within these INDEL, we defined twelve regions that are larger than ten kbp in *Xanthomonas axonopodis* pv. *citri* and seven regions in *Xanthomonas campestris* pv. *campestris*. Except for both type II and type III secretion systems which are similar at ~90 % nucleotide level but have switched places around the origin of replication, all other genomic islands were different in gene

content between the two genomes All these 19 INDEL regions, referred to as genomic islands, carry proteins related to genetic mobile elements such as transposases and integrases

Phages, plasmids and transposable elements are considered important sources of genetic variability for bacteria genome evolution (Boucher *et al* 2003, Canchaya *et al* 2004; Mira *et al* 2002) Most of the prokaryotic organisms experience clonal cellular division in which genetic exchange can only occur by invasive DNA. Recent reports disclose the impact of these mobile elements in the differentiation of closely related species and strains bringing into discussion the concept of bacterial species How similar and conserved should a bacterial chromosome be in order to define that two strains are from the same species? What is the minimal percentage of invasive DNA that would differentiate two strains? These questions can be raised at both species level and strain level and human pathogenic bacteria have been the main focus of these questions (Canchaya *et al* 2004; Casjens 2003, Nakagawa *et al* 2004; Perna *et al* 2001) Genome sequences of plant pathogens are coming to light and support the observation that invasive genetic elements are tools for genome diversification.

Materials and methods

Xanthomonas axonopodis pv. *citri*, strain 306 and *Xanthomonas campestris* pv. *campestris,* ATCC33913 genomic DNA was prepared from freshly grown cultures using standard procedures Sequencing strategy and annotation of *Xac* and *Xcc* are described in da Silva *et al* (2002) and comparative studies were mostly carried manually at http://www lbi ic.unicamp.br/ and in a relational database as described in Van Sluys *et al* (2002)

Results and Discussion

Genomic island distribution

Xac genome was composed of circular chromosome (5,175,554 bp) and two plasmids (pXAC33 and pXAC64) while *Xcc* contains one circular chromosome of 5,076,187 bp. Considering the main chromosome, there are 99,367 bp present in *Xac* and absent in *Xcc* distributed as discrete regions around the chromosome backbone (table 1) The region encompassing the terminus of replication accounts for 30% of this difference and was named LR1 (Large Rearrangement 1) This region was composed in the two genomes of three specific genomic islands and some rearrangements in gene order The interesting aspect in these islands is the presence in *Xac* of the *syr*E locus that is responsible for the synthesis of syringomycin. In *Pseudomonas syryngaeae,* this nonapeptide is associated with a long unbranched 3-hydroxy fatty acid chain that has an antifungal activity (Menestrina and Semjén 1999) The largest INDEL in *Xac* corresponds to a 61,169 bp insertion where RTX toxin and most of the non-

Table 1: *Xac* genomic island distribution and content

Genomic Island [1]	Genes involved	G+C	Xac[2]	tRNA[3]	Xcc
1 (31,494)	Type III secretion system	62.30%	461744 - 493822	Not present	Present and inverted
2 (17,170)	Type II secretion system	64.90%	819406 - 838208	Not present	Present and inverted
3 (22,234)	Phage with muramidase	59 50%	1209345 -1229179	Lys	n.d.
4 (23,616)	cvgSY	63%	1908626 -1932532	Ser	n.d
5 (27,635)	Haemaglutinnas	63%	2083162 - 2113080	Arg	n.d.
6 (15,483)	Seringomycin - syrE	61.40%	2443729 - 2458844	Leu	syrE not present
7 (61,169)	Hemolysins	65.60%	2542422 - 2675446	Not present	rearranged
8 (30,094)	Pseudomonas plasmid recombinase	58 90%	2818374 - 2847359	Ala	partial
9 (20,301)	Type IV secretion system	56.10%	3070365 - 3090908	Val	partial
10 (27,184)	Phage P2/CTX Pilin genes	62.70%	093912 - 3134268 (3121308)	Asn	partial
11 (13,723)	avrPphE	61.60%	3795496 – 3811352	Not present	partial
12 (13,385)	cvgSY	58 90%	3877719 – 3827899	Gly	n.d

1 size in bp of the genomic island
2. genomic position of the limits of the island.
3 tRNA potential integrating site
4. n.d. not determined

gamma bacteria genes are found. Also in the LR1, there was a region corresponding to 19 genes from a discrete region also found in *Xylella fastidiosa* CVC (*Xf*-9a5c) strain and not present in *X. fastidiosa* PD (*Xf*-Temecula) strain (Van Sluys *et al* 2003) In *Xf*-9a5c, this region encompasses a DNA methyltransferase that harbors an group II intron not present in *Xac* (Simpson *et al* 2000; da Silva *et al* 2002)

Three specific regions with a particular interest also compose the equivalent region in *Xcc*. First of all, an insertion of ΦLf filamentous phage, which is specific from *X.campestris* pv. *campestris* strains was identified. Inside the delimited region, a cyclic beta 1,2 glucan synthetase was present This protein is associated with virulence in some bacteria such as the members of the Rhizobiaceae family (*Agrobacterium*, *Rhizobium* and *Brucella*) for which, mutants in this locus present reduced virulence (Breedveld and Miller 1994; Briones *et al* 2001, Thomashow *et al* 1987) The second genomic island identified has two main features probably involved in plant-pathogen interaction. These are the presence of the *avrBs1* gene not found in *Xac* flanked by two transposases and a tannase encoding gene. The latter is considered to be an important enzyme for microorganisms to overcome growth inhibition induced by tannins synthesized by plants The third region harbors three genes similar to those involved in antibiotic biosynthesis of *Streptomyces* (macrolides and streptomycin)

Outside the terminus region, other genomic islands could be found scattered along the chromossome backbone. In *Xac*, genes encoding pennicilin modifying enzymes, acriflavin resistant protein, hly activator protein and most important another avirulence gene known as *avrPph*E were found and were not present in *Xcc*. In *Xac* the most similar gene to *avrPph*E is at

the side of IS*Xac3*. Also, it is interesting that 1030 genes coming from non-gamma bacteria are specifically present in *Xac* genome most of them located within the genomic islands described here.

In *Xcc* a full length copy of P2/CTX phage was found while in *Xac* only a truncated version was present In *Xcc,* along with this full length phage copy was located the *umu*C and *umu*D genes which are absent in *Xac*. This would suggest that, probably, a major rearrangement in *Xac* genome involving the loss of the phage region also resulted in the loss of these proteins involved in repair

Transposon and IS elements

Xac and *Xcc* genomes were invaded by several IS elements and each genome has an element that is most represented (table2) IS*1478* belongs to IS5 family of transposases and was present in 16 copies in *Xcc*. IS*Xac3* belongs to the IS3 family of transposases and was present in 21 copies in *Xac* and has not been previously detected in *Xanthomonas*. The impact of these elements on these genomes can be glimpsed by the fact that only 4 IS elements are shared by *Xac* and *Xcc* (IS*1478*, IS*1479*, IS*Xcd1* and IS*Xac3*) and all the other 13 elements are particular elements to either of the two genomes Table 2 presents the list of the 17 full-length elements identified, but it is important to mention that *Xac* genome carries 10 extra transposases not fully characterized.

Tn5045 is a composite transposon already described in *X. campestris* as IS*XC5* (Tu *et al* 1989) This transposon was originally described to be born in pXW45J plasmid from *X.campestris* pv *citri* strain XW45 In *X. axonopodis* pv *citri* a full lenght copy of the element was present in the pXAC33 while in pXAC64 the IS unit (IS*Xac1*) was missing. In both plasmids the

Table2. Transposons and IS elements found in *Xac* and *Xcc* genomes

Transposon and IS element (size)	IS family[1]	Accession number[2]	Number of copies[3]	
			Xac	*Xcc*
IS*Xac1* (1,352 bp)	IS4	Z73593	8	-
IS*Xac2* (1,195 bp)	IS1	AF263433	8	-
IS*Xac3* (1,255 bp)	IS3	AF327445	21	7
IS*Xac4*	IS1	AF034211	2	-
IS*1389*	IS3	U77781	1	-
IS*1404*	IS3	U45994	-	6
IS*1477*	IS3	M28557	-	7
IS*1478*	IS5	U59549	1	16
IS*1479*	IS5	AF077016	1	10
IS*1480*	IS5	U61260	-	7
IS*1481*	IS4	AF090837	-	11
IS*1595*	ISNCY	AF225215	-	1
IS*Xcd1*	IS1	AF263433	2	2
IS*Xcc1*	IS3	AF047478	-	5
ISD1	IS3	AF047478	-	1
Tn5041	Tn3	Z73593	-	1
Tn5044	Tn3	AF174129	2	-
Tn5045 (6,938 bp)	Tn3	Z73593	2	-

[1] as defined Mahillon, J and Chandler, M. (1998)

[2] Accession number of the most similar element (AA level) identified at (http:// www-is.biotoul.fr/)

[3] Copy number represent full length copies of the IS elements present in the chromosome and in the plasmids.

element is in closely associated with the *pthA* gene. The transposon was present, between positions 5223-5235, a sequence (gccatcgccagca) that was also found in *pth*A repeats This region could have an impact on the variability in the numbers of repeats We also found the same sequence as part of genes of unknown function in *Xylella* (71% AA identity) and *Xanthomonas campestris* (68% AA identity) In *Xylella*, this coding region is present in the pXF51 plasmid and in *Xanthomonas* is associated to Tn5053 family transposons The IS component (IS*Xac1*) of the composite Tn5045 has invaded the circular chromosome and was present in 8 identical copies in *Xac*. IS*Xac2* is similar to a previously described insertion sequence from *Xanthomonas axonopodis* pv. *dieffenbachiae* ISXCD1 Most of the elements related to this sequence are translated by programmed frameshifting as has been described previously for *Desulfovibrio vulgaris* Hildenborough ISD1 IS*Xac3* is closely related to an *Erwinia amylovora* transposon born in the ubiquitous plasmid pEA29 and to an IS-like element present in the *Agrobacterium tumefaciens* pTI plasmid from the AB2/73 limited host range strain. IS*Xac2* and IS*Xac3* were present in *Xac* generally associated with genomic islands listed in table 1 In *Xcc,* IS*Xac1* and IS*Xac2* are very poorly represented being identified only as highly degenerated copies or truncated elements while IS*Xac3* was identified in 7 copies all of them harbouring 26 bp internal deletion. It is temptimg to speculate that these IS elements may be drivers of the differentiation of the *Xanthomonas* genomes probably as a result of phage transduction or plasmid conjugation events

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Species recognition and identification of agriculturally important fusaria: current status and future prospects

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Fusarium is a large genus of filamentous fungi that represents the single most important group of mycotoxigenic plant pathogens Fusaria have also emerged within the past two decades as opportunistic and often fatal pathogens of severely immuno-compromised patients Members of this genus produce an amazing diversity of toxic secondary metabolites such as trichothecenes, fumonisins and estrogenic compounds which pose a serious threat to human and plant health and food safety. In order to develop molecular epidemiological tools for the rapid detection and identification of the most important fusarial pathogens, multilocus sequence typing is being used to investigate species boundaries Knowledge of species limits is essential for understanding each pathogen's geographic distribution, host range and toxin potential, and to establish successful molecular surveillance programs for economically devastating plant diseases such as Fusarium Head Blight of small grain cereals Because globalization of trade in agricultural commodities will continue to result in the inadvertent movement of foreign fusarial pathogens worldwide, a global network of plant disease specialists is needed to meet this continuing threat

Introduction

Fusarium collectively represents the single most important genus of mycotoxigenic phytopathogens (Marasas *et al* 1984) Moreover, due to the dramatic increase in the number of artificially immuno-suppressed and immuno-compromised patients over the past twenty years, there has been an increased recognition of fusaria as opportunistic and life threatening pathogens of humans (O'Donnell *et al* 2003, 2004a) Although morphological species recognition (MSR) has formed the foundation of *Fusarium* taxonomy (Wollenweber and Reinking 1935, Booth 1971, Gerlach & Nirenberg 1982; Nelson *et al* 1983), only approximately 120 morphospecies are currently accepted in the taxonomies cited above, including species descriptions published up to July, 2004. By way of contrast, preliminary estimates using genealogical concordance phylogenetic species recognition (GCPSR) (Taylor *et al* 2000), which employs multilocus sequence typing (MLST), suggest that *Fusarium* comprises over 300 phylogenetically distinct species Unfortunately, relatively few medically important (Fisher *et al* 2002; Kasuga *et al* 2003, Koufapanou *et al* 1997; Litvintseva *et al* 2003) and agriculturally important fungi (Carbone *et al* 1999; Couch & Kohn 2002; Craven *et*

al 2001, Geiser *et al* 1998a, 1998b, 2000, 2001, O'Donnell 1997, 2000; O'Donnell *et al* 1998a, 1998b, 2000, 2004b; Steenkamp *et al* 2002) have been subjected to GCPSR.

A recent study of the *Fusarium graminearum* species complex, which includes a number of economically devastating etiological agents of Fusarium head blight (FHB) and scab worldwide (Windels 2000), provides a clear example of how MSR underestimates species diversity (O'Donnell *et al* 2004b) Because this study conclusively shows that morphology alone may be insufficient for mycotoxicologists and plant pathologists to accurately report on the toxin potential and pathogenicity of *Fusarium* species, we have developed a multi-institutional collaborative network to investigate species boundaries using a combination of MSR and GCPSR. In addition, we have teamed with mycotoxicologist Dr Gretchen Kuldau, Department of Plant Pathology, The Pennsylvania State University to reassess the toxin potential of all fusaria within a robust phylogenetic framework and molecular plant pathologist Dr H. Corby Kistler, USDA, St Paul, MN for pathogenicity testing and mycotoxin analyses of head blight fusaria within the *F. graminearum* complex. Results of these studies, which include identification of molecular markers for long-term epidemiological studies, will be made freely available to the global scientific community via the Internet (Geiser *et al* 2004; Kang *et al* 2002)

Materials and methods

Researchers from around the world send fusaria to Agricultural Research Service Culture Collection (NRRL), Peoria, IL, the National Institute of Agrobiological Sciences (MAFF), Tsukuba, Japan and the Fusarium Research Center (FRC), University Park, PA to be characterized phylogenetically using DNA sequence data from one or more loci and/or phenotypically. Each strain accessioned is given a unique accession number and they are stored in liquid nitrogen vapor at -175°C or by lyophylization for future reference and distribution to the scientific community upon request

Procedures for the polymerase chain reaction (PCR)-based amplification of target loci and DNA sequencing are described in O'Donnell *et al* (2004b) and references therein. Subsequent to the publication of the first set of PCR primers and protocols for the amplification and direct sequencing of fungal nuclear and mitochondrial ribosomal genes (White *et al* 1990), single copy protein coding genes interrupted by large and/or numerous introns, such as translation elongation factor (1α), generally have been shown to be more informative for species level studies (Geiser *et al* 2004; O'Donnell *et al* 2004b) Use of the internal sequencing primer EF-3> 5'-GTAAGGAGGASAAGACTCACC-3', which is nested just within the EF-1α amplicon, makes it possible to generate a nearly complete sequence of this locus for most fusaria with just one sequencing primer

Sequencing reactions are currently being run on Applied Biosystems-Hitachi 3100 or 3730 capillary sequencers which yield high quality sequences of 800 base pairs or more. All sequences are edited with Sequencher version 4.1.2 (Gene Codes, Ann Arbor, MI) and then exported for maximum parsimony phylogenetic analysis using PAUP* version 4.0b10 (Swofford 2002) To identify unknown strains to species or species complex, partial EF-1α sequences are used as a query to BLAST the FUSARIUM-ID v.1.0 database (Geiser *et al* 2004) at http //fusarium.cbio.psu.edu housed at The Pennsylvania State University, University Park, PA.

Species limits are assessed using a combination of MSR (Aoki & O'Donnell 1998, 1999a,b; Aoki *et al* 2001, 2003, Hirata *et al* 2001) and GCPSR (Taylor *et al* 2000; O'Donnell *et al* 2004b)

Results and Discussion

Substantial progress has been made over the past decade at elucidating the systematics, evolution, biogeography, host range and mycotoxin potential of agriculturally important fusaria within a phylogenetic framework. Results of these morphological and molecular systematics studies, which may be generally applicable to other agronomically important fungi, are outlined below.

1) Progress in *Fusarium* systematics has been greatly facilitated by intensive collaborations among scientists at the FRC, MAFF, NRRL, BBA (Berlin, Germany) and CBS (Utrecht, The Netherlands) culture collections, all of which house extensive collections of *Fusarium,* including many ex-type strains and strains important from a mycotoxicological perspective (Marasas *et al* 1984)

2) Support of and the continued accessioning of novel germplasm in publically accessible international culture collections is critical to continued progress in *Fusarium* systematics

3) Coordinated research involving scientists using MSR and/or GCPSR is essential in order to help bridge the enormous gap between the number of morphospecies (~120) and the estimated number of phylogenetic species (~300; O'Donnell, unpubl) However, as recently discovered in a study of the *F. graminearum* species complex (O'Donnell *et al* 2004b), it is reasonable to assume that many phylogenetically distinct species resolved by GCPSR may not be diagnosable using MSR.

4) The terms macro- and microconidia obscure the morphological diversity of conidia and should be replaced with more informative descriptors as discussed in Aoki *et al* (2003)

5) Discrete DNA sequence data possesses considerably more information for investigating species limits than other types of molecular markers However, DNA sequence data from the nuclear ribosomal internal transcribed spacer (ITS) region and the 5' end of the nuclear large subunit spanning domains D1 and D2, the two most widely used loci for species level systematics in the Fungi, are too conserved to resolve most *Fusarium* species However, nuclear genes such as EF-1α and β-tubulin, which are interrupted by phylogenetically informative introns, have enormous utility in GCPSR (Geiser *et al* 2004; O'Donnell *et al* 2004b and references therein)

6) DNA sequence-based phylogenetics has shown that the subgeneric, morphological-based sectional classification of *Fusarium* is mostly non-monophyletic. Fortunately, results of the molecular phylogenetic studies have identified several monophyletic species complexes, including *Gibberella fujikuroi* (O'Donnell *et al* 1998a; 2000), *F. oxysporum* (O'Donnell *et al* 1998b), *Fusarium graminearum* or the dominant B-trichothecene mycotoxin producers (O'Donnell *et al* 2000, 2004b; Ward *et al* 2002), and *F. solani* (O'Donnell 2000; Aoki *et al* 2004)

7) Molecular evolutionary genetic studies of the trichothecene toxin gene cluster (Kimura *et al* 2003) have shown that the evolution of these 12 clustered genes within the *F. graminearum* species complex is discordant with the species phylogeny (Ward *et al* 2002) Furthermore, the phylogenetic evidence suggests that the toxin gene cluster is under a novel form of balancing selection such that three reciprocally monophyletic chemotype clades (i.e., 3ADON+DON, 15ADON+DON and NIV) have been maintained over multiple speciation events Results of this study provide a phylogenetic framework for investigating the role of each trichothecene chemotype in plant pathogenesis In addition, multiplex PCR assays targeting the ends of the gene cluster make it possible to accurately predict the trichothecene chemotype of each strain within the *F. graminearum* species complex (Ward *et al* 2002)

8) While genealogical concordance appears to be the norm (O'Donnell *et al* 2004b), discordances have been detected within all of the major species complexes a) ITS2 paralogs or xenologs are uniformly distributed across the *G. fujikuroi* and *F. oxysporum* species complexes where their evolution has been homoplastic (O'Donnell and Cigelnik 1997; O'Donnell *et al* 1998a, 2000), b) highly divergent β-tubulin sequences within the *F. solani* complex confound

phylogenetic reconstruction, and c) a nuclear ribosomal intergenic spacer region phylogeny of the *F. graminearum* species complex does not track with the multi-locus phylogeny (O'Donnell *et al* 2004b) Fortunately, through the power of GCPSR one natural hybrid was detected within the *F. graminearum* complex which possesses some alleles of *F. asiaticum* (Asian parent) in a mostly *F. meridionale* (South American parent) background (O'Donnell *et al* 2000)

9) The Internet-based tool FUSARIUM-ID v.1.0 (Geiser *et al* 2004) for the identification of fusaria represents a significant step towards accurately correlating host range, geographic distribution and toxin potential with species limits

10) The recent completion of the whole genome sequence for *F. graminearum* (http //www.broad.mit.edu/annotation/fungi/fusarium/), coupled with plans to sequence a representative of the *G. fujikuroi* (i.e., *F. verticillioides*), *F. oxysporum* (Di Pietro *et al* 2003), and *F. solani* species complex (H Corby Kistler, pers commun.) should result in the identification of a plethora of phylogenetically informative loci for use in high resolution MLST genotyping of agriculturally important fusaria, thereby facilitating global epidemiology via the Internet (Geiser *et al* 2004; Kang *et al* 2002; Taylor and Fischer 2003)

11) Molecular surveillance of the FHB fusaria and their toxin potential is one of our top research priorities, given that hybridization following the global transposition of foreign pathogens may give rise to super pathogens (Brasier 2001) Because of the globalization of world agriculture, we are collaborating with Dr Maarten van Ginkel of CIMMYT, Juárez, México to develop of a global network of plant disease specialists to meet the continuing threat of FHB

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Functional analysis for pathogenicity-related genes of xanthomonads using mutagenesis approach

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Gram-negative bacteria *Xanthomonas campestris* pv. *campestris* (*Xcc*) and *X. oryzae* pv. *oryzae* (*Xoo*) are casual agents of black rot of crucifers and bacterial blight of rice, respectively. They cause severe yield loss in each crop every year Understanding the genetic basis of essential steps during pathogenesis may lead to innovative approaches to control these two diseases Here we analysed two random transposon insertional libraries, which contain 16,512 (*Xcc*) and 16,712 (*Xoo*) transformants, respectively, for their ability to cause disease in susceptible host plants Seventy-five non-redundant, single-copy mutants defective in pathogenicity of *Xcc* and 73 mutants of *Xoo* were identified. The mutated genes belong to multiple functional categories, including undetermined pathways, function-unknown genes and three genes specific to *Xcc* 8004 strain that were not found in published *Xcc* ATCC 33913 genome database. Our results suggests that *Xcc* and *Xoo* evolved with similar strategies to attack their hosts, but adopted different pathway regarding their host specificity. Our work represents one of the largest scales in screening for bacterial pathogenicity-related genes and provides new insight into the global view of pathogenesis of xanthomonads

Introduction

Almost all species belong to the genus *Xanthomonas* are plant-associated bacteria, and most of them are plant pathogens with definite host range. Among them, *Xcc* and *Xoo* represent two typical plant-bacterium interaction relationships *Xoo* evolved so-called "gene-for-gene" relationship with rice cultivars where genetic relationship of matched specificity was developed between a bacterial avirulence gene (*avr* gene) and a plant disease resistance gene (*R*-gene) (Shen and Ronald 2002) As for *Xcc*, this kind of relationship has not been fully evolved so that *Xcc* has the ability to infect most cruciferous plants (Alvarez 2000) Recently, the completion of three strains of *Xanthomonas* whole-genomic sequences [*Xcc* ATCC 33913, *X. axonopodis* pv. *citri* 306 (da Silva *et al* 2002) and *Xcc* 8004 (our unpublished data)] provided a profile of genetic information and valuable predictions to explore pathogenesis of these important phytopathogens However, genomic annotations have reflected inadequacy in our current knowledge. For example, though it has predicted that more than 250 genes (about 6 % of genes in the the genome) being involved in pathogenicity of *Xcc*, many of them remain undefined. In addition, of the some 4,100 protein-coding genes annotated in each genome, only a small group of genes (< 320) has been identified experimentally in xanthomonads, and there are approximately one third (> 1,300 genes) that even haven't been assigned to any functional category (da Silva *et al* 2002) Therefore, high-throughput genomic techniques are required to

prove the functions of these genes, which are applicable not only to confirm the predictions generated by *in silico* studies, but also to gain further insight into *Xanthomonas* pathogenesis by discovering novel genes or biochemical pathways involved in infection.

Construction of two mutant libraries and screening of pathgenicity-deficient mutants

The mutant library was constructed as described previously using EZ: TN <KAN-2> transposome (Sun *et al* 2003) Two stable pathosystems, *i e*. *Xcc*-cabbage (*Brassica oleracae* cultivar Jingfeng 1) and *Xoo*-rice (*Oryza sativa* cultivar IR24) were set up to screen pathogenicity-deficient mutants Large-scale plant inoculation was conducted clone-by-clone under optimized conditions (temperature of 28~35°C, relative humidity > 95 %) To avoid false-positive result caused by phase variation or other unpredictable factors, at least four rounds of independent verification were repeated to exclude these possibilities One hundred seventy two *Xcc* and 200 *Xoo* pathogenicity-deficient mutants were obtained. Copy numbers of transposon insertion in pathogenicity-deficient mutants were detected by Southern blotting and flanking sequences of transposons were obtained by TAIL-PCR (thermal asymmetric interlaced PCR) (Liu and Whittier 1995) The identified insertion sites and flanking sequences were then analysed by searching against local or public databases

Preliminary analysis of pathogenicity-related genes

From 172 *Xcc* pathogenicity-deficient mutants, 75 non-redundant disrupted ORFs/intergenic regions were identified. Each of these mutants contains a single-copy of Tn5 insertion. As for *Xoo*, though its whole-genomic sequence has not been published, database search and alignment with similar genes indicates that at least 73 are inserted within ORFs or in intergenic regions Additionally, there are five insertions (one in *Xcc* and four in *Xoo*) located in transposase genes However, since these genes maintain as multi-copies in the genomes, accurate insertion sites of them are uncertain.

Rapid development of genomics brings a paradigm shift in studies of plant-pathogen interactions To elucidate all of the pathogenicity-related components encoded in bacterial genome has become a prevailing subject for pathologists in the near future. Based on the available genomic information on xanthomonads, our study represents one of these efforts in systematical understanding of the essential aspects during pathogenesis

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Genetic diversity of *Ralstonia solanacearum* strains in Japan and the Southeast Asian countries

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Bacterial wilt caused by *Ralstonia solanacearum* is one of the most important diseases distributed in worldwide. Japanese strains were divided into four pathogenic groups Groups I to III were pathogenic to mainly solanaceous crops, and corresponded to race 1 Group IV was pathogenic to potato, and corresponded to race 3 A dendrogram constructed based on the rep-PCR analysis of Japanese strains defined 35 fingerprint types Each strain that differed by race or biovar represented a distinct fingerprint type. The strains were separated into two main groups, one with all race 1, and the other with only race 3 Race 1 strains were further subdivided. The rep-PCR fingerprints of strains from Japan and various countries revealed two main clusters Cluster 1 comprised all biovar 3, 4, and 5 from Asia and Australia as well as biovar N2 race 1 from Japan Cluster 2 included most of the biovar 1, 2, and N2. The 16S rDNA sequence of Japanese race 3 biovar N2 strains was homologous to that of a certain strain from Indonesia. Two newly identified race 4 biovar 4 strains responsible for bacterial wilt of Zingiberaceae plants might presumably invade from either Thailand or China

Introduction

Ralstonia solanacearum is the causal organism of bacterial wilt of more than 200 species and 50 families of plants in tropical, subtropical and warm regions in the world. This bacterium is a heterogeneous species, which shows phenotypic and genetic variability. For more than four decades, *R.solanacearum* strains have been classified according to binary systems, race and biovars, based on host range and utilization of carbohydrates, respectively.

In Japan, bacterial wilt disease caused by *R. solanacearum* has been reported on more than 40 species and 20 families of plants However, the genetic background and the systematic relationship among strains have been poorly investigated.

Recent changes in agricultural systems as well as global trade in production of seeds and saplings have brought unexpected occurrences of new host plants or invasive strains Improvement of detection and identification methods is important by elucidating genetic diversity of foreign strains and indigenous *R. solanacearum* strains from the viewpoints of not only taxonomic problems but also plant quarantine matter

Recent advanced molecular techniques have been effectively applied to analyze genetic diversity or genetic relationship in certain plant pathogens as well as plant-associated bacteria.

Under these circumstances studies of genetic diversity of *R. solanacearum* strains from Japan and diverse geographical origins were investigated.

Phenotypic characteristics and pathogenicity of Japanese strains

R. solanacearum is heterogeneous and divided into five races and five biovars based on host range and utilization of carbohydrates, respectively. In Japan, races 1 and 3, biovars N2, 3 and 4 have existed so far, which were pathogenic to mainly solanaceous crops such as tomato, tobacco and eggplant for the former, and potato for the latter

Based on pathogenicity tests, the Japanese strains examined were divided into four pathogenic groups Three groups (I to III) were pathogenic to many solanaceous plants such as tomato and eggplant, which corresponded to race 1 Group IV was pathogenic to potato, weakly pathogenic or nonpathogenic to tomato, and corresponded to race 3 Race 1 strains were isolated from various plants in most parts of Japan, whereas race 3 strains were found only in cultivated potato fields in limited regions including Nagasaki Prefecture.

In biovar determination tests, strains were divided into three biovars (N2, 3 and 4) Biovars 3 and 4 were most common. Japanese N2 strains were distinct from foreign biovars 2 and N2 strains in several phenotypic traits

Genetic diversity of Japanese and foreign strains

A comparison of 16S rDNA sequences separated the Japanese strains into two groups, group 1 with strains of biovars N2, 3 and 4, which belonged to race 1, and group 2 with strains of biovar N2 that corresponded to race 3 Group 1 strains all had identical sequences, and strains representing the three biovars within the group did not differ from each other Group 2 strains had characteristic nucleotides that differed at seven positions from group 1 strains In a comparative analysis of Japanese and foreign strains based on 16S rDNA sequences, Japanese group 1 was closely related to Asian and Australian biovars 3, 4 and 5, and belonged to division 1 Japanese group 2 was homogeneous with Indonesian biovars 2 and N2 in subdivision 2b, suggesting a close relationship between them.

A dendrogram was constructed based on rep-PCR genomic fingerprints of Japanese strains The three primer sets, REP, ERIC and BOX, defined 35 fingerprint types at the 95% similarity level Each strain that differed by race or biovar represented a distinct fingerprint type. The strains were separated into two main groups one with all race 1 and the other with only race 3 Race 1 strains were further subdivided into six groups at 80% similarity. Within race 1,

each biovar (N2 and 4) separated into a single group, with high similarity within each biovar On the other hand, biovar 3 strains were divided into five groups, with low average similarity among the strains

Comparative analysis of the rep-PCR fingerprints of *R. solanacearum* strains, including six biovars from Japan and various countries, revealed two main clusters Cluster 1 comprised all strains of biovars 3, 4, and 5 (races 1, 4 and 5) from Asia and Australia, and strains of biovars 1 and N2 (race 1) from Reunion and Japan. Cluster 2 included most strains of biovars 1, 2, and N2 (races 1, 2 and 3) from 13 countries (Fig. 1) In cluster 2, the average similarity within biovar 2 from eight countries and biovar N2 strains from Brazil was 94 and 65%, respectively, whereas average similarity between biovar N2 strains from Brazil and Japan was 21%

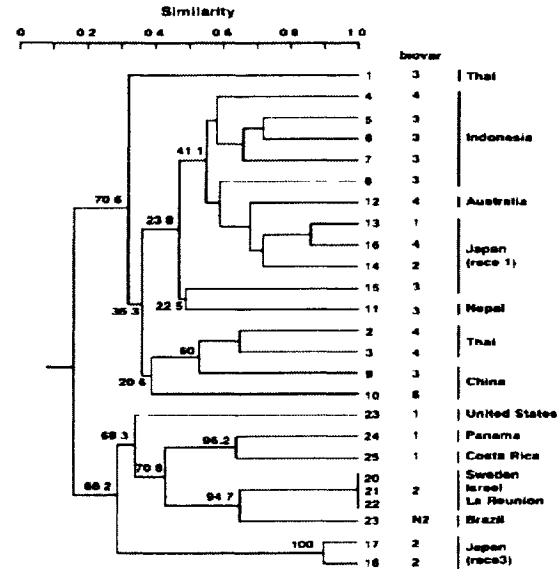

Fig. 1 Dendrogram showing genetic diversity of *R. solanacearum* isolated from Japan and foreign countries based on rep-PCR

New *R. solanacearum* strains parasitic to Zingiberaceae plants in Japan

Ginger (*Zingiber officinale*) and mioga (*Z. mioga*) are important sources of spice or medicinal crops, which have long been cultivated mainly for food in Japan. Curcuma (*Curcuma* spp.), ornamental plants for cut flower also belonging to the same Zingiberaceae family, was introduced from Thailand to Japan. In 1995, a bacterial wilt disease of *C. alismatifolia* caused by *R. solanacearum* occurred in the cultivated fields of a few localities in Kochi Prefecture, the leading production center in the country. Subsequently, the outbreak of this disease has spread to ginger fields in 1997, and since 1999 it has expanded successively to mioga plantations in the neighboring cities within the same Prefecture. Bacterial wilt of zingiberaceous plants are caused by *R. solanacearum* race 4 strain and have been reported in several countries, but no such disease had been recorded among 14 families containing 29 species of host plants reported before 1995 in Japan.

Thus new bacterial strains above mentioned was examined to characterize from pathological, physiological and molecular biological viewpoints in comparison of indigenous strains as well as those obtained from geographically different countries

The disease symptoms of three plants were almost similar, of which yellowing and

wilting started from lower leaves, quickly spread upwards until the whole plant became entirely golden brown and wilted.

All isolates from the diseased plants were identified as *R. solanacearum* based on physiological and biochemical tests and were proved to be biovar 4. On the other hand, those from Thailand and Indonesia consisted of both biovars 3 and 4, and those from Australia and China were biovar 4.

Strains from zingiberaceous plants caused wilting of potato and marigold severely besides ginger, while tomato, tobacco and sweet pepper did not wilt but showed vascular discoloration. HR was induced in tobacco. On the other hand, neither of representative Japanese races (1 and 3) nor indigenous strains isolated from eggplant and tomato in Kochi Prefecture wilted ginger Based on the pathogenicity tests these isolates were designated as race 4 —a race previously unknown in Japan (Table 1)

Genetic diversity of the strains was tested by rep-PCR analysis using BOX, REP and ERIC as primers Analysis was carried out using selected curcuma, ginger and mioga strains from Japan, Thailand, Indonesia, Australia and China as well as representative Japanese races and biovars

Table 1 Comparison of pathogenicity between Zingiberaceae strains and indigenous race 1 and race 3 strains in Japan

Plant	Pathogenicity (wilting)		
	race 4 (ginger strain)	race 1 (tomato)	race 3 (potato)
tomato	0-M	M-H	0-L
eggplant	L-M	M-H	0
sweet pepper	L-M	M-H	0
tobacco	HR	L-M/HR	0
potato	L-H	M-H	M-H
ginger	M-H	0	0
mioga	M-H	0	0

HR hypersensitive reaction

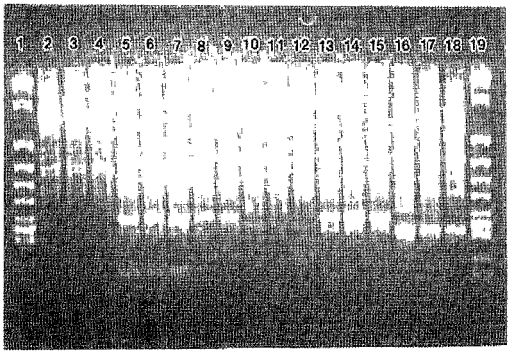

Fig. 2. BOX-PCR profiles of strains isolated from ginger and representative strains of Japanese races and biovars

Lanes 2-7 ginger strain, 8, 9, race 1/bv 4, 10,11, race 1/bv 2, 12, 13, race 1/bv 3, 14,15 race 1/ bv 4, 16-18, race 3/bv 2, 1, 19, DNA marker

The DNA profiles obtained were highly reproducible. In rep-PCR analysis of the strains, two types (Type I and Type II) of DNA fingerprint patterns were obtained. However, neither the DNA patterns of indigenous *R. solanacearum* strains isolated from various plants in Kochi Prefecture nor those of representative Japanese races nor biovars were identical to any of

the two types (Fig. 2) The DNA pattern of Type 1 strains was identical to that of several ginger and *Curcuma* spp. strains from Thailand, and that of Type 2 was identical to ginger strains, which were originated from Australia and China (Fig. 3)

A dendrogram constructed based on the rep-PCR analysis also revealed genetic diversity as well as their relationships among strains Group A (containing Type 1) consisted of all *Curcuma* strains, some ginger strains and all mioga strains, whereas group B (containing Type 2) contained mostly ginger strains (Fig. 4)

Based on the results obtained, it was considered that a possibility that Type 1 and Type 2 pathogenic strains from either curcuma or ginger were introduced independently through contaminated seed materials imported around 1995, and remained undetected until the first outbreak in *Curcuma* sp. In 1995 and subsequently in ginger in 1997. Furthermore, Type 1 strains were found in mioga after 1999. It was therefore concluded that the disease caused by these two exotic *R. solanacearum* strains has started from different origins and has been spreading in epidemic proportions through separate routes

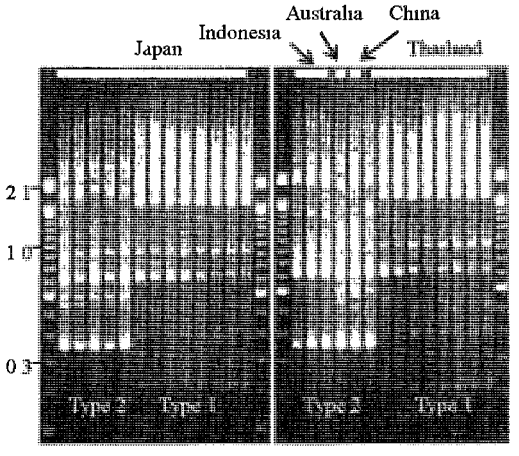

Fig. 3 BOX-PCR profiles of Zingiberaceous strains from different countries

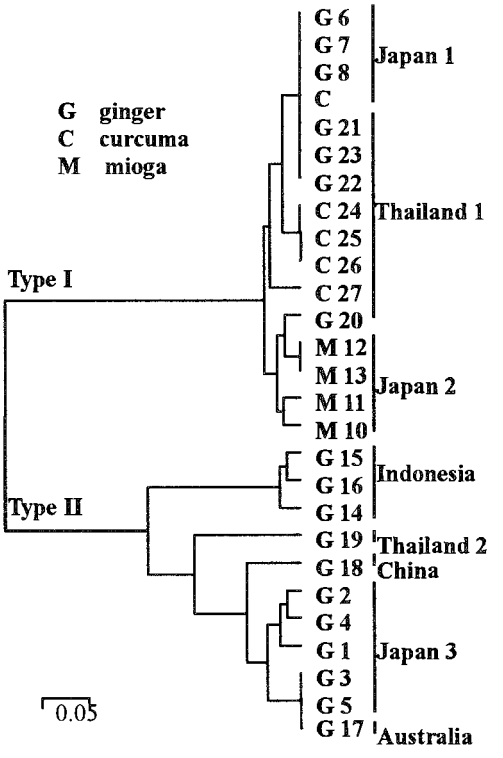

Fig 4. Dendrogram showing genetic diversity of *R. solanacearum* isolated from Zingiberaceae plants on the basis of rep-PCR

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Taxonomic analyses of soybean SDS and dry bean root-rot pathogens based on a MAFF - NRRL Collaboration

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Taxonomic and phylogenetic analyses of soybean sudden death syndrome (SDS) pathogens and dry bean root-rot pathogens were performed based on a MAFF - NRRL collaboration. Isolates of these pathogens from the United States, Argentina, Brazil and Japan were accessioned in both culture collections Detailed phenotypic comparisons of macro- and microscopic features and phylogenetic analyses of multilocus DNA sequence data, including those on the nuclear ribosomal intergenic spacer region and the single copy nuclear gene translation elongation factor 1-α, indicated that they comprised five distinct species Soybean SDS in North and South America was found to be caused by four distinct species *Fusarium virguliforme*, *F. tucumaniae* and two undescribed species of *Fusarium*. Further, dry bean root-rot in North America and in Japan was found to be caused by *F. phaseoli* and an undescribed species of *Fusarium*. Among them, an undescribed species was a common pathogen to both soybean SDS in Brazil and dry bean or mung bean root-rot in the United States, Canada and Japan.

Introduction

Soybean sudden death syndrome (SDS) has been reported from all major growing regions in North and South America (Nakajima *et al* 1993, Ploper 1993, Anderson and Tenuta 1998, Rupe *et al* 2001) and this disease has recently become a serious constraint to the commercial production of this crop. Since its discovery in Arkansas in 1972, the etiological agent of this disease has been reported as *F. solani* (Mart) Sacc. or its forma specialis, f. sp. *glycines* Roy (Roy *et al* 1997) Recent molecular phylogenetic analyses of DNA sequences, however, revealed that the *F. solani* species complex or section *Martiella* comprises at least 26 phylogenetically distinct species (O'Donnell 2000), many of which have not been described. North American isolates of the SDS pathogen have been shown to be genetically homogeneous and closely related to a root-rot pathogen of *Phaseolus vulgaris* L., *F. solani* f. sp. *phaseoli* (Burkh) W. C. Snyder & H. N. Hansen (O'Donnell 2000, Li et al 2000, Rupe *et al* 2001) In 2000 and 2001, field surveys were conducted in Argentina for the soybean SDS pathogen as part of a collaboration between INTA-EEA, Marcos Juárez, Argentina and JIRCAS, Japan (Aoki et al 2003) Twenty strains of *Fusarium* were recovered from soybean plants exhibiting typical symptoms of the disease. These isolates were deposited to the Microorganisms

Division of the MAFF Genebank System (MAFF) at the National Institute of Agrobiological Sciences (NIAS, Tsukuba, Ibaraki, Japan) and in the Agricultural Research Service Culture Collection (NRRL) at the National Center for Agricultural Utilization Research (NCAUR, USDA/ARS, Peoria, Illinois, USA) A systematic and phylogenetic study on soybean SDS pathogens and related fusaria was initiated. In addition to the new soybean SDS isolates from Argentina, strains of soybean SDS pathogens in the United States and a dry bean root-rot pathogen, *F. solani* f. sp. *phaseoli* in the United States and Japan stored at NRRL, FRC (The Pennsylvania State University, USA) and MAFF were examined. Strains of *F. solani* f. sp. *phaseoli* were included in the study because of their close phylogenetic relationship to the SDS pathogen (O'Donnell 2000) Also in 2002 and 2003, new accessions of soybean SDS pathogens from Brazil and Argentina, mung bean (*Vigna radiata* (L.) Wilczek) root rot pathogens from Canada were made at MAFF and/or NRRL and included in the study. The objective of this research was to compare North and South American isolates of the soybean SDS pathogens and their allies morphologically and molecularly using genealogical concordance phylogenetic species recognition (Taylor *et al* 2000), to understand their genetic diversity and species limits

Materials and methods

In total, 24 Argentinean, 14 Brazilian and 8 USA isolates of soybean SDS pathogens, 3 USA and 2 Japanese isolates of dry bean root-rot pathogens, 2 Canadian isolates of mung bean root-rot pathogens, and 1 isolate from an unknown host from the United States were examined and compared systematically and phylogenetically. All strains included in the study are stored by lyophilization or in liquid nitrogen vapor at the Agricultural Research Service Culture Collection (NRRL), National Center for Agricultural Utilization Research and at the MAFF Genebank System (Microorganisms Division), National Institute of Agrobiological Sciences Typical strains of *F. solani*, including biological species within the *F. solani* species complex comprising mating populations I-VII, preserved at NRRL and MAFF were also examined for comparison.

Detailed procedures for investigating morphological characters were described in Aoki *et al* (2003) *Fusarium* strains were incubated on potato dextrose agar (PDA) and SNA (Nirenberg 1990) in 9-cm plastic Petri dishes at 20°C in complete darkness, under continuous fluorescent light or under daylight to study morphological and cultural characteristics Average and standard deviation (S.D.) in the size of conidia were based on the measurement of 50 randomly selected conidia, based on the number of septa and cultural conditions Molecular phylogenetic analyses of DNA sequences from the strains followed O'Donnell (2000) and Aoki *et al* (2003) DNA sequences from the entire nuclear ribosomal intergenic spacer

(IGS) region, the nuclear ribosomal internal transcribed spacer (ITS) region and domains D1 and D2 at the 5' end of the nuclear large subunit rDNA (28S), and a portion of the translation elongation factor (1-α) gene were obtained using an Applied Biosystems-Hitachi Prism 3100 or 3730 genetic analyzer Sequence data were edited with Sequencher version 4.1.2 (Gene Codes, Ann Arbor, MI) and then the aligned sequences were analyzed phylogenetically in PAUP* version 4.0b10 (Swofford 2002), using maximum parsimony.

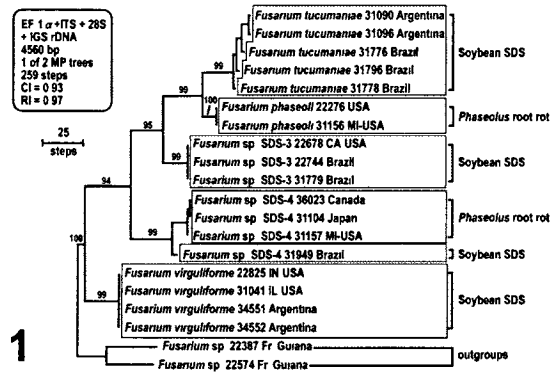

Fig 1 One of 2 most-parsimonious phylograms showing evolutionary relationships among the soybean SDS and *Phaseolus* root rot pathogens The combined data set of 4560 aligned base pairs was rooted with sequences of *Fusarium* spp. NRRL 22387 and NRRL 22574. Strain numbers are those of NRRL

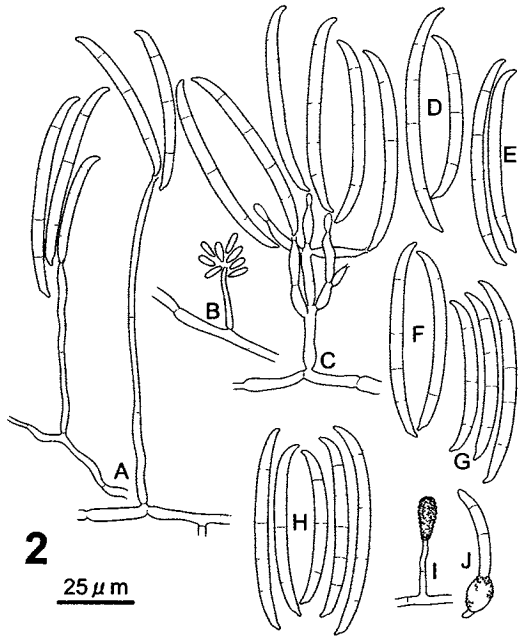

Fig. 2. Microscopic morphology of *Fusarium tucumaniae* isolated from soybean plants exhibiting typical SDS symptoms in Argentina. A: Aerial falcate conidia with a foot-cell formed on tall conidiophores B: Aerial minute conidia formed on a short conidiophore C-H: Sporodochial falcate conidia with a foot-cell and a branched conidiophore I-J Chlamydospores A, H-J on SNA; B-G on PDA A-C, H-J NRRL 34546 = MAFF 239252; D: NRRL 34547 = MAFF 239253, E: NRRL 34548 = MAFF 239254; F: NRRL 34549 = MAFF 239255, G NRRL 34550 = MAFF 239256.

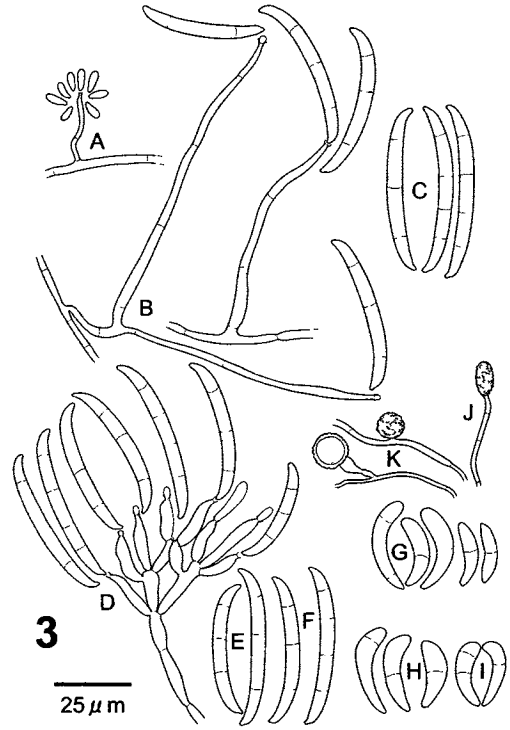

Fig. 3 Microscopic morphology of *Fusarium virguliforme* isolated from soybean plants exhibiting typical SDS symptoms in Argentina A: Aerial minute conidia formed on a short conidiophore B: Aerial falcate conidia with a foot-cell formed on tall conidiophores C-F: Sporodochial falcate conidia with a foot-cell and a branched conidiophore G-I Comma-shaped sporodochial conidia J-K: Chlamydospores A-C, J on SNA; D-I, K on PDA. A-G, J, K: NRRL 34551 = MAFF 239257; H: NRRL 34552 = MAFF 239258, I NRRL 34553 = MAFF 239259

Results

Parsimony analysis of the combined DNA sequence data (4560 bp) indicates that SDS of soybean is caused by four phylogenetically distinct species while strains causing dry bean root-rot in the United States and in Japan were resolved as two phylogenetically distinct species (Fig 1) One of these latter two species also contains strains that are responsible for soybean SDS in Brazil and for mung bean root-rot in Canada.

Detailed comparisons of conidial morphology revealed that the soybean SDS and dry bean root-rot pathogens share unique, aerial conidial features in that they all form septate conidia with a foot-cell on tall and mostly simple aerial conidiophores (Figs 2A & 3B) and nonseptate minute ellipsoidal conidia (so-called microconidia) on short aerial conidiophores (Figs 2B & 3A) These features were not observed in typical strains of the *F. solani* species complex represented by mating populations I-VII Most (21 strains) of the Argentinean soybean SDS pathogens produce diagnostic septate sporodochial conidia (so-called macroconidia; Fig. 2C-H) that are longer than those of the other soybean SDS pathogens in the United States and Brazil and those of the dry bean root-rot pathogens in the United States and Japan. The Argentinean pathogen was described as *F. tucumaniae* T. Aoki, O'Donnell, Yos Homma & Lattanzi (Aoki *et al* 2003; Fig. 2), which was also discovered in Brazil causing soybean SDS (7 strains) The soybean SDS pathogen in the United States (8 strains) was described as *F. virguliforme* O'Donnell & T. Aoki (Aoki *et al* 2003) This species also contains 3 strains of soybean SDS pathogens in Argentina (Scandiani *et al* 2004; Fig. 3) and forms unique comma-shaped sporodochial conidia (Fig. 3G-I) together with septate sporodochial conidia (macroconidia; Fig. 3D-F)) One of the other species, *Fusarium* sp. 3, which causes soybean SDS in Brazil (6 strains), produces septate sporodochial conidia (macroconidia) with rounded ends that differentiate this species The other soybean SDS species, *Fusarium* sp. 4, causes soybean SDS in Brazil (1 strain), as well as dry bean root-rot in the United States and Japan (3 strains) and mung bean root-rot in Canada (2 strains) This species is diagnosed by the production of septate sporodochial conidia (macroconidia) with a rostrate apical cell like a wedge-shaped beak and a distinct basal foot cell In addition, a dry bean root-rot pathogen (2 strains), previously known as *F. solani* f. sp. *phaseoli*, was recombined as *F. phaseoli* (Burkh) T. Aoki & O'Donnell at the rank of species (Aoki *et al* 2003) It is characterized by shorter septate sporodochial conidia (macroconidia) with an acuate apical cell and a distinct basal foot cell that is typically hooked ventrally. For the two undescribed *Fusarium* spp., i.e. spp. 3 and 4, formal descriptions are in preparation.

Table 1 Diagnostic morphology of *Fusarium* species responsible for soybean SDS, dry bean and mung bean root-rot

Species	*F. tucumaniae*	*F. virguliforme*	*Fusarium* sp 3	*Fusarium* sp 4	*F. phaseoli*
Aerial conidia					
1) Multiseptate	+	+	+	+	+
2) Minute nonseptate	+	+	+	+	+
Sporodochial conidia					
3) Multiseptate					
Average length	> 60 µm	50-60 µm	45-60 µm	50-65 µm	< 55 µm
width	< 5 µm	5-5 5 µm	5-5 5 µm	5-5 5 µm	5-5 5 µm
Apical cell	Acuate	acuate	typically rounded	wedge-shaped beak	acuate, hooked
Basal foot cell	Distinct	typically indistinct	typically rounded	distinct	distinct
4) Non(-2)septate	+/-, short-clavate	+, comma-shaped	+/-, short-clavate	+, short-clavate	+, short-clavate
Host	Soybean	soybean	soybean	soybean, dry bean, mung bean	dry bean
Distribution	Argentina, Brazil	USA, Argentina[a)]	Brazil, USA	Brazil, USA, Japan, Canada	USA,(cosmo-politan?)

[a)] Scandiani *et al* (2004)

Discussion

For differentiating the four *Fusarium* species responsible for soybean SDS and the two species that cause dry bean or mung bean root-rot, diagnostic morphological features are summarized in Table 1 The known distribution of each of the five species within this monophyletic group is as follows *F. virguliforme* – United States and Argentina, *F. tucumaniae* – Argentina and Brazil, *F. phaseoli* – United States, *Fusarium* sp. 3 – United States and Brazil, and *Fusarium* sp. 4 - United States, Canada, Japan and Brazil The discovery of a second soybean SDS pathogen in Argentina, *F. virguliforme*, was reported recently by Scandiani *et al* (2004) *Fusarium virguliforme* also appears to be present in Canada as a soybean SDS pathogen (Terry Anderson, pers commun.)

The causal pathogens of soybean SDS and dry bean root-rot have been called and described as *F. solani* f. sp. *glycines* and *F. solani* f. sp. *phaseoli*, respectively, based primarily on host pathogenicity (Roy 1997, Roy *et al* 1997) As discussed in Aoki *et al* (2003), and further demonstrated in the present study, neither the four soybean SDS pathogens nor the two dry bean root-rot pathogens form exclusive or monophyletic groups, strongly suggesting that pathogenicity to each host may have independent evolutionary origins This finding challenges the *formae speciales* naming system because it obscures the fact that genetically diverse species are being referred to by the same *forma specialis* name. Results of the present study provide a taxonomic and phylogenetic framework for understanding the genetic diversity of pathogenic *Fusarium* species that have had strong negative economic impact on soybean and green or dry

bean production within North and South America.

Results of the present systematic study serve to illustrate the collective benefit of scientific collaboration among scientists at different international culture collections who bring complementary scientific tools to bear on plant pathological problems of importance to world agriculture. Moreover, it is through the continued deposit and phenotypic and genetic characterization of novel germplasm within publically accessible microbial collections that applied agricultural science will continue to progress

Ackowledgments

Special thanks are due to Dr Y. Homma, JIRCAS, Japan and members of the soybean research group at INTA-EEA, Marcos Juárez and at INTA-EEA, Famailla, and members of the Phytopathology Section, EEAOC, Tucumán, Argentina for their assistance in obtaining fresh isolates of soybean SDS pathogens in Argentina. Cordial thanks are due to Dr J. T. Yorinori, Embrapa Soja, Londrina, Brazil, Dr M.M. Scandiani, Laboratorio Agrícola Río Paraná, San Pedro, Argentina for depositing Brazilian and Argentinean isolates of soybean SDS pathogens in NRRL and MAFF. Thanks are due to Dr Terry Anderson, Agriculture and Agri-food Canada for depositing the mung bean root-rot pathogens from Canada.

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Systems biology Initiatives in the rice blast fungus, *Magnaporthe grisea*

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Magnaporthe grisea, the causal agent of rice blast, is an economically devastating fungal plant pathogen. Whole genome sequence data has been released and over 8,800 unique ESTs of this fungus are available in public databases Recently we generated 20,000 mutants using *Agrobacterium tumefaciens*-mediated transformation (ATMT) and developed a high-throughput phenotype assay system. Thus far, over 1,000 loss of virulence and several hundred transformants, including auxotrophs, developmentally-defective and oleate-nonutilizing mutants, were obtained from an ATMT mutant library The T-DNA tagged sequences from the mutants are being rescued by TAIL-PCR technology. As the first step to establish a system for this pathogen, we developed databases to manage all of the phenomics and genomics data on these transformants Furthermore, a web-based portal system has been established for efficient data acquisition and analyses, utilizing BLAST and INTERPRO searches, gene ontology (GO) classification, expression profiling, phylogenetic and comparative analyses, and tools for additional functional genomics research. Fungal systems biology initiatives provide a platform for a system-level understanding of the biology of *M. grisea*, and they are also applicable to other fungal pathogens

Introduction

Rice blast, caused by *Magnaporthe grisea*, was developed as a model plant disease to study plant-fungi interactions This is due not only to the economic significance of this disease worldwide but to the genetic and molecular tractability of this pathogen. These features include classical genetic crossing using strains of two different mating types, extensive genetic maps, and development of transformation and gene knock-out technologies Extensive research has been conducted to understand infection mechanisms of the pathogen and defense mechanisms of the host at the cellular and molecular biological levels during the past decade. Through an elegant series of studies, the environmental cues and related signaling systems involved in infection of the host plant by the fungus have been elucidated. More than a dozen genes have been identified as pathogenicity determinants through insertional mutagenesis using REMI (restriction enzyme mediated integration) or reverse genetic strategies However, the precise mechanisms required to complete the disease cycle are not fully understood. Recent advances in genomics research are making great progress towards an ultimate understanding of pathogenesis at the biochemical and molecular biological levels In 2002, whole genome drafts of rice and *M grisea* were completed and made publically available. Currently much effort is being focused on accurate annotation of genes in both organisms

Agrobacterium tumefaciens-mediated transformation (ATMT) has long been used to transfer genes to a wide variety of plants and has also been used extensively as a tool for

insertional mutagenesis in *Arabidopsis thaliana*. More recently, several fungi have been transformed using *A. tumefaciens*. For insertional mutagenesis, this technique offers enormous potential as an alternative tool to REMI One of the principal advantages of ATMT over conventional transformation techniques is the versatility in choosing which starting material to transform Applicable to several fungi, *A. tumefaciens* can transform protoplasts, hyphae, spores, and blocks of mushroom mycelial tissue. Furthermore, ATMT generates a high percentage of transformants with a single insert of T-DNA in the fungal genomes, which will facilitate the subsequent isolation of tagged genes especially from those fungi lacking a sexual stage. Recently we developed ATMT technology in *M grisea*.

To dissect the function of potential fungal pathogenicity genes throughout the genome in the rice blast fungus, we initiated a project that includes 1) generation of mutants using ATMT 2) development of a high throughput screening technology for mutants 3) high throughput technology for DNA extraction, and finally 4) rescuing flanking sequences from mutants of interest Moreover, we are establishing an infrastructure of bioinformatics tools for systems biology.

Fungal strain used in this study

We assessed the genetic make-up of *M grisea* KJ201, a Korean field strain, using different transposons, avirulence genes, growth rate, and mating ability. Strain KJ201 is quite different from strain 70-15, for which the whole genome was sequenced, based on the copy number of transposons and avirulence genes These elements are generally useful for comparing the genetic diversity of strains

Development of high throughput screening technology

To characterize phenotypes of many transformants (mutants) at the same time, development of a high throughput screening (HTS) system is required. The concept and technology of HTS have been developed and are widely used in the pharmaceutical industry to evaluate potential candidates for new drugs However, HTS systems are not well developed in the plant sciences One bottleneck is the lack of specific sites we can target However, HTS systems have been developed by companies that develop pesticides

All single-spored mutants were grown in a 24-well tissue culture plate in liquid medium to obtain mycelia. Using these plates, we measured growth rate, pigmentation, and the colony morphology of each mutant By changing the composition of media, we can use this technology to measure the ability of each strain to conidiate.

In addition to HTS for phenotypes, we developed a high throughput technology for DNA extraction.

High throughput pathogenicity assay

Traditionally plants are grown in soil in pots to test fungal pathogenicity. However, it is not always easy to replicate the same environmental conditions in every experiment As we are all aware, disease development is the outcome of a complex combination of host plant, pathogen, and the environment We developed a protocol for growing rice plants on a tissue culture medium in the laboratory. Further, we removed rice seed coats Removing seed coats provide several advantages including efficient surface sterilization, more homogeneous germination and accelerated plant growth. It is important to pre-treat dehulled rice seeds in 100% ethanol before surface sterilization. This pretreatment reduces surface tension on the rice seed surface, rendering more thorough sterilization of the seed surface. Further, we can control the composition and concentration of ingredients in the tissue culture medium as well It is well known that most rice cultivars become more susceptible when higher levels of nitrogen are supplied in the culture medium (soil)

Mutants selected for further study

We obtained more than two thousand transformants showing defects from the primary HTS procedure. These mutants were further tested for pathogenicity defects on rice seedlings Once defects were verified on rice seedlings, these mutants were grown in liquid medium and genomic DNA was isolated. By Southern blot analysis, T-DNA insertion into the chromosome and its copy number was verified for each mutant

Identification of flanking sequences of T-DNA insertion

Pathogenicity defective mutants, verified as a single integration of T-DNA, were subjected to TAIL-PCR to rescue flanking sequences Since the genome draft of *M grisea* is available, we were able to identify ORFs based on sequences flanking the insertion site. Thus far, we have identified more than 200 loci where T-DNA was inserted. To confirm phenotypic changes by T-DNA insertion, we knocked out the same loci using a gene replacement technique.

Development of infrastructure for systems biology

In addition to our genomics research, we are constructing databases to manage the enormous amount of genomics data. Presently, a web-based portal system has been established for efficient data acquisition and analyses, utilizing BLAST and INTERPRO searches, gene ontology (GO) classification, expression profiling, phylogenetic and comparative analyses, and tools for additional functional genomics research.

Acknowledgements

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2. NEW PARADIGMS OF BIOLOGICAL RESOURCE CENTERS

Preface

WFCC-MIRCEN World Data Centre for Microorganisms (WDCM) organized the WDCM symposium entitled "Microbial Resources Centers in the 21st Century: New Paradigms" on February 16th, 1999. The symposium was immediately followed by "OECD Workshop Tokyo '99 on Scientific and Technological Infrastructure – Support for Biological Resource Centres (BRCs)" on 17-18 February, 1999.

Since these events, the term of BRCs has beard a new meaning, especially for the microbial culture collections Based on the efforts made by the Task Force on BRCs chaired by Professor Hideaki Sugawara (Japan) in the Working Party for Biotechnology of OECD, OECD published an official report entitled "Biological Resource Centres – underpinning the future of life sciences and biotechnology" in 2001

In 2002 and 2003, the Task Force on BRCs led by Dr Louis Rechaussat (France) has developed standards to be called BRCs and also a strategy to realize the Global System for BRCs In the meantime, NITE BRC was established in Japan; the EBRCN project was activated in Europe; ATCC trademarked "The Global Bioresource Center", African countries, China and Thailand organized international meetings on BRCs, and other relevant activities were carried out in many countries

Therefore, it is the opportune time for us at ICCC-10 to reach consensus about the concept of BRCs and also implementation of the concept We will revisit the new paradigm of BRCs in the special symposium II and the panel discussion to have concrete plans toward BRCs in the 21st century.

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The new paradigm of the Biological Resource Centres (BRCs): challenges and opportunities of culture collections or BRC's

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Different collections or BRC's are in different financial and environmental conditions and do not necessarily have the same challenges and opportunities In general however, three important cornerstones can be distinguished. As a first cornerstone, BRCs organize the professional conservation and distribution of biological materials and the data related to them. Key values are authenticity, genetic integrity and validity of the information provided. There is a need for global standards to assure top quality of biological materials and data through appropriate Total Quality Management The second cornerstone is the linking of BRC's to scientific centres in taxonomy, molecular genetics, biochemistry, cellular biology, biochemistry or genomics The third cornerstone, common for all BRCs is the valuation and thus demonstrable utilization of its holdings and its data. The bioeconomic logic of industry, healthcare and the research related to it is here clearly the driving force of a mainly economic reality.

Biological Resource centres (BRCs) are an essential part of the infrastructure underpinning life sciences and biotechnology. The OECD (Organization for Economic Co-Operation and Development) think tank on BRCs (1999-2004), has done a great effort of thought to define the new Biological Resource Centre and forms the basis for the future development of the actual culture and reference collections The effort, which has taken so many years and was from the beginning inspired by many WFCC members, has resulted in an important visionary document ('Biological Resource Centres, underpinning the future of life sciences and biotechnology' (http //oecdpublications.gfi-nb.com/cgi-bin/oecdbookshop. storefront))

Different cornerstones can be distinguished in any vision developed starting from the actual situation of Culture collections It is obvious that the actual culture collections are in different financial and environmental conditions and consequently at different stages of cutting their cornerstones Perfect situations do not occur We can think of a grading system for BRCs according to a number of clearly defined criteria. A tiered structure can be envisaged. In general however, the challenges and opportunities of culture collections for the next 5 years are situated in the areas discussed below.

BRCs organize the professional preservation and distribution of biological materials and the data related to them. The biological materials concerned are of all kinds bacteria, viruses, fungi, tissues, animal cells In different fields of the bioeconomy, materials are used and generated that have to be conserved together with the data and the literature references BRCs are repositories of high quality biological material from industry, research, from clinical

centres and also from the great biodiversity study efforts The quality and the stability of the materials need to be maximally guaranteed. Therefore, key values of BRC's are authenticity and genetic integrity of the material and validity of the information provided. There is a need to achieve global standards to assure top quality of biological materials and data related to them (including traceability) Appropriate Total Quality Management therefore forms a first important cornerstone. Concerning the Information Technology, culture collections are moving to a data model integrating all scientific, administrative, literature and strain data. In the last decades, bioterrorism affected microbiology on a high degree (cfr. Antrax, The Butler Case) Biological Resource Centres therefore face continually increasing Biosafety and biosecurity demands

As a second cornerstone, BRCs are, and will continue to be, centres of excellence in taxonomy, preferably linked to university departments In the future, especially genomic taxonomy and taxonomy of unculturable organisms will be of importance. Indeed, there is a traditional link between culture collections and taxonomy of bacteria and fungi This basic link needs fortification. The present developments in phylogeny, population genetics and genomics require from the taxonomist that he adapts himself and rethinks his science. The future genomic taxonomy will synthesize microevolutionary, phylogenetic and genomic data in a new synthesis

Since today only a very small part of the microbial diversity has been studied, we can expect that in the future, there will be massive incorporation of biodiversity items The needed absorption capacity of BRC's will be huge. New Technologies as automatization, robotisation and miniaturisation need to be increasingly applied.

Besides their preservation role, culture collections are important for the conservation of biodiversity. At the Earth Summit in Rio de Janeiro in 1992, world leaders agreed on a comprehensive strategy for "sustainable development" One of the key agreements adopted at Rio was the Convention on Biological Diversity (www.biodiv.org)

An important part of the biodiversity debate involves access to and sharing of the benefits arising out of the commercial and other utilization of genetic material, such as pharmaceutical products Often, the products would be sold and protected by patents or other intellectual property rights, without fair benefits to the source countries This stresses the importance of Property Rights related to the biodiversity data, as well as data and database protection.

Furthermore, culture collections also play a capacity-building role, to help biodiversity-rich countries to better understand and utilize their microbial diversity, and an important resource for public information and policy formulation. They are bodies that the public and the policy makers can call upon for objective help in developing regulations and guidelines for the safe and ethical use of biological resources

Finally, another important cornerstone common for all BRCs is the demonstrable utilization of its holdings and its data. The bioeconomic logic of industry, healthcare and the research related to it is here clearly the driving force of a mainly economic reality. Thus, the valorisation of BRC's will be the use of its treasures for the biodiscovery in the developing bio-economy, taking into account the IPR and CBD. It is clear that WFCC has a role to play in the further development of a BRC concept and in the standardizations needed. The OECD document forms the basis for further developments because of its overarching vision. Electronic linkage of the WFCC and individual BRCs to international organisations and Global networking will be of major importance to provide an effective voice in international initiatives and science policy development, e.g. the GBIF initiative, WIPO,

The critical role of Biological Resource Centers in public health

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Designing appropriate mechanisms for the security of infectious agents and biodefense-related pathogens requires the availability of centralized Biological Resource Centers (BRCs) and collaboration between government agencies, academia, industry and the public health community. Of particular relevance to meeting these requirements is the American Type Culture Collection (ATCC), which has garnered a reputation for neutrality, integrity, quality, service, and personnel with comprehensive bioresource expertise since its establishment in 1925. In recognition of its success in global surveillance for public health, The National Institute of Allergy and Infectious Diseases (NIAID) of the National Institutes of Health (NIH) has recently established the Biodefense and Emerging Infections Research Resources Repository (*bei*resources) and the Malaria Research and Reference Reagent Resource Center (MR4 Center) at ATCC, where qualified, registered scientists can obtain quality-assured materials and information they need to study organisms or reagents that might be used as agents of bioterrorism or that cause diseases such as malaria, SARS, West Nile virus, and hepatitis C. Also, in 2002 the Center of Veterinary Medicine (CVM) at the U.S. Food and Drug Administration (FDA) awarded the ATCC a three-year contract to study the mechanisms and speed of development of microbial resistance to veterinary antibiotics once they are in common use for domestic animals The goal is to test susceptibility patterns of 5,000 strains in total, which include *Escherichia coli, Salmonella,* and *Campylobacter,* identified in the last five decades

Introduction

As a consequence of recent bioterrorism events and the threat of emerging infectious diseases, the U.S. government and the scientific community recognized a specific need for a central resource of these threatening agents to enhance research activities, while controlling their quality and access to them. Possession of all these agents and training to identify the most likely ones require reference standards that are based on the complexity of analytical testing, with the more complex tests requiring the most stringent standards for personnel, research tools, quality control, quality assurance, proficiency testing, and documentation, as well as regular inspection to assure compliance with all U.S. and international regulations regarding safety, security, access, and distribution of the materials and associated data. Of particular relevance to meeting these requirements are Biological Resource Centers (BRCs) that specialize in coordinating the shared use of quality-assured biological materials and information among government agencies, industry, academia, and the public health community.

One of the established BRCs supporting the public health infrastructure is the American Type Culture Collection (ATCC) Since its founding in 1925, the ATCC, in conjunction with other public health partners, has been involved in global health services to

control, improve, and protect community health. Recognizing the organization's success in assuring the security of infectious reagents and standards, the U.S. government recently awarded the ATCC more than 130 million dollars in contracts to carry out the following programs

1) The Biodefense and Emerging Infections Research Resources Repository (***bei***resources), established by NIAID/NIH in 2003
2) The Malaria Research and Reference Reagent Resource Center (MR4 Center), established by NIAID/NIH in 1998
3) The Use of Existing Microbiological Collections to Examine Historical Susceptibility of Pathogens to Antimicrobial Agents (Drug Resistance Program), established by CVM/FDA in 2002

The Biodefense and Emerging Infections Research Resources Repository (*bei*resources)

The use of biological agents in acts of terrorism or war dates to ancient times Long before the germ theory of disease was understood, potentially toxic material from ill people or dead bodies were used against armies, catapulted into cities, or placed in water supplies in attempts to demoralize and kill perceived enemies When microbes were discovered to cause infectious diseases, research into the potential use of microbes as weapons began. In the United States, for example, President Franklin Roosevelt publicly denounced in 1942 germ warfare as "an inhuman form of warfare." Privately, he approved a top-secret plan for the U.S. to develop biological warfare capability. A year later the U.S. had a four-pound anthrax bomb. In 1950 the U.S. Army tested the spread and survival of "simulants," which were actually *Serratia marcescens* bacteria, by spraying them over San Francisco. Within days one San Franciscan was dead and many others were ill with unusual *Serratia* infections, but the Army called this "apparently coincidental " Similar tests were conducted in the New York City subway system, at the Washington National Airport, and elsewhere. The anthrax attacks in the U.S. mail in the fall of 2001 are a reminder that other potential targets of bioterrorism exist, including the food and water supplies At least 21 attacks against food/agriculture have been documented in the last 100 years Federal health agencies are evaluating and accelerating measures to protect the public from health consequences of such an attack.

Homeland security is a multifaceted endeavor, of which biodefense is a critical component The National Institute of Allergy and Infectious Diseases (NIAID) of the National Institutes of Health conducts and supports much of the research aimed at developing essential tools to detect and prevent infectious diseases caused by a wide variety of emerging pathogens, including agents that could intentionally be introduced. In September 2003 the NIAID established the Biodefense and Emerging Infections Research Resources Repository

(*bei*resources) at ATCC, where qualified, registered scientists can obtain quality-assured materials and information they need to study organisms that might be used as agents of bioterrorism or that cause emerging diseases such as SARS, West Nile virus, and hepatitis C. The targeted agents have been categorized and prioritized by the U.S. Public Health Service based on their severity in causing widespread disease, public panic and social disruption and the need for special public health preparedness and response. NIAID has also set research priorities and goals for each organism in the broad area of biodefense and emerging infections The new center named "*bei*resources" helps facilitate the understanding of the pathogenesis of NIAID Category A, B, and C Priority Pathogens and emerging infectious agents, and aid in the development and evaluation of diagnostics, therapeutics, and vaccines against these organisms It offers an alternative for universities and other institutions that no longer want to store or distribute select agents

In order to facilitate relevant research and product development, *bei*resources collects information about biodefense-related reagents and standards and disseminates this information through print, electronic media, and workshops, enhances technology transfer through development and publication of methods, and facilitates commercial development of reagents through proactive communication with biotechnology and pharmaceutical companies In addition to securing acquisition, storage, and distribution of biological agents, *bei*resources also generates new reagents as scientific advances are made. It is anticipated that, in the long-term, *bei*resources will become the Federal government's national resource and clearinghouse for specimens, reagents, and information on these organisms, reflecting a concerted effort by NIAID, CDC, USDA, and ATCC. By centralizing this function, access to and use of these materials can be monitored under strict biosurety practices and procedures

A list of currently available materials that are essential for studying the mechanisms, detection, prevention and treatment of infectious diseases may be obtained from ATCC or accessed at the *bei*resources website, www.beiresources.org.

The Malaria Research and Reference Reagent Resource Center (MR4 Center)

Malaria, the most deadly of all tropical parasitic diseases, has been undergoing a dramatic resurgence. The World Health Organization (WHO) estimates that between 300 and 500 million new cases of malaria occur each year, and that annual deaths from the disease number between 2 and 3 million. In 1997, approximately 1,800 cases of malaria in the United States were reported to the Centers for Disease Control and Prevention (CDC) Increasing resistance of the malaria parasite to effective drugs presents problems for the treatment of active infections At the same time, increasing resistance of mosquitoes to standard insecticides makes control of transmission difficult to achieve. At the International Conference on Malaria in Africa

held in Dakar in 1997, malaria experts from 35 countries and representatives from major malaria research funding agencies identified a specific need for a new biological resource center for standardized reagents and methods to ensure the comparability of results for malaria research and control

In response to this stated need, the Malaria Research and Reference Reagents Resource Center, known as MR4, was established at ATCC in 1998 as one of the four components of the Multilateral Initiative on Malaria (MIM), a cooperative effort of agencies involved in malaria research, control and development assistance sponsored by the U.S. National Institute for Allergy and Infectious Diseases The mission of the MR4 is to provide a central source of quality-controlled malaria-related reagents and information to the international malaria research community, free of charge, to qualified, registered users The reagents include parasites, mosquito vectors, molecular biology items, recombinant proteins, synthetic peptides, antibodies, antigens, molecular probes and constructs, and human reagents A process to help with a 3-part material transfer agreement between a depositor and a recipient, through MR4 as the second party, as well as a statement of the terms and conditions under which reagents are used, is in place to meet the legal requirements of registration and ownership of the material that is in the MR4 Center

In order to promote technology transfer and interaction between malaria investigators, as well as to encourage better linkage between field-based and laboratory-based research programs, MR4 also provides an electronic bulletin board for users, workshops and training programs, comprehensive online databases and printed catalogs describing the available reagents An International Advisory Committee has been established to provide guidance to the center in prioritizing reagent acquisition and serving as a liaison to the malaria community. The Noguchi Memorial Institute for Medical Research at the University of Ghana has recently been selected as the first MR4 satellite in Africa, which will increase its ability to communicate and serve the African malaria community.

As of this date, MR4 has more than 400 registered users (PIs) In 2003, MR4 acquired almost 320 new reagents, distributed approximately 850 items to scientists all over the world, and conducted two workshops Over 800 reagents are now available, including antibodies, antigens, plasmids, EST clones, and genomic DNA and cDNA libraries A complete list of current reagents may be obtained from the MR4 at ATCC or accessed at the MR4 website, www.malaria.mr4.org. The updated "Methods in Malaria Research Manual", a "working-document" of malaria protocols, is available for downloading as a Microsoft word (*.doc) or PDF (*.pdf) file. The manual is intended to give enough detail in the methodology that someone who is not an expert in the field of malaria will still be able to make use of these protocols

The Use of Existing Microbiological Collections to Examine Historical Susceptibility of Pathogens to Antimicrobial Agents (Drug Resistance Program)

Few issues evoke as much discussion and disagreement as the use or misuse of antibiotics in feed/animal production systems Increased concern over the development of antimicrobial resistance (AR) in human microbial pathogens that are also carried by feed animals has led many public health and medical professionals to advocate the elimination of subtherapeutic antimicrobials from feed animal production. However, many veterinarians and animal production specialists have been concerned that eliminating too many antimicrobials would lead to poor animal health and increased microbial pathogen loads in feed animals and thus adversely impact human health. In 1967 the National Academy of Sciences reported that the practice of adding antibiotics to animal food, while producing greater yields, might leave traces of antibiotics in meat, thus increasing drug resistance among bacteria. The Centers for Disease Control and Prevention (DCD) calls antimicrobial resistance (AR) one of its top concerns Infections caused by resistant microorganisms are becoming more serious and difficult to treat, and the cost in the United States may be as high as $4.5 billion per year An additional problem is the development of multiple antimicrobial resistance (MAR) Since 1996 more than 30,000 isolates originating from animals or their production environment have been analyzed for AR and MAR. MAR has emerged in foodborne and commensal bacteria. Many factors, including serotype, species, resistance to compounds other than antimicrobials, and movement of mobile genetic elements, influence the development of MAR

In 2002, the Center of Veterinary Medicine (CVM) at the U.S. Food and Drug Administration (FDA) awarded the ATCC a three-year contract to study the mechanisms and speed at which microbes develop them after antibiotics are introduced and become widely used on the farm The goal is to test the susceptibility patterns of 5,000 strains in total, which include isolates of *Escherichia coli*, *Salmonella*, and *Campylobacter*, identified in the last five decades Antimicrobial panels comprised of several antibiotics in different concentrations specified by the FDA are custom-manufactured. ATCC acquires and characterizes resistant strains, studies the mechanisms and degrees of resistance, and makes cultures available to medical researchers around the world. The CDC is a valuable source for this work; they provide isolates dating back to before 1950. In addition, ATCC has also obtained isolates from universities, veterinary diagnostic services, and public health laboratories To date, more than 3,000 strains have been tested, and a database has been constructed for analysis

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http //www.beiresources.org/
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The National Institute of Allergy and Infectious Diseases http://www.niaid.nih.gov/biodefense/
The Centers for Disease Control and Prevention: http://www.bt.cdc.gov/agent/
The World Health Organization: http://www.who.int/

National Biological Resource Center to establish the intellectual infrastructure for life sciences and biotechnology

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Recent progresses in microbiology have revealed that the extent of diversity of microorganisms will be much greater than previously thought and most of them are as yet unknown. The roles of biological resource centers (BRCs) are expanding into various fields: one of its most important roles will be, of course, a reference center of living microorganisms described in literature and standard organisms for specific quality control including taxonomic type strains, as culture collections have been serving till now. However, BRCs are expected to go several steps further to catch up with the technical advancement to maintain and characterize novel fastidious microorganisms such as extremophiles and others as much as possible. Needless to say, most of the reference organisms common to world-wide culture collections have to be preserved with the quality high enough to meet the global standard. In addition, new approaches to bioresources are envisaged by emphasizing their possible applications in industries based on the government's policy to stimulate industrial use of microorganisms. Screening of new drugs including lead compounds will be supported by supplying a large number of newly isolated diverse microorganisms. Also, international collaborations amongst researchers will create bioresources of novel characteristics. At the same time, technology transfer will make it possible to isolate desired novel microorganisms and supply of specific gene clones derived from genome analysis accompanied by sequence information will be of value for seeking methods of application of enzymes and other proteins encoded. Biosafety and intellectual property right are also the key issues for BRCs. The roles of BRCs will thus become diversified by the expansion of biotechnology.

Introduction

As defined in the OECD report issued in 2001, biological resource centers (BRCs) are an essential part of the infrastructure underpinning life sciences and biotechnology. Life sciences and biotechnology, including agriculture, food production, drug discovery, etc., cover diverse fields extending from the advanced research to our daily life. In addition to supplying materials, BRCs are expected to play various other roles in the related fields and the knowledge and technology of BRC staff members in handling bioresources are required to be as high as those of the scientists at various research institutes. BRCs may sometimes be affected by the government's policy in biosafety and intellectual properties and concerned with the social consensus and agreement among industries. Considering such significant roles of BRC, the Ministry of Economy, Trade and Industry of Japan decided to establish NITE Biological Resource Center (NBRC) which was inaugurated in 2002 as the first output after the OECD report on Biological Resource Centers. I shall discuss below on the roles of BRCs from various points of view.

Essential functions of a culture collection

1. As a supplier of reference organisms to maintain the standards

Examinations such as sterilization performance, activities of aseptic reagents or antibiotics as well as quality control of media are described in each of the official guidance in which the microbial "strains" to be used are specified. Strains are generally designated by national laws or regulations by the numbers in the domestic culture collection. These locally authorized data are desirably valid under the international regulations or those of other countries. This is in accordance with the certification system of BRCs that has been discussed in the BRC Task Force of OECD that the certificated BRCs to be the international official supplier for global standard for smooth international trading.

2. Depository of taxonomic type strains

Type strains of culturable species of prokaryotes have to be deposited in culture collections to show the availability. Prior to the proposal of a new species, the type strain of the species is to be deposited in appropriate "public" culture collections. This rule is employed in Bacteriological Code (1992) and spreading in other microorganisms such as yeasts and fungi. The number of validly published species is rapidly increasing as shown in Fig 1. According to the data of Dr. J. P. Euzeby, the number of prokaryotic species (including subspecies) is almost 6,000 (as of July 2004) and this is three times the number in January 1980.

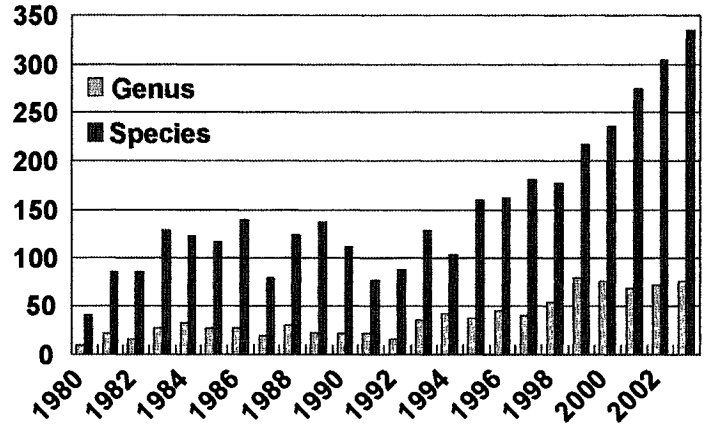

Fig. 1. The numbers of genera and species of prokaryotes published validly. (Data based on J. P. Euzeby)

In addition to the increase in number, cultivation and preservation of these organisms are becoming difficult in both techniques and equipments. One of the reasons is the recent trend of studies on extremophiles. If a problem be found in the culture preserved, the proposed taxon may lose the status. Furthermore, culture collections are requested to issue a certificate of maintenance and availability of a new species. Consequently, culture collections are heavily loaded with the responsibility for preservation.

3. Considerations of the intellectual property right

Biological specimens have been regarded as international heritages that are made accessible to all who are interested in. However, considering the potential benefit of bioresources, BRCs have to clearly indicate the limit of their use as the conditions for their deposit. CBD and related laws of the country of origin sometimes control the use of biological materials. The access procedure and conditions should be indicated in the catalogue with the name of country of origin.

4. Biosafety

Microorganisms are classified into four biosafety levels on the basis of their infectivity. The containment levels and choice of laboratories in BRCs are dependent upon those of microorganisms. Several lists of microorganisms based on the biosafety level are available. However, BRCs are requested to be able to classify microorganisms to accept newly proposed species or unidentified materials. This is closely related to the ability of identification of microorganisms. Packaging for shipment is an international matter dependent upon the biosafety level specified by the regulations of IATA and postal services. Plant quarantine is controlled by national laws. BRCs are expected to show the appropriate information on biosafety to users' community. Needless to say, highest attention should be paid to protect microorganisms from illegal use of bioterrorism. Local BRCs must have a function to reduce the number of unnecessary international transfer of biological materials by ignorant users and support their easy access with correct information attached.

5. Identification services and quality control

Correct identification of microorganisms is essential for BRCs and is indispensable for the evaluation of depositors' data and for their classification by biosafety level, in addition to giving scientific names to them. Microbial cultures are examined and their identity is confirmed every time ampoules are renewed by referencing to the data obtained at the time of deposit. Whenever possible, identification service will be made available to customers. Scientists in charge of each group of microorganisms are expected to enhance their capability to improve the quality of preserved bioresources by performing their own research in taxonomy and related fields of science.

New Approaches of Biological Resource Centers

The bioresources preserved at BRCs are mostly reference organisms such as the standard strains for various tests, taxonomic types, organisms used in scientific papers, etc. It seems that the time ripe for BRCs to develop bioresources of their own and add values to them.

Following approaches are ongoing at NBRC.

1. Development and supply of biological resources difficult to access

Restriction in acquisition of bioresources has recently become stricter. Export of biomaterials from resource-rich countries is controlled by laws stipulated on the basis of the Convention on Biological Diversity. Those who are interested in microorganisms useful in various applications seek the way to obtain desired resources. The barrier is, however, generally difficult to break for those in private sectors. Therefore, BRCs are expected to establish a system to legally transport and supply materials to users with clear guidance with respect to the range of their use. Sometimes BRCs are involved in the benefit-sharing and further contract for their industrial uses. NITE-DOB is promoting collaborations to explore novel microorganisms with researchers in South East Asian countries under the memorandum of understanding (MOU) with the institutes in individual countries in accordance with CBD. The isolated microorganisms are taxonomically characterized, provided for the assay of production of bioactive compounds for evaluation. The resultant microorganisms are deposited in the culture collections for further utilization with a certain material transfer agreement (MTA). These organisms are potentially good resources for further investigations.

2. Clones derived from genome analysis

The genome of some two hundred microorganisms has been analyzed to date. The data are valuable with a large amount of information. The clones constructed for genome analysis are made available to basic and applied fields of research and NBRC is prepared to supply clones especially in the case of those organisms that are difficult in cultivation or DNA extraction such as hyperthermophiles or eukaryotes such as the Koji mold (*Aspergillus oryzae*).

3. Collaborations with private sectors to explore research seeds by using biological resources and the associated information

While a large number of microorganisms are preserved at BRCs, most of them are scarcely used. To stimulate use of such sleeping bioresources, the resources of NBRC will be offered for screening of useful functions to the cooperative research teams including scientists from universities and private sectors. The results will be exploited for their industrial applications and the data obtained will be added to the NBRC database as they are valuable for our planning of bioresource collection. Applications for cooperative researches of this type are open to the public.

Establishment and strengthening of a local BRC network

Formation of a global BRC Network will be one of the subjects of the second term BRC Taskforce of OECD. A regional network will, therefore, be important to discuss in this connection by raising some specific problems.

1. Roles of the scientific society, JSCC

Japan Society for Culture Collections is a scientific society to promote the activities of culture collections in Japan and the related studies such as taxonomy, preservation, informatics, etc. One of the most important activities of JSCC was the recommendation of the establishment of a national biological resource center. This was drafted by JSCC in July 1999, and approved by the Science Council of Japan through the Liaison Committee on Microbiology. The annual meeting of JSCC was the place to discuss on the matters about the management of culture collections. Practical matters, such as CBD, plant quarantine, biosafety, etc., common to individual collections were also discussed in business meetings of JSCC.

2. Publication of a combined catalogue

JSCC has been publishing combined catalogues of cultures of the member collections since 1962. The latest edition was the sixth edition of JSCC Catalogue of Cultures published in 1998 that contains 8,470 species of bacteria, archaea, fungi & yeasts, microalgae, protozoa, and virus. By exploiting the opportunity to revise the Catalogue, JSCC is currently planning to establish a combined database of microorganisms preserved in the JSCC member collections and consequently a catalogue will be published.

3. Establishment of a consortium to supply microbial resources with research-quality

The diversity of microorganisms and their scientific background are so wide and diverse that a single BRC is not able to cover the entire field even. Twenty-five member collections affiliated with JSCC have their own specialty and characteristics in the collection. The total number of strains maintained in these 25 collections is 229,840 as of the end of March 2004 (Anon. 2004). Cultures distributed by these collections were 22,554. Affiliated collections are classified into five groups as shown in Fig. 2. Almost 50 percent of the total distribution is shipped by the top three collections, although the organisms preserved by them are approximately 17% of the total. These collections have personnel of administration for accession and distribution. Therefore, if they would function as virtual centers of the member collections, they will contribute to the efficient distribution of microorganisms.

Capacity building

Not only the techniques of handling materials, but the knowledge of operating BRCs has become important in laws and regulations. To perform a cooperative research is a good occasion to transfer technology and knowledge, which can be realized through workshops, training courses, etc. to be held in resource-rich countries. Essential techniques in the isolation and characterization of microorganisms as well as knowledge in their taxonomy and biosafety are important to control the management and transfer of biomaterials. Recent progresses in bioinformatics have made it possible to analyze a large amount of sequence data for the identification of various genes and their protein products. Data management is thus important to join in the BRC network as well as for in-house database construction. It should also be stressed that technology transfer will be important for global standardization that may be regarded as a non-monetary benefit for resource-rich countries.

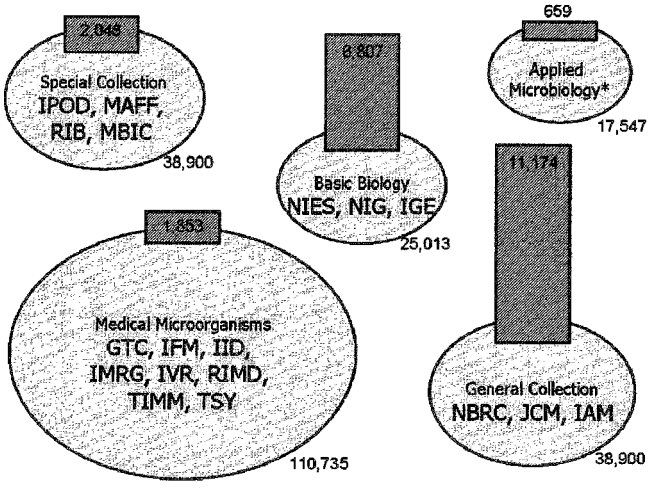

Fig. 2. Numbers of holdings (area of ovals) and distribution (length of bars) of cultures from JSCC Affiliated collections. "Applied Microbiology" corresponds to AHU, ATU, HUT, NRIC, OUT, and RIFY.

Conclusion

BRCs are envisaged as serving for an essential function within the community of life sciences and biotechnology. Industries expect national BRCs to take the leadership in the establishment of code of conduct for handling of bioresources. The biosafety level for hazardous and/or genetically modified organisms requires social consensus. One of the roles of BRCs will be to coordinate industries and consumers. An equally important role will be to expand the users of bioresources. Supply of qualified materials and appropriate information will surely support the activities of users. BRCs have to provide materials used in popular research subjects. However, it is quite common that a microorganism is not used for several decades until it is re-focused for its certain function. Materials, therefore, have to be maintained even if they are not used for a long time. The existence of a stable financial backup system makes it possible to work for the establishment of an infrastructure for the community, as mentioned in the OECD Report. In addition, each BRC is a component of the global BRC network. The long term strategy and perspectives are required for the management of national BRCs.

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Microbial diversity and pharmaceutical industry culture collections

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Pharmaceutical industry culture collections constitute extensive archives of microorganisms of a limited number of phylogenetic groups. Although industrial collections may lack taxonomic or phylogenetic breadth, they often benefit from taxonomic depth. Major public collections typically provide broad phylogenetic diversity, but may limit the depth of the collection to a few representative strains for each taxa. These differences in collection strategy reflect the functions which the collections are to serve. Pharmaceutical industry collections are biased to provide chemical diversity of small molecular weight compounds, and more comprehensive taxonomic breadth may not effectively support pharmaceutical discovery efforts. For this reason, industrial and public culture collections should not be interpreted as redundant of each other. The pharmaceutical industry cannot realistically depend on the strains found in public culture collections to serve their needs, nor can the public collections realistically under-represent industrially important taxa in their collections because industry overrepresents these. Industrial culture collections will continue to have value to the pharmaceutical, biotech and animal health industries, but the provision of collection services is likely to be by smaller more focused research organizations in the future. These smaller organizations can provide the specialized expertise of maintaining and utilizing the collections more efficiently than the pharmaceutical companies themselves.

Introduction

During the 1940's, pharmaceutical companies began to conduct microbiological research in earnest to exploit the newfound successes in antibiotic discovery. A few companies began to collect and hold microbial cultures to support this research. Since that time, many of the major pharmaceutical companies have abandoned their collections used for generation of bioactive secondary metabolites (Baker 2004). Collections of microorganisms used for genetic studies or as assay targets generally would not be included in those lost from the industry.

Qualities of industrial culture collections

Industrial culture collections developed for drug discovery purposes differ from company to company, but there are some features in common among them. In general, these industrial culture collections are not extensively characterized to taxonomy, phylogeny or to even physiology or metabolism. The majority of strains held in such collections are initial isolates from diverse sources, although a number of the smaller combinatorial biology companies, e.g. Kosan Biosciences, Maxygen, Diversa, etc., will have collections of genetically modified strains bearing metagenomic DNA within a common host. For the most part,

pharmaceutical company collections will be dominated by strains belonging to the actinomycetes and filamentous fungi, with much fewer representatives from other phylogenetic groups (Table 1). This bias is a result of the predominance of bioactive secondary metabolites within these two major groups. The Chapman and Hall Dictionary of Natural Products (Buckingham 2004) provides a compendium of identified natural product compounds, and if one reviews the number of compounds by selected phylogenetic groups, it becomes very clear why industrial collections will be rich in the actinomycetes and filamentous fungi. Because the industrial collections are strong in a limited number of phylogenetic groups, the number of closely related strains within a single group will be greater than in most public access microbial repositories. For example, if one looks at the data available on the internet for two major repositories, the American Type Culture Collection (ATCC) or the Centraalbureau für Schimmelcultures (CBS), the representation of industrially relevant fungal strains by taxonomic name is very different than that represented in Chapman and Hall (Fig. 1.). Similar differences would be observed for comparisons among taxa of the actinomycetes, eubacteria, etc.

Table 1. Representation of microbial phylogenetic groups within industrial collections.

Phylogenetic group	Relative number of strains
Actinomycetes	+++++
Filamentous fungi	+++++
Yeasts	++
α-proteobacteria	++
β-proteobacteria	++
δ-proteobacteria	++
γ-proteobacteria	+++
Firmicutes	++
Archaebacteria	+
Cyanobacteria	±

Industrial culture collections can be better characterized as safe-keeping operations rather than centers for pro-active and systematic curation. Typically there are less rigorous standards for curating the collection of microorganisms in an industrial setting than there would be at a microbial resource center. Unless a strain is actively being utilized or studied, preserved cultures will not be regularly refreshed.

Risks to industrial culture collections

Industrial culture collections are under siege for a number of reasons. Firstly, the golden years of antibiotic and natural product discovery programs are over. Secondly, mergers within the industry have made culture collections redundant and/or financial liabilities to successor companies. The majority of the largest pharmaceutical companies have discarded or sold their culture collections (Baker 2004). With the loss of these collections, a great wealth of resources has also been lost. Thirdly, the BioDiversity Treaty (Rio Convention) has also added uncertainty in the use of microbial resources and this has biased industry away from their use. Other risks to the holding or use of industrial collections continue to arise.

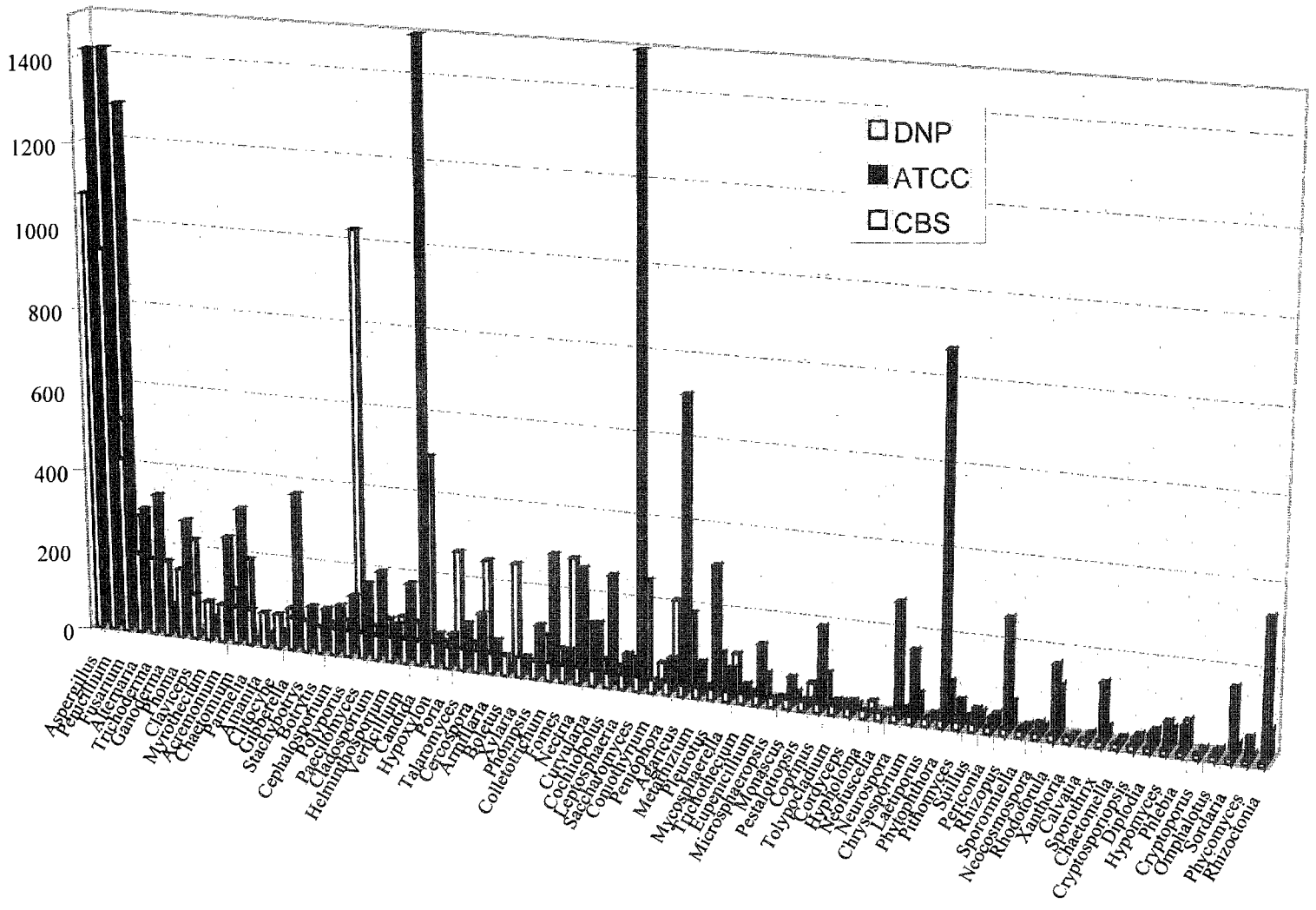

Fig. 1. Taxonomic breadth of public collections represented by numbers of strains for selected fungal genera

Bio-terrorism regulations

Since 2001, greater scrutiny has been placed on access to and use of microbial collections both in the public and private sector. Within the United States, most industrial collections are required to hold regulatory permits for many of their strains. This was required prior to 2001 for certain plant and animal pathogens, but the stringency of the regulations has increased greatly. The regulatory agency overseeing pathogenic microorganisms, the Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture, is now jointly administered with the Department of Homeland Security. The added regulatory burden associated with holding a diverse microbial collection, that most likely contains some pathogenic species, further biases pharmaceutical companies from continuing their use.

Cost of strain purchases from public repositories or creating new collections

Building a new library of secondary metabolites for drug discovery purposes from strains purchased from public access repositories is cost prohibitive. The current rate for microbial cultures, ~U.S.$150-200 per individual strain, would mean that creating a strain collection of approximately 5000 strains by acquisition would cost close to U.S.$1 million, not counting the cost of use, storage and maintenance of the strains after acquisition. Baker (2004) estimated the costs associated with creating a new microbial collection by *de novo* isolation of strains from Rio-compliant environmental samples to be less costly than the commercial acquisition costs identified above, but much more expensive than retaining existing microbial collections. Therefore, the least expensive option for utilizing microbial resources for industrial purposes, and also the most opportunistic and flexible for future uses, would be to retain existing industrial strain collections and provide access to these for scientific research supporting human, animal and plant health. How this might be accomplished logistically is the primary question.

Intellectual property limitations

Creating industrial repositories for microbial collections separate from, or distinct within, public repositories or microbial resource centers may impose additional risks associated with perceived lower value. In general, pharmaceutical companies desire to have ownership of patent or producing strains because it allows them to preserve their intellectual property. If the microbial strains were owned by a contract services provider, the pharmaceutical company would typically only be able to license the strains for patent purposes. However, with a bit of creative business development, the liabilities of this approach could probably be managed. The difficulty is that having a relatively large microbial collection held by a contract services provider may not be seen as self-sustaining in the long term. Subsidies or semi-exclusive

access privileges for interested industrial partners may be able to maintain viability of the collection in the short term, but the collection must yield some tangible product for which it would need to receive a continuing stream of royalties.

Having industrial culture collections acquired by public repositories

Although it might seem to be an ideal mutually beneficial opportunity for protecting industrial collections as well as providing greater access to those resources, the incorporation of industrial collections into microbial resource centers is unrealistic. Microbial resource centers are themselves increasingly coming under financial scrutiny, and acquiring a relatively poorly characterized collection with limited immediate use would seriously jeopardize even the most efficient culture collection. The majority of strains in an industrial culture collection will rarely be accessed over a decade of maintenance, and the microbial repository will be forced to prune the collection with no adequate rationale for selecting strains for disposal.

Recommendations for protection and utilization of industrial culture collections

The historical model of public corporations maintaining and using their own microbial collections for their exclusive use in pharmaceutical development seems to be obsolete, or at least on the wane. Two scenarios for the continued maintenance and utilization of industrial culture collections appear to be the most likely for success. The first, in which a contract services provider maintains a collection and provides access to materials in the collection for a fee, is the current business model for a number of natural product discovery collections arising from industry, e.g., MerLion Pharmaceuticals, Albany Molecular Research, Inc., and Cerylid Biosciences. The second scenario, in which a not for profit organization maintains a collection and provides access to organizations via continuing semi-exclusive memberships, is not at all common, yet has been promoted as a mechanism for building natural product discovery programs. One of the difficulties of this latter scenario is that it is subject to fluctuations in financial support depending on membership participation.

Cross-communication between public and industrial collections

One criticism of industrial collections is that they limit access to important taxa, even for academic purposes. However, in reality the breadth of diversity of taxa represented in industrial collections is probably not of that great importance to basic science. Greater breadth of diversity of most microbial phylogenetic groups will more likely be represented within microbial resource centers than in industrial collections. Also, the depth of taxonomic diversity which might be better represented within industrial collections can probably be replicated in an academic setting with a limited effort. Truly unique or metabolically

interesting strains from industrial collections routinely get deposited in public repositories for patent purposes, and in this manner can precipitate new interest in that taxon. The Budapest protocols for patent deposits will ensure that cross-communication between industrial and public culture collections will continue to occur into the future.

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Biosafety demands and the self-image of modern BRCs – Global challenges

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If we were asked to identify the most prominent demands for a modern vital Culture Collection or to fix the general outline for a new paradigm of BRCs, we would certainly mention profound long-term expertise of staff, high technical standards and high standards of quality control measurements, well-functioning management systems, reliable long-term funding and, among further characteristics, staff having a constant look at all the complex bio-legislation aspects, many of which are more or less affecting the heart of a BRC in that access to and handling and despatch of biological resources are governed by a number of different and often confusing national and international regulations. Whenever we talk about bio-legislation, we have in mind the "destiny" of an isolate/culture/strain: it is accompanied from the very first process of isolation/sampling (see CBD) through all the lab procedures (health and safety requirements) until its long-term storage and finally its despatch to serve science, application or teaching. The transportation chain with all export and shipping regulations are at the final point of this "destiny" when the strain is supplied in order to function as a valuable tool in the recipient's lab, maybe after a long and highly-paid journey, maybe in another continent.

Introduction

The complex framework of bio-legislation requires a system of concepts which did not receive such special emphasis in the past. An overall picture on the situation, aims, opearation and problems of Culture Collections has been nicely drawn by Sigler (2004) and Smith (2003). However, already in 1990 the WFCC had established and published the Guidelines for the Establishment and Operation of Collections of Cultures of Microorganisms (WFCC Standards Committee 1990). These guidelines already emphasized that Culture Collections are embedded in regulatory mechanisms to be adhered to. Issues the PQSR Committee dealt with reflect the changed global demands which also receive constant attention by the WHO of course (see WHO 1993). Over the last decades and especially over the last few years, BRCs had and have to familiarise themselves with this progressively branching out system of concepts. However, the user of the BRC, this means the bio-scientific community, is often not aware of all this and is consequently surprised about administrational expenditure, paperwork, costs and delay in delivery of the ordered cultures. Therefore, BRCs need adequate support in monetary terms for personnel and their training in order to fulfil all these aims, in keeping with the times and promoting these requirements. It is the understandable spirit of the times to develop common harmonised standards on different levels including biosafety. In that respect, BRCs are ideal

examples of scientific institutions because of the large versatile spectrum of biological resources they hold and because of their attitude to be up-to-date with the regulations (Smith *et al.* 1999). The OECD Global Forum on Knowledge Economy: Biotechnology focuses in its guidance document for the operation of all BRCs on all the above mentioned premises and key features (See OECD 2001).

An example of action in Europe

In order to harmonise standards and practice of implementation of regulations, the European EBRCN project has focused on the development of helpful basic documents that can be used as working documents with the attitude to make BRC-internal implementation easier. Whereever a BRC is located, independent of the size and whatever its main domains are: the documents cover the most relevant legal aspects having a high relevance for all Culture Collections. A BRC should comply with:

- Classification of microorganisms on the basis of hazard
- Quarantine regulations
- Ownership of intellectual property rights
- Convention on Biological Diversity (CBD)
- Safety information provided to the recipient of microorganisms
- Regulations governing packaging and shipping
- Control of distribution of possibly dangerous microorganisms
- Health and safety requirements

This list is certainly not complete but offers a basis and the respective documents remain working documents as they have to be adapted to national legislation or to the BRC-specific resources and activities.

Prevention of *malafide* use of microorganisms: bioterrorism and biological weapons discussions

Unfortunately, BRCs also have to focus their special emphasis on biosecurity: in contrast to all other biosafety aspects, the term biosecurity evolved and means precautionary measures against misuse of certain biological resources with a potential to be used as bio-weapons. Biosecurity involves protection of facilities, dependent on the resources they hold and reliable despatch/access control in order to make sure the recipient is registered or authorised to work with the material. However, certain pathogens are valuable and frequently used reference or research strains on the one hand but fall under dual-use regulations or the

Biological and Toxin Weapons Convention (BTWC) on the other hand because they were reported in one or more cases of misuse. Among such species, we find toxin producing microorganisms like *Staphylococcus aureus* or *Clostridium perfringens*, verotoxigenic *Escherichia coli* or several fungi allocated to Risk Group 1. Bio-weaponry in the context of mass destruction had sadly been already known whereas bioterrorism had only existed in abstract thinking, but unexpectedly became an incision-like issue for WFCC and the member collections. WFCC Newsletter no. 34, January 2002, was dedicated to this issue (Swings *et al.* 2002, Smith & Rohde 2002). Since a while, new projects on bioterrorism and bio-weaponry defence are evolving. One of the new ones is the New Defence Agenda (NDA) Bioterrorism Reporting Group (www.newdefenceagenda.org), a project funded by the European Union. NDA has the subtitle "A neutral platform for discussing NATO and EU Security Policies". Upon invitation, at the initial NDA meeting in Brussels, June 2004, the chance was taken to represent the voices of WFCC and the EBRCN project and to add to the discussion a scientifically based ductus: how do we deal with exchange of highly pathogenic biological material needed for research? How would we define scientific freedom and how far can this go? How do we ensure tracking and traceability of consignments containing dangerous organisms? Who are the recipients of pathogens ordered from Culture Collections? And how do we deal with transfer of knowledge? What knowledge is required to produce designed bio-weapons? Which control mechanisms can be realistically verified by Culture Collections? Of course, all these questions do not cover the existing "black market". There are probably several similar projects elsewhere and it is very important that these do not just work in parallel without a feedback to the respective (national) authorities: to the export offices, ministries of export control etc. A common line is highly desired and at last the reflux to the United Nations where international experts delegations for disarmament regularly meet.

BRC services beyond "usual" curator activities: intermediary consulting service on the laws

High quality requires sufficient control mechanisms, biosafety requires responsibility on many levels of human action including transfer of knowledge. The WFCC is a wonderful forum for Culture Collections, for detection of their problems and so WFCC functions as an organ for communication between the individual members and the world-wide scientific community. As a result of the communication with the user/customer, the BRCs function as mirrors and can articulate where the problems are. BRCs ideally follow their self-image in that they also offer intermediary consultation service in the bio-legislation area which can be quite time-consuming: due to the complexity, this goes far beyond the FAQ level and demonstrates that BRCs are recognised to be institutions carried by true expertise. Such activities are moving

away from scientific work but are terribly needed. Furthermore, BRCs send experts to conferences and meetings dealing with matters of bio-legislation and on the other hand offer seminars or individual training on these issues so that they are centres of information and advice.

The complexity of transport regulations and new shipping highlights

The ever-changing postal, packaging and transport regulations for microorganisms have been in the limelight since years and our efforts towards certain changes in the UN Model regulations ("Orange Book") only recently culminated in a successful decision process rewarding our input. This happens in times when shipping regulations are becoming stricter and controls tighter, when costs of dangerous goods transport (Risk Group 2 organisms) are climbing extraordinarily and when letter post (containing harmless organisms) is being irradiated. This success of WFCC made clear that a constant and positive contact with national and international regulatory bodies is of high relevance and can lead to fruitful co-operation and the development of new pathways while contributing considerably to the distribution of information and raised awareness. With regard to our paradigm question, this co-operation with the authorities could even be defined as one of the missions of modern BRCs since some of the worlds leading scientists with profound special knowledge are working with BRCs. Due to their daily work, they have hands on practical knowledge of implementation of bio-legislation and address the problems so that they are trustworthy co-operative partners for regulatory authorities. A very outstanding example for this aspect is that WFCC has been granted observer status to the UN Sub-Committee of Experts for the Transport of Dangerous Goods (UNSCETDG). This UN Committee publishes the recommendations for dangerous goods shipping for all modes of transport. An overview on all questions on packaging and international shipping of biological material is given by a DSMZ brochure (Rohde 1999). Another example of WFCC activities is that WFCC goals were highlighted during the UN BTWC Meeting of Experts for disarmament in August 2003, UN, Geneva, where tracking and traceability of packagings containing infectious substances was a topic among others. So, WFCC as a global initiative with a very broad basis of expertise and with member scientists who have good contacts to the large national or international specialist societies like IUMS, FEMS, ASM, SGM or VAAM has best chances to be heard and we have experienced how deeply recognised this input is. A prerequisite, however, is that we have active individuals who keep going.

Summary: consequences for designing a new paradigm of BRCs

Because a new paradigm of BRCs cannot be seen without the international context, key recommendations for modern BRCs should no doubt include global, co-operative thinking

together, not thinking alike, towards harmonisation of standards, procedures and good practices and positive thinking towards careful but active development of models for shaping the future. BRCs are expected to be exemplary prototype institutions being concerned about being on "the safe side" and not outside the law. Due to growing responsibility and the necessity of professional staffing including the bio-legislative area and in order to maintain the credibility of Culture Collections/BRCs, their status within the individual countries should be upgraded where necessary as funding is the outstanding most crucial problem in many cases. The role of the Culture Collections/BRCs cannot be overestimated whether we look at the precious biological resources or at the precious expertise of staff.

Abbreviations used

ASM	American Society for Microbiology
BTWC	Biological and Toxin Weapons Convention
CBD	Convention on Biological Diversity
EBRCN	European Biological Resource Centres Network
FEMS	Federation of European Microbiological Societies
IUMS	International Union of Microbiological Societies
PQSR	Postal, Quarantine and Safety Regulations Committee of WFCC
SGM	Society for General Microbiology
VAAM	Vereinigung für Allgemeine und Angewandte Mikrobiologie, Association of General and Applied Microbiology

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3. PROGRESS IN THE RESEARCH ON AGRICULTURAL MICROORGANISMS

Overview of the MAFF Genebank project

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Keywords: culture collection, database, genetic resource, microorganism, workshop

Introduction

The MAFF (Ministry of Agriculture, Forestry and Fisheries in Japan) Genebank Microorganisms Section, established in 1985, consists of a central bank (Genebank, NIAS) and 12 sub-banks (NARC, NIFTS and 10 institutes). The Genebank contains various microorganisms including fungi, bacteria, yeasts, viruses, phages, nematodes, protozoa, and insect cells. These microorganism resources are distributed not only within Japan but all over the world. Here we describe the activities of the MAFF Genebank

Activities of the project

1. **Preservation and Distribution of the MAFF strains:** MAFF Genebank preserves 20,472 microbial strains that are preserved by cryopreservation for fungi, by freeze-drying for bacteria and yeasts and by L-drying for plant viruses (Fig. 1). The MAFF Genebank is characterized by a wide collection of plant pathogens, e.g., *Pyriculalia grisea* and *Xanthomonas oryzae* pv. *oryzae*. The numbers of strains deposited and distributed amount to 500-800 (Fig. 2) and 700-800 a year, respectively.
2. **Exploration for Collecting of Various Microorganisms within Japan:** Three to four explorations within Japan are done every year. We have explored many areas in Japan from Okinawa and the Bonin Islands to Hokkaido for useful microorganism resources including bacteria that produce polysaccharide-degrading enzymes, fungicide-resistant phytopathogenic fungi, mushrooms, viruses causing diseases in fruit trees and so on. In 2003 the Hokuriku, Shikoku and Hokkaido areas were searched for nematodes and pathogenic fungi. Fourteen nematodes, 17 fungal isolates of a *chrysanthemum* wilt disease pathogen and 25 fungal isolates of some herbaceous leaf blight diseases pathogens were obtained and characterized. These isolates were deposited into the MAFF Genebank and will be available to the public.
3. **Characterization of the MAFF strains:** In 2003, 12,738 data on characterization were

collected. Items of characterization included pathogenicity, sequencing (rDNA and ITS region), gene (toxin and antibiotic production), host range, microscopic morphologies, race, production of enzymes, and others. These data are stored in the MAFF database, and some data were presented in papers.

4. **Publications:** To promote the use of our strains, MAFF Microorganism Genetic Resources Manuals are published every year and the latest is No. 16, which describes enzymes produced by filamentous fungi. The manuals can be retrieved from our web site as PDF files. We also publish annual reports on exploration and on activities of the MAFF Genebank; the former are available on our web sites (http://www.gene.affrc.go.jp/micro/).
5. **Construction of Web Sites and Databases:** All of the data about deposited strains are stored in a database, and users can access with them via the Internet. Information needed to access the Genebank (how to deposit your strains or to get the strains you want) is also obtained from our web pages.
6. **Provision of Financial Support to Several Microorganism Studies:** The Genebank provides domestic institutes studying microorganisms with financial support. In 2003, 4 projects were supported.
7. **Organization of the International MAFF Genebank Workshops:** Every 3 or 4 years, international workshops are held, where studies of agricultural microorganisms are presented and discussed actively. Under the latest theme "Diversity and Use of Agricultural Microorganisms", 19 topics were presented by researchers from China, Korea, Philippines, Thailand, USA and Japan.

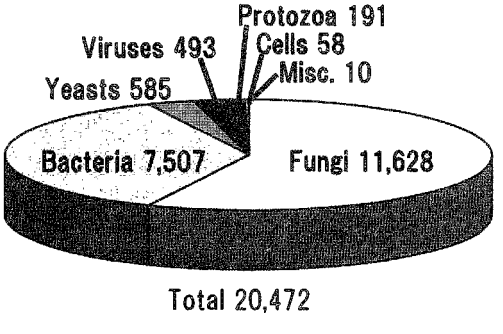

Fig.1 Details of MAFF strains in 2003

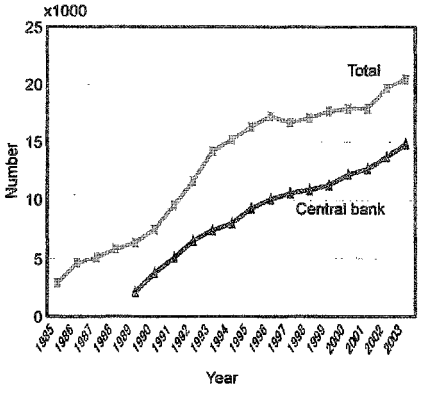

Fig. 2 The number of preserved strains in the central bank and total number in the MAFF Genebank

Current status and future prospects of collection and use of microbial resources in ACCC

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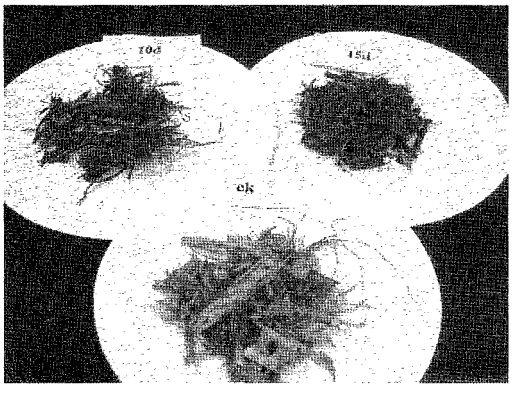
Key Words: biodegradation, biodiversity, bioferlilizers, microorganisms inoculation

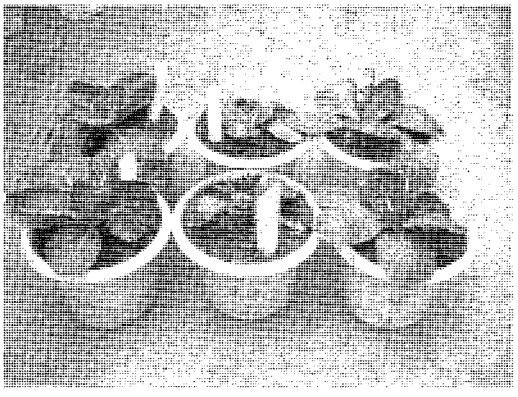
In China, research and application of agricultural microbial resources were mostly focused on mushrooms rhizobium inoculation, biofertilizers, biocontrol, microbial foods, and marsh gas. ACCC's mission includes the collection of microbial resource diversity, the zoology of soil microorganisms after microbial inoculation, isolation and screening of strains which degrade pesticides, faeces and straws, rapid identification of mushrooms, and biofertilizer manufacture.

Part 1: Straw Biodegradation

Table 1: Content of humic acid and humic substance, WSC/organic N, T value

Treatment	Humic Acid		Humic Substance	WSC/OrgN		T(C/N terminal:original)	
	10d	15d	10d	10d	15d	10d	15d
1 CK	0.337a	1.173b	1.835d	2.293cd	2.113a	0.95a	0.63a
2 A	0.343a	1.376ab	2.613bc	2.532bcd	1.486ab	0.74abc	0.48ab
3 B	0.375a	1.231ab	2.252cd	1.851d	1.529ab	0.61c	0.41bc
4 H	0.347a	1.266ab	2.302cd	1.890d	1.232b	0.69bc	0.37bc
5 A+B	0.294a	1.367ab	2.094d	3.783a	2.008ab	0.87ab	0.44bc
6 B+H	0.332a	1.372ab	2.839ab	3.139abc	1.919ab	0.77abc	0.39bc
7 A+H	0.364a	1.274ab	2.843ab	3.260ab	1.824ab	0.73abc	0.41bc
8 A+B+H	0.374a	1.628a	3.313a	2.610bcd	1.845ab	0.58c	0.31c





—3 highly effective straw-degrading strains have been isolated and identified to be *Trichoderma viride*, *T. harzianum* and *Paenibacillus pabuli.*

—Straw Inoculated with these 3 strains has been tested with the parameters like the content of humic acid and humic substance and the value of WSC/organic N and T value(Terminal C/N:Original C/N).

Part 2: Biocontrol of Soilborne Diseases in Vegetable Production

In our research, two bacteria and two Actinomyces against *Ralstonia solanacearum* were selected after pot experiments, in which the protect efficiency was 70% or 50%. One fungus and three Actinomyces against *Fusarium oxysporum* were selected after pot experiments, in which the protect efficiency was 50%. Two bacteria, two Actinomyces and three fungi against *Phytophthora capsici* were selected after pot experiments, in which the protect efficiency was 90% or 70%.

Part 3: Isolation and Application of Rhizobium

In the past 50 years, ACCC have obtained more than 1500 strains from almost 5000 portions of indigenous legume-plant rhizobium samples collected from 20 provinces across the country. In 2002, more than 15 institutes and 60 persons of microbiology were organized by ACCC to isolate and screen legume rhizobium all over China. In our study, we have used 26 strains of *R. fredii* and 25 soybean cultivars for symbiotic association formative efficiency. The nitrogenase activity is usually 3-6nmol$L^{-1}g^{-1}h^{-1}$(CZ142). The nitrogen fixation ability of *R. fredii* to soybean cultivars, Yuejin-5, Shanning-2, Ludou-4, Minquan round bean, Shanghai early bean is similar or superior to that of *B. japonicum* 15006. Compatibility of *R. fredii* towards soybean cultivars is stricter than that of *B. japonicum*. The average nodulation rate of 26 strains against 203 soybean cultivars is 49%, among which strain 15067(USDA 191) gives 89% in nodulation. Summer soybean cultivars, Yuejin 5, Ludou 4 and Shaming 2 were inoculated with 4 strains of *R. fredii*. The seed production was increased by 9.4%-31.4%.

Part 4: Rapid Identification of *Lentinus edodes* and *Pleurotus* spp.

Antagonism, isozyme, intergenic spacer (IGS) and random amplified polymorphic DNA (RAPD) have been chosen for quickly identification of *L. edodes*. In the present investigation, 43 *L. edodes* strains including 41 cultivated strains cultivated on a large scale and 2 wild strains were collected from China.

On the basis of the fruit-body morphology, antagonistic test, isozyme and RAPD analysis were used in the study of genetic diversity of 79 strains which were classified into *P. sapidus, P. ostreatus, P. florida, P. sajor-caju, P. pulmonarius* and *P. cornucopiae,* respectively.

It makes possible to identify species, clear synonyms of strains and give the basis for genetics and breeding of oyster mushrooms.

Part 5: Isolation and Evaluation of Phosphate-dissolving Microorganisms

Phosphate-dissolving microorganisms in soils play an important role in phosphorous cycling, converting insoluble phosphate into soluble forms. Three strains of P-dissolving fungi were obtained. Two of them, P8 and Pn1 were identified as *Penicillium oxalicum*, and the other one *Aspergillus* sp. In plate assay, the strain of *P. oxalicum* P8 showed higher capability to dissolve $Ca_3(PO_4)_2$, $Ca_8H_2(PO_4)_6$ $5H_2O$, $CaHPO_4$, $FePO_4$ and bone meal than ATCC20851 and ATCC14581. However, in broth assay, P8 consistently demonstrated higher efficiency to dissolve Morocco rock phosphate (MRP) than ATCC20851. Results of incubation of MRP with P8 and Pn1, using NO_3-N and NH_4-N as N sources, indicated that both NO_3-N and NH_4-N in broth enhanced the release of P in MRP, and that NO_3-N form was more efficient than NH_4-N.

Collection and identification of plant pathogenic *Fusarium* in Thailand

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Introduction

Fusarium is one of the most devastating plant pathogen ever causes economic crops diseases in countries. In Thailand, this pathogenic genus also causes major plant diseases. Many crops severely destroyed are field crops, tuber crops, vegetables, ornamental plants, and fruit crops. Having many kinds of plant hosts, survival in soil habitat for years, natural genetic variation, and toxic substances production, *Fusarium* collection and identification would ideally be the useful and important task to reach the knowledge of its origin and epidemic as well as distribution resources. Each identified *Fusarium* species culture has been preserved in the Culture Collection of Department of Agriculture (DOA) for 3 years. The culture, which its viability and variation have been checked every 6 months, is very useful for further valuable researches such as the natural morphological variation, molecular structure study, DNA identification, morphological identification and also toxic substances production

Materials and Methods

Plant pathogenic *Fusarium* was isolated from plant disease specimens by tissue transplanting method on WA (Water Agar) and PDA (Potato Dextrose Agar). Each specimen cut into 3x3 mm pieces were immersed in 10% Chlorox and placed on WA. After incubated at 26-28℃ for 24-26 hrs. mycelial tip was transferred to PDA. Pure culture of *Fusarium* was selected by single spore technique by transferring *Fusarium* spores into sterile distilled water in vial, streaking spore suspension on WA and incubating at 26-28℃ for 16-24 hrs. Spore germination was checked and collected under 10x objective lens of a compound microscope and transferred to PDA. Morphological identification of *Fusarium* was based on pure culture, followed by Nelson *et al.*'s method (1983). Colony characteristic as well as pigment, sclerotial and sporodochial formation was investigated on PDA and CLA (Corn Leaf Agar). Slide culture method was used to study sporogenous cells, phialides, microconidia and macroconidia of each *Fusarium* species. Morphology of macroconidium, conidiophore and chlamydospore were investigated after placing culture on CLA under NUV (Near Ultraviolet) light at 26-28℃ for

10-14 days. Four practical preservation techniques of *Fusarium* culture, lyophilization, 10% glycerin at -80℃, dried filter papers at 8-10℃ and dried garden soil at 8-10℃ were studied. Viability and variation of isolates were checked on PDA every 6 months.

Results and Discussion

From a hundred and five of plant-diseased specimens were collected from plantation areas of Thailand during October 2000 - September 2003, eighty-eight isolates of *Fusarium* were obtained from tissue transplanting method isolation. Species identification based on pure culture revealed 7 species and 8 formae speciales (f. sp.) as follows; *F. graminearum* from ear scab disease of wheat, *F. moniliforme* from stalk rot of corn, *F. proliferatum* from Bakanae disease of rice, *F. solani* from sudden death disease of soybean and black spot of potato, *F. subglutinans* from fusarium wilt of sugarcane, *F. semitectum* from dirty panicle disease of rice.

Most of *F. oxysporum* isolated from plant disease was classified systemically into 8 formae speciales (f. sp.). They were *F. oxysporum* f. sp. *asparagi*, *F. oxysporum* f. sp. *cubense*, *F. oxysporum* f. sp. *gladioli*, *F. oxysporum* f. sp. *lycopersici*, *F. oxysporum* f. sp. *phaseoli*, *F. oxysporum* f. sp. *tracheiphilum*, *F. oxysporum* f. sp. *vasinfectum* and *F. oxysporum* f. sp. *zingiberi*. Colony characteristic and morphology of species derived from Thailand resemble to those from *Fusarium* keys of Nelson *et al.* but sometimes there was a difference. That's because of variations of *Fusarium* occurring naturally. This occurrence would be emphasized for identification study on *Fusarium*.

The culture preservation of *Fusarium* species has been studied for its maintenance for 3 years. According to the long viability and no variation after preservation, lyophilization has been the best preservation compared to 10% glycerine at -80℃ which has been also better than preservation on dried filter papers at 8-10℃ and dried garden soil at 8-10℃. Dried preservation method, when preserved more than 2 years, sometimes have a lost of viability and contamination so that lyophilization and 10% glycerin at -80℃ method would a good alternatives for long term *Fusarium* preservation.

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Maintenance of microbial genetic resources by cryopreservation and freeze-drying

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Keywords: culture collection, viability test

Introduction

MAFF (Ministry of Agriculture, Forestry and Fisheries of Japan) Genebank was established in 1985. The microorganisms section consists of the central bank (NIAS) and 12 sub-banks (NARC, NIFTS, NIFS and 9 institutes) mainly located in Tsukuba. The central bank of the section preserves microbial strains using cryopreservation and freeze-drying techniques. The preserved microorganisms are tested for viability at regular intervals. The data from viability tests are useful for selecting a preservation procedure for a new deposited strain if they are summarized to a table in an appropriate format. In this study, we described the viability of microbial strains after one-month and one-year of preservation.

Materials and Methods

Freeze-drying: Cells suspended in 10% skim milk with 1.5% monosodium glutamate were dispensed to ampoules, frozen at -40℃ overnight and freeze-dried. To revive the freeze-dried cells, they were re-suspended in 100 μl of water. The suspension was transferred onto an appropriate plate medium and incubated. Visible growth on the plate was assessed as successful preservation.

Cryopreservation: Mycelial discs (6 mm diam.) were cut out of an agar plate on which mycelia were growing. Five or ten discs were put into a vial containing 1ml of 10% glycerol. After cold-hardening and freezing (-70℃), the vial was transferred to an atmosphere of liquid nitrogen (-165℃). To revive the frozen mycelia, a vial containing frozen cells was thawed quickly in a water bath at 30℃. The discs were put on an appropriate plate medium and incubated. Visible growth in at least 80% of discs was assessed as successful preservation

Results and Discussion

In 2003, the microorganisms section and the central bank maintained 20,472 and

14,836 microbial strains, respectively. At regular intervals preserved samples are tested for viability and the records of the tests are input to the database of the MAFF Genebank. The viability data have been accumulated since 1985, and 27,877 bits of data were stored in 2003. These data were processed to give a table showing genus names, numbers of tested strains, numbers of strains that survived one-month of preservation, and numbers of strains that survived one-year of preservation.

In the preservation of yeasts, 99.0% (100 strains /101 tested strains) of strains could be preserved successfully; only a *Candida* strain failed to survive for one month. Of the 1,996 bacterial strains, 1,992 strains survived one-year preservation; three *Lactobacillus curvatus* and one *Pseudomonas fluorescens* could not be preserved. As for actinomycetes, 237 tested strains could survive one-year of preservation. Although a few microorganisms could not be preserved by the freeze-drying method, the method was found to be an excellent one.

In the MAFF Genebank, fungal and oomycetous cells are preserved using cryopreservation. Of 6,681 fungal strains, 6,631 (99.3%) and 6,578 (98.5%) strains survived one-month preservation and one-year preservation, respectively (Table). In the preservation of oomycetes, 72.5% (300/414) and 63.8% (264/414) strains are could be preserved successfully for one month and one year, respectively (Table). Oomycetous strains are difficult to preserve by cryopreservation as known previously (Smith 1982). Our results also show low viabilities of oomycetes, and that successful preservation depended on the strain. Cells that can not survive cryopreservation are reluctantly maintained by subculture on slant.

Table. Cryoprservation of fungi and oomycetes.

Genus	Tested	1M	1Y	%	Genus	Tested	1M	1Y	%
Fungi	**6,681**	**6,631**	**6,578**	**98.5**					
Ascomycota	**1,118**	**1,115**	**1,107**	**99**					
Halosphaeria	18	18	17	94.4	*Morchella*	3	3	2	66.7
Peziza	5	5	3	60	*Roselinia*	30	29	27	90
Scleroderris	9	9	8	88.9	*Sclerotinia*	49	49	48	98
Thermoascus	2	0	0	0	Others	1,002	1,002	1,002	100
Basidiomycota	**1,615**	**1,587**	**1,552**	**96.1**					
Amanita	3	3	2	66.7	*Amauroderma*	10	10	9	90
Armillaria	56	53	53	94.6	*Auricularia*	17	16	14	82.4
Ceratobasidium	44	41	39	88.6	*Coprinus*	13	13	12	92.3
Corticium	46	45	45	97.8	*Eichleriella*	10	10	8	80
Exobasidium	38	35	34	89.5	*Ganoderma*	28	27	27	96.4
Gloeoporus	5	4	4	80	*Grifola*	9	9	8	88.9
Hebeloma	5	5	4	80	*Helicobasidium*	15	12	11	73.3
Inonotus	13	13	12	92.3	*Ischnoderma*	5	3	3	60
Laccaria	3	3	2	66.7	*Lampteromyces*	14	14	13	92.9
Lepista	6	5	5	83.3	*Leucoagaricus*	5	3	3	60
Leucopaxillus	3	3	2	66.7	*Lyophyllum*	17	17	16	94.1
Naematoloma	38	37	35	92.1	*Oudemansiella*	20	20	19	95
Panellus	30	30	29	96.7	*Pholiota*	122	122	121	99.2
Pluteus	1	1	0	0	*Polyporus*	24	24	23	95.8
Protodaedalea	2	2	1	50	*Psilocybe*	5	5	4	80
Rhizopogon	1	0	0	0	*Rigidoporus*	9	9	8	88.9
Rozites	1	0	0	0	*Serpula*	4	2	2	50
Suillus	9	8	7	77.8	*Tricholoma*	88	88	83	94.3
Ustilago	21	20	20	95.2	*Volvariella*	1	1	0	0
Others	874	874	874	100					
Zygomycota	**74**	**73**	**73**	**98.6**					
Choanephora	2	1	1	50	Others	72	72	72	100
Anamorphic fungi	**3,874**	**3,856**	**3,846**	**99.3**					
Cercospora	209	209	207	99	*Fusarium*	776	767	767	98.8
Pestalotiopsis	144	144	143	99.3	*Rhizoctonia*	258	250	245	95
Rhynchosporiu	8	7	7	87.5	*Selenophoma*	4	4	3	75
Sphaceloma	7	7	6	85.7	Others	2,468	2,468	2,468	100
Oomycetes	**414**	**300**	**264**	**63.8**					
Achlya	15	1	0	0	*Aphanomyces*	6	2	2	33.3
Dictyuchus	6	0	0	0	*Phytophthora*	114	97	88	77.2
Pythium	266	196	170	63.9	*Thraustotheca*	4	1	1	25
Others	3	3	3	100					

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Long term preservation for 20 years of entomogenous fungi

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Introduction

The effective long term preservation of the microbial isolates is indispensable for the study of microorganisms. Many efforts had been spent for the maintenance and preservation of them, however, there is few information about preservation of entomogenous fungi, which are important as microbiological control agents of insect pests. In this study, we described the viability of the major entomogenous fungal isolates after 20 years preservation by freeze-drying and isolates after 8 years stored at 5°C on slants, and the pathogenicity of survived isolates after preservation by freeze-drying.

Materials and Methods

Fungi: Seven species of entomogenous fungi were preserved by freeze-drying or on slants, *Ascosphaera apis, Beauveria bassiana, B. brongniartii, Paecilomyces farinosus, P. fumosoroseus, Metarhizium anisopliae, Nomuraea rileyi* and *Verticillium lecanii.*

Freeze-drying and reconstitution: Conidia suspended in 10% skimmed milk with 1% sodium glutamate were dispensed into ampules in about 0.5ml portion. And the ampules with conidial suspension were frozen below -50°C for 30 min before lyophilization. The freeze-dried ampules were sealed and reserved at 5°C. After 20 years, the conidia were reconstituted by re-suspending in sterile distilled water.

Storage at 5°C: The fungi were cultured on the pupal-extract sucrose agar slants at 25°C for two weeks and stored at 5°C for 8 years.

Viability tests: The conidia from the ampules (freeze-dried) were transferred to the silkworm pupal-extract sucrose liquid medium. The cultures on the slants (stored at 5°C) were transferred to the fresh agar slants of the pupal-extract sucrose medium. Both transferred isolates were cultured at 25°C for two weeks.

Pathogenicity: The fourth or fifth instar larvae of the silkworm, *Bombyx mori* (C146×J137), and adults of the yellow-spotted longicorn beetle, *Psacothea hilaris*, were applied with conidia of the revival cultured isolates after freeze-drying. The insects treated were reared at room temperature for 14-20 days and the number of dead insects from the fungi were scored.

Results and Discussion

The viability of total isolates freeze-dried was 74.6% (Table 1). The viability of the four species, *A. apis, B. bassiana, B. brongniartii* and *P. farinosus* were all over 80%. However, the viability of *N. rileyi* was 28.6%. Furthermore, Table 2 showed that high viability of the entomogenous fungi stored at 5°C on agar slants except *M. anisopliae* and *N. rileyi*. The viabilities of *A. apis, B. bassiana, B. brongniartii, P. farinosus*, and *V. lecanii* were all over 90%. The tendency of the viability of each species was similar in these two preservation methods. Thus, the viabilities after long term preservation are considered to depend on the fungal species tested in this study. Further investigation is necessary for the preservation of *N. rileyi* and *M. anisopliae*.

We investigated the pathogenicity of survived isolates after 20 years preservation by freeze-drying (not shown). The tested isolates of *N. releyi, M. anisopliae* and *B. bassiana* were all kept their pathogenicity against the silkworm larvae. And the isolates of *B. brongniartii* exhibited pathogenicity against host insect, *P. hilaris*. These results showed that the preserved isolates have kept the pathogenicity even after 20 years.

This is the first case exhibited that major entomogenous fungi can be preserved for 20 years by freeze-drying. Maintenance of the properties of isolates is essential for economically important fungi, for example, microbiological control agents. The freeze-drying was considered to be a useful method for long term preservation and maintenance of fungal genus *Beauveria* and *M. anisopliae*.

Table 1 Viability of the fungal isolates after 20 years preservation by freeze-drying

Fungal species	Preserved isolates	Viable isolates	Viabilities (%)
Ascosphaera apis	3	3	100.0
Beauveria bassiana	25	20	80.0
B. brongniartii	17	14	82.4
Metarhizium anisolpliae	10	7	70.0
Nomuraea rileyi	7	2	28.6
Paecilomyces farinosus	3	3	100.0
P. fumosoroseus	6	4	66.7
Total isolates	71	53	74.6

Table 2 Viability of the fungal isolates on agar slant culture stored at 5°C for 8 years

Fungal species	Stored isolates	Viable isolates	Viabilities (%)
Ascosphaera apis	3	3	100.0
Beauveria bassiana	72	66	91.7
B. brongniartii	55	50	90.9
Metarhizium anisopliae	8	4	50.0
Nomuraea rileyi	1	0	0.0
Paecilomyces farinosus	4	4	100.0
P. fumosoroseus	6	4	66.7
Verticillium lecanii	2	2	100.0
Total isolates	151	133	88.1

Grouping of bacteria isolated from leaf sheaths and panicles of intact rice plants

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Keywords: bacterial flora, rice plants

Introduction

Bacterial flora of rice plants in a field was investigated to construct microbe inventory (a microbe list) including species name, sampling site, function, etc. The information on these microorganisms and their diverse function is useful for disease control of crops and bioremediation. However, little information is available on bacteria inhabiting crops. Although there is a report on bacterial flora on a rice seed (Cottyn *et al.* 2001), bacterial flora on leaf sheaths of intact rice plants has been lacking. Our objective is to isolate bacteria from leaf sheaths and panicles of cultivated, intact rice plants (cv. Koshihikari) cultivated in a paddy field and to classify them based on sequences of 16S rDNA for bacteria.

Materials and Methods

Ten uppermost leaf sheaths (LS), one LS per hill, were sampled in a field 1 month before heading time (A) and at heading time (B). Both ends of leaf sheaths were covered with paraffin to separate epiphytic bacteria and other bacteria including endophytic bacteria.

For five leaf sheaths sampled at two sampling times (A and B) and four panicles sampled at heading time, 16S rDNA region of the isolates (485 strains) were amplified by PCR, and the PCR products (about 650bp) were sequenced (Fig).

Results and Discussion

The percentage of *Microbacterium* and *Sphingomonas* was about 30% each of total bacteria in A samples, and that of genus *Sphingomonas* was about 70% in B samples. The genus

Pantoea was about 70% in panicles (Table). However, plant pathogenic bacteria were not isolated at all. This result suggests that genus *Sphingomonas* inhabits dominantly in the leaf sheaths of rice plant in a field.

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Table. Bacterial flora on leaf sheaths and panicles of intact rice plants

	Genus	Frequency (%)
Leaf sheaths (Washing solution and washed leaf sheaths)	*Sphingomonas*	30–70
	Microbacterium	5–30
	Acidovorax	5–10
	Methylobacterium	5–10
Panicles	*Pantoea*	74

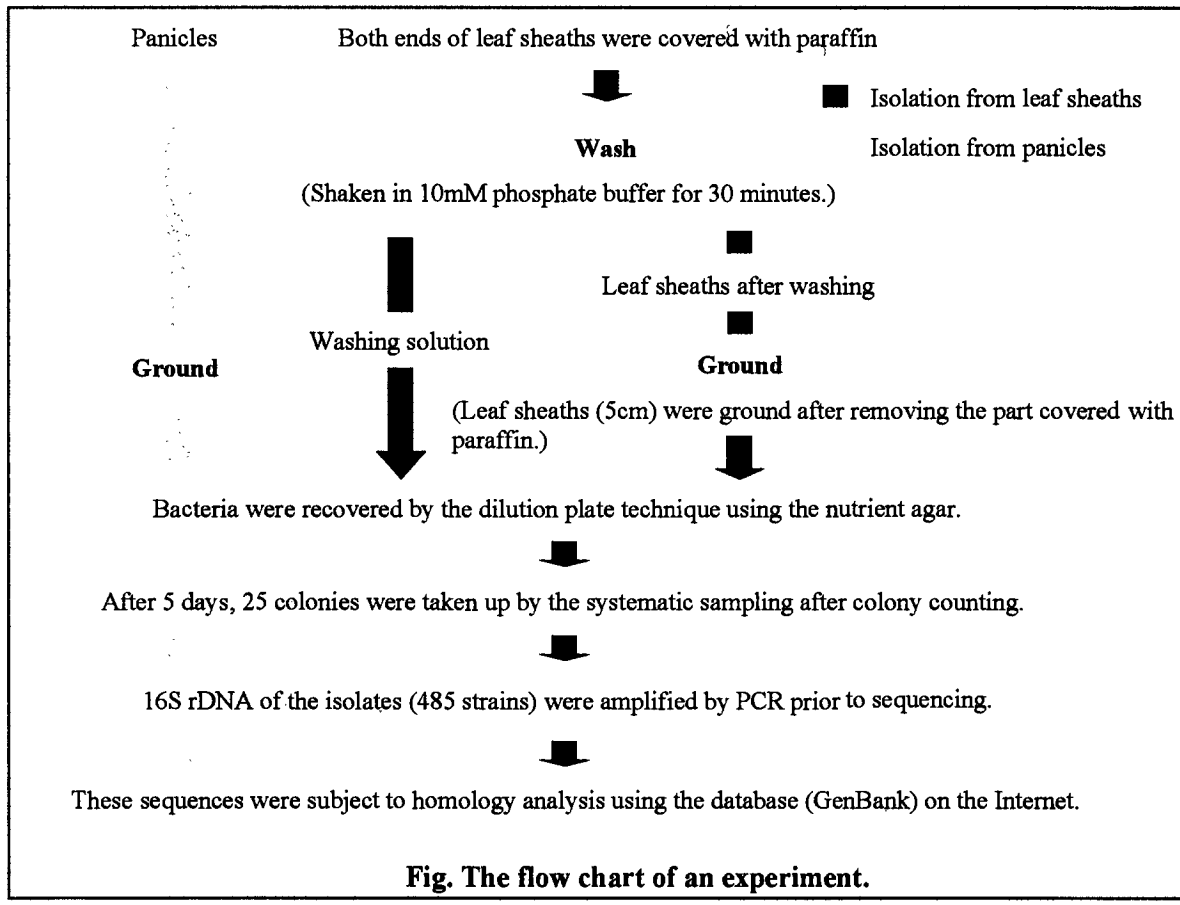

Fig. The flow chart of an experiment.

Pathogenic diversity of *Xanthomonas oryzae* pv. *oryzae* in Myanmar

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Keywords: bacterial blight, race, rice, *Xanthomonas oryzae* pv. *oryzae*,

Introduction

Rice is widely grown throughout Myanmar. Bacterial blight of rice (BB) caused by *Xanthomonas oryzae* pv. *oryzae* is one of the major constraints in rice production. Occurrence of the disease has been informed every year from various rice growing regions all over Myanmar. Growing resistant cultivars is the most safe and feasible control measure against the disease (Ezuka and Kaku 2000). To control the disease by growing resistant varieties, determination on pathogenic diversity of *X. oryzae* pv. *oryzae* is a prerequisite. Evaluation of resistance in rice germplasms and incorporation of the resistant genes into improved varieties may then be investigated. The present study aims to determine the pathogenic diversity of *X. oryzae* pv. *oryzae* in major rice growing areas of Myanmar and to evaluate the resistance of rice germplasms to different races of the pathogen.

Materials and Methods

Forty-four isolates of *X. oryzae* pv. *oryzae* collected from eight locations of Myanmar during 1988 were evaluated for their virulence on five rice varieties namely Taichung Native 1 (TN1), Kogyoku, IR 1545-339, Java 14 and Kheisaba (Lead rice).

One hundred and twenty rice varieties were tasted for resistance to five representative isolates of five races of *X. oryzae* pv. *oryzae* differentiated in the previous experiment.

Based on the results of the previous experiment, five varieties namely Bagolonethwe, IR 1545-339, Yenet 7, Java 14 and Sinekari 3 were selected as Myanmar differentials and the representative isolates of the five races were tested for their virulence on selected varieties to confirm the differential interactions. TN 1 and IET 8955 were used as a susceptible check and a resistant cheek variety, respectively.

Fifty-two isolates of *X. oryzae* pv. *oryzae* collected from 8 locations during 2000, and 11 isolates collected from 6 locations during 2003 were analyzed for their virulence on proposed international differential rice varieties for *X. oryzae* pv. *oryzae* namely IRBB 1, IRBB 2, IRBB 3, IRBB4, IRBB 5, IRBB 7, IRBB 8, IRBB 10, IRBB 11, IRBB 13, IRBB 14 and IRBB 21 (Ogawa 1993). TN 1 was applied as a checked variety.

Results and Discussion

Virulence of *X. oryzae* pv. *oryzae* Isolates Collected During 1988

Isolates of *X. oryzae* pv. *oryzae*, collected during 1988, were distinctly different in virulence on 5 rice varieties. According to the reaction types, 44 isolates were classified into 5 races designated as race 1, 2, 3, 4 and 5 (Table 1). The virulence on each race on the 5 varieties was qualitatively different from that of other races. This kind of differences in pathogenic capacity could be accounted for screening and breeding program for BB resistance.

Table 1. Disease reactions of forty-four isolates belonged to five races on five rice varieties

Races	No. of isolates belonged to each race	Disease reaction[a]				
		TN 1	Kogyoku	IR 1545-339	Java 14	Kheisaba
Race 1	5	S	R	R	R	R
Race 2	23	S	S	R	R	R
Race 3	2	S	S	S	R	R
Race 4	10	S	S	S	S	R
Race 5	4	S	S	S	S	S

[a]R = Resistant, 0 to 12% leaf area infected
S = Susceptible, 13 to 100% leaf area infected

Varietal Resistance of Rice to Different Races

The distinct disease reactions were observed between the interactions of 120 rice varieties and 5 races. Based on these reactions, 120 test varieties could be placed in 8 groups. Twenty-nine varieties in Group-I were susceptible and 30 varieties in Group-VI were resistant to all races, respectively. Varieties in other groups were resistant to some races and susceptible to others (Table 2).

Table 2. Disease reactions of 120 varieties belonged to 8 varietal groups to 5 races of *Xanthomonas oryzae* pv. *oryzae*

Varietal Group	No. of varieties belonged to each group	Disease reaction				
		Race 1	Race 2	Race 3	Race 4	Race 5
Group I	29	S	S	S	S	S
Group II	15	R	S	S	S	S
Group III	11	S	S	S	R	R
Group IV	2	R	R	R	S	S
Group V	31	R	R	R	R	S
Group VI	30	R	R	R	R	R
Group VII	1	R	R	S	S	S
Group VIII	1	S	S	R	R	R

Confirmation on the Differential Reactions of Selected Varieties

For the development of a differential set to identify races of *X. oryzae* pv. *oryzae* in Myanmar, 7 varieties were selected based on the results of previous experiment and retested for their virulence on the representative isolates of the five races. The results indicated that the interaction between the varieties and the races was highly significant at $P<0.01$. This suggested that there was a distinct differential interaction between the selected varieties and the races.

Among 7 varieties tested in this study, 5 varieties namely Bago-lonethwe, Yenet 7, IR 1545 -339, Java 14 and Sinekari 3 could be used as differentials to identify the races of *X. oryzae* pv. *Oryzae* in Myanmar. TN 1 and IET 8955 should be included in the differential set as a susceptible and a resistant check, respectively (Table 3).

Table 3. Disease severities (% leaf area infected) between 7 rice varieties and 5 races of *X. oryzae* pv. *oryzae*[x]

Varieties	% Leaf Area Infected[y]				
	Race 1	Race 2	Race 3	Race 4	Race 5
TN 1	80.00 a A	73.00 a B	79.33 a A	57.33 a C	75.33 a AB
Bagolonethwe	1.07 c C	75.33 a A	71.67 b A	55.00 a B	60.33 b B
Yenet 7	47.33 b A	44.00 b A	3.73 d B	3.07 d B	3.93 f B
IR 1545-339	4.33 c C	3.93 c C	29.00 c B	29.67 c B	51.33 c A
Java 14	0.43 c C	4.67 c C	4.53 d C	38.67 b A	27.00 e B
Sinekari 3	3.67 c B	2.67 c B	2.80 d B	2.73 d B	44.33 d A
IET 8955	1.73 c A	2.00 c A	2.33 d A	2.07 d A	2.53 f A

CV = 9.2%

[x] Inoculated 90 days after sowing and evaluated 21 days after inoculation

[y] Means followed by the same letter are not significantly different at 1% level by DMRT. Small letters indicate comparison of means in each column while capital letters indicate comparison in each row.

Virulence of *X. oryzae* pv. *oryzae* Isolates Collected during 2000 and 2003

To investigate the pathogenic diversity of *X. oryzae* pv. *oryzae* from Myanmar, 52 isolates collected from 8 locations during 2000 and 11 isolates collected from 6 locations during 2003 were analyzed for their virulence on proposed international differentials for *X. oryzae* pv. *oryzae* (Ogawa 1993).

Based on the virulence analysis, 12 races were detected among 52 isolates collected during 2000. Another 3 races could be identified by the virulence analysis of 11 isolates collected during 2003 on the differentials (Table 4).

Table 4. Virulence of 63 isolates of *X. oryzae* pv. *oryzae* on 13 rice varieties

Race	No. of isolates	Disease Reaction												
		TN 1	IRBB 1	IRBB 2	IRBB 3	IRBB 4	IRBB 5	IRBB 7	IRBB 8	IRBB 10	IRBB 11	IRBB 13	IRBB 14	IRBB 21
1	5+(2)	S	R	R	R	R	R	R	R	R	R	R	R	R
2	4	S	R	R	R	R	R	R	R	S	R	R	R	R
3	3	S	R	R	R	R	R	R	R	S	S	R	R	R
4	2	S	R	R	R	S	R	S	R	S	S	R	R	R
5	4+(2)	S	S	S	R	R	R	R	R	S	S	S	R	R
6	11	S	S	S	R	R	R	R	S	S	S	S	R	R
7	7	S	S	S	R	R	R	S	R	S	S	R	R	R
8	2	S	S	S	R	R	R	S	R	S	S	S	R	R
9	1	S	S	S	R	S	R	R	R	S	S	S	R	R
10	6	S	S	S	R	S	R	S	R	S	S	R	R	R
11	6	S	S	S	S	R	R	S	S	S	S	R	R	R
12	1	S	S	S	S	S	R	S	R	S	S	R	R	R
13	3	S	S	S	R	R	R	R	S	S	S	S	S	S
14	-3	S	S	S	S	R	S	S	S	S	S	R	S	S
15	-1	S	S	S	S	S	S	S	S	S	R	R	S	S

Numbers in parenthesis were number of isolates collected during 2003

Conclusion

In the present study, 15 races were identified from 63 isolates collected from 14 locations of Myanmar during 2000 and 2003. These results do not show a complete nature of existing races of *X. oryzae* pv. *oryzae* in Myanmar because a limited number of bacterial isolates collected from some locations only were tested. More races could be found if nationwide collection of isolates were made. It is, therefore, necessary to carry out further research on identification and distributions of races by using isolates collected nationwide. Furthermore, it is also essential to evaluate the genetic diversity of *X. oryzae* pv. *oryzae* from Myanmar to understand the population structure of the pathogen. The awareness of the national level of pathogenic and genetic diversity of *X. oryzae* pv. *oryzae* will be a great help to the national level of screening and breeding program for rice bacterial blight resistance.

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Phylogenetic analysis of *Xanthomonas* species based on the nucleotide sequences of 23S rRNA gene and 16S-23S rDNA spacer region

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Keywords: ITS, phylogeny, 23S rDNA, *Xanthomonas*

Introduction

The genus *Xanthomonas* consists of plant pathogenic bacteria attacking a variety of important crops. To date, *Xanthomonas* was classified into 20 genomic species on the basis of DNA-DNA reassociation (Vauterin *et al.* 1995). Until recently, analyses of 16S rDNA sequence and 16S-23S rDNA intergenic spacer sequence were employed for comparative analyses within *Xanthomonas* strains. Analysis of 16S rDNA sequences has revealed a small divergence and high conservation among xanthomonads (Hauben *et al.* 1997). 16S-23S rDNA intergenic spacer sequences has showed a higher level resolution than 16S rDNA sequences (Goncalves and Rosato 2002). In this study, we analyzed the phylogeny of *Xanthomonas* based on sequences of 23S rRNA gene and 16S-23S rRNA intergenic spacer region (ITS), and compared them with previous study of 16S-23S rDNA intergenic spacer sequences.

Materials and Methods

Bacterial strains. A total of 109 strains including 18 *Xanthomonas* species and two strain of *Stenotrophomonas maltophilia* (previously, designated as *Xanthomonas maltophilia*) used in this study.

PCR amplification of 23S rDNA and ITS. The PCR primers for 23S rRNA and ITS were designed from the conserved regions in the 16S rRNA and 23S rRNA genes. PCRs were carried out with the following temperature profile: an initial denaturation at 94℃ for 5 min; 30 cycles of denaturation at 94℃ for 1 min, annealing at 55℃ for 1 min, and extension at 72℃ for 2 min; and final extension at 72℃ for 7 min.

Direct sequencing. The PCR products purified from agarose were directly sequenced using a BigDye Terminator Cycle Sequencing FS Ready Reaction kit, and the sequencing products were analyzed with a model ABI 3100 genetic analyzer.

Data analysis. Phylogenetic analysis was performed by using the CLUSTAL X program. Phylogenetic trees were constructed according to the neighbor-joining method.

Results and Discussion

Based on partial sequences of 23S rDNA, *Xanthomonas* species were divided into two major clusters. Members of each cluster were similar to those of 16S rDNA analysis. Cluster I was a major group which was composed of 14 species, while cluster II was consisted of 4 species, *X. albilineans*, *X. hyacinthi*, *X. sacchari* and *X. translucens*. The similarities between two clusters were ranged from 96.6 to 97.8 %, but those within same group were more than 98.8 %. Cluster I consisted of two subgroups, with each subgroup having heterogeneous species. The obtained results were similar to those of 16S rDNA sequence analysis, and suggested that rDNA (16S or 23S) analyses were not sufficient to delineate xanthomonads at species level.

ITS was divided into three regions ITS 1, 2, and 3 by existence of two tRNAs, $tRNA^{Ala}$ and $tRNA^{Ile}$. The length of each ITS region was 85-96 nt, 14-76 nt, and 223-228 nt, respectively. The length of ITS was very diverse among *Xanthomonas* species. Although two tRNAs were highly conserved, they were classified into two types by slight differences in sequences. In $tRNA^{Ala}$, two nucleotides were different between two types, and three nucleotides were different in $tRNA^{Ile}$. On the basis of ITS sequences, *Xanthomonas* species were also divided into two clusters. Members of each cluster were the same as those of 23S clusters, but there were slight differences in subgroup level. Comparative analysis between subgroups and tRNA types in cluster I indicated that subgroups were defined by tRNA types. The tRNA typing was useful for clustering of *Xanthomonas* species by ITS sequences. The results obtained by ITS analysis suggested that phylogenetic relationships and lineages among *Xanthomonas* species could be reflected by the topology of the ITS tree.

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Development of genome database system for *Xanthomonas oryzae* pv. *oryzae*

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Keywords: BLAST search form, ORF viewer

The genome database system has been developed for the complete genome sequence of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). The system provides sequence and gene information obtained from our genome sequencing project of Japanese representative race I strain T7174 (MAFF311018). We analyzed a data structure relative to genome information on *Xoo* and designed a relational database schema, thus eliminating any inconsistence of data mutuality. The database will be accessed through the internet. Two kinds of search system, BLAST search form and viewer of gene information, have been prepared in the homepage of the database.

Sequence data is registered to table "fragment". This table is linked to table "cron", "contig", "genome", "orf", and "cron_microarray" through reference relationships. The schema of database is shown in Fig. 1. The database provides gene information with ORF viewer and BLAST search form. Moreover, the database system is used for microarray and gene knock-out experiment.

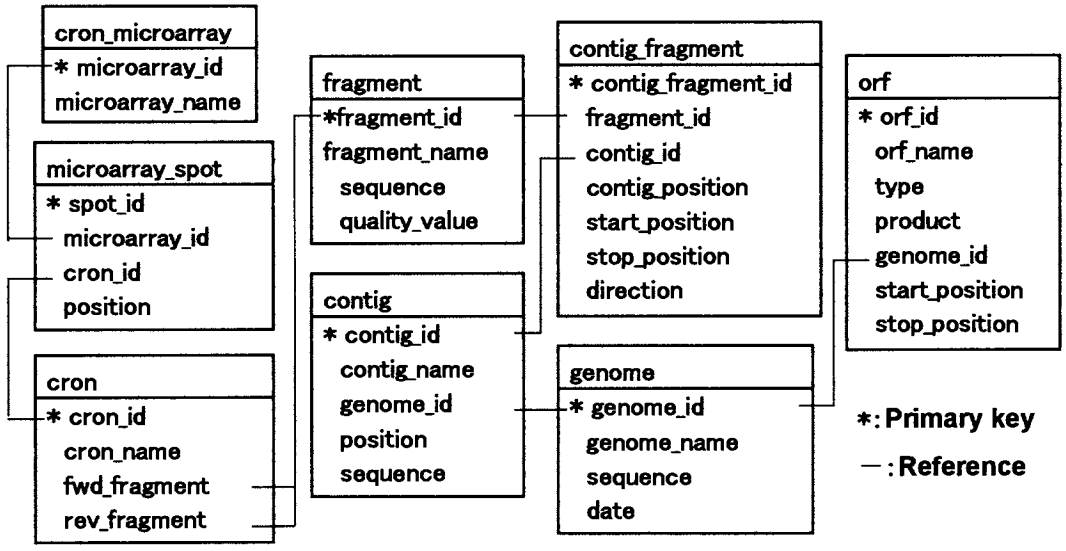

Fig. 1. Schema of *xoo* database

In the BLAST search form, complete genome or ORF sequences can be chosen as a target database, and the BLAST starts based on inputted sequence of nucleotide or amino acid when the "Search" button is pushed (Fig. 2).

In the viewer of gene information, gene information such as gene name, length, and product is shown by a clicking of target gene in the genome maps (Fig. 3). A keyword search is available to the gene information. The display range of the genome map can be changed by a choice of magnification level. The latest information on gene analysis of *Xoo* can be sent to researchers interested in this study by using the database system.

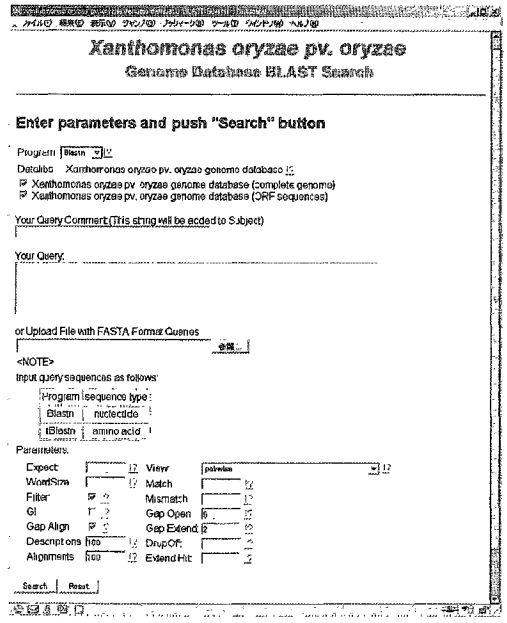

Fig. 2. BLAST search form

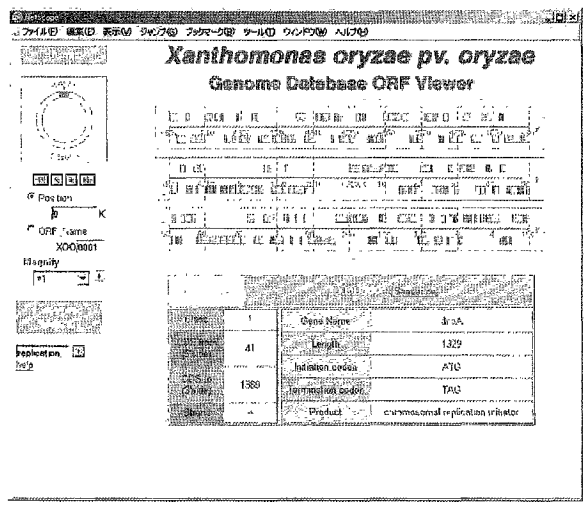

Fig.3. ORF viewer

PCR-based genomovar identification and characterization of *Burkholderia cepacia* complex strains isolated from diverse origins

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Keywords: plant pathogen, opportunistic human pathogen, genetic analysis, environmental risk

Introduction

Burkholderia cepacia, first described in 1950 as the pathogen of onions, has emerged in recent years as an important human opportunistic pathogen that causes numerous outbreaks, particularly among cystic fibrosis (CF) patients. *Burkholderia. cepacia* is also broad-spectrum antagonist through the production of various antibiotics, such as pyrrolnitrin and could be considered as biopesticides in the control of plant diseases as well as in bioremediation of a wide range of recalcitrant compounds. Taxonomic studies have revealed that *B. cepacia* consists of genetically distinct species or genomovars, which has become known as the *B. cepacia* complex (Bcc). Despite the acknowledged significance of the Bcc strains, little is known about their genomovar distribution of natural strains, particularly related to agro-ecosystem.

The objective of this study was to determine the genotypic identification and characterization of the 119 Bcc strains recovered from clinical and environmental sources in Japan and Thailand.

Methods and Results

One hundred nineteen strains of the Bcc and reference strains, namely, *B. cepacia* genomovar I (ATCC 25416^T and ATCC 17759), *B. cenocepacia* III-A (ATCC 25609, ATCC 17774 and ATCC 17460), *B. cenocepacia* III-B (ATCC 17765), *B. stabilis* (ATCC 27515), *B. vietnamiensis* (ATCC BAA-248^T), and *B. pyrrocinia* (LMG 14191 and K1112) were used. Genomovar determination of the Bcc strains was performed by a combination of PCR-RFLP of the 16S rRNA and *recA* genes, and genomovar-specific PCR. Furthermore, the distribution of the epidemic marker (BCESM) encoded by *emsR* and pyrrolnitrin biosynthetic locus encoded by *prnC* among strains were investigated. All procedures in preparation of bacterial DNA, PCR

amplification, digestion of PCR products with endonucleases, detection of the BCESM and *prnC*, and others were carried out as described elsewhere (Seo and Tsuchiya 2004).

Based on the results of analysis by 16S rDNA RFLP generated after digestion with *Dde* I , the Bcc strains were differentiated into two groups (Fig. 1): group 1 (including *B. vietnamiensis*) and group 2 (including *B. cepacia* genomovar I , *B. cenocepacia* and *B. stabilis*). All strains belonged to group 2 except for one strain. In the RFLP analysis of the *recA* gene using *Hae*Ⅲ, strains were separated into eight patterns designated as A, D, E, G, H, I, J and K, of which pattern K was new (data not shown). When they were compared with those obtained in previous studies, where patterns designated A-J were reported, 73 strains were identified *B. cepacia* genomovar I (D, E and K), 33 as *B. cenocepacia* (G, H and I), three as *B. stabilis* (J) and one as *B. vietnamiensis* (A). These strains belonged to pattern K were identified as *B. cepacia* genomovar I based on genomovar-specific PCR.

Burkholderia. cepacia epidemic strain marker (BCESM) encoded by *emsR* and the pyrrolnitrin biosynthetic locus encoded by *prnC* were present in 22 strains (18%) and 88 strains (74%) from all sources, respectively. All *emsR* positive strains belonged to *B. cenocepacia*, whereas most *prnC* positive strains belonged to *B. cepacia* genomovar I (Table 1). Comparing the *emsR* and *prnC* strains, we observed a good match between *emsR* positive and *prnC* negative strains. All of the *emsR* positive strains were found to belong to the *prnC* negative group except for some cymbidium strains (*B. cenocepacia* Ⅲ-B).

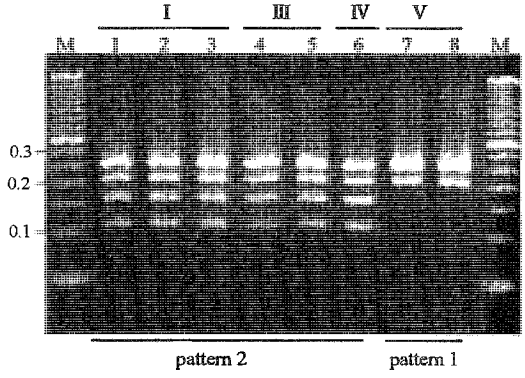

Fig. 1 Electrophoresis patterns obtained after digestion of amplified *Burkholderia cepacia* complex 16S rDNA with restriction enzyme *Dde* I . M, DNA size standard (50-bp ladder). The genomovar status of each strain is indicated by the Roman number

Table 1 *prnC* and *emsR* in different genomovars or species among clinical and environmental isolates of the *B. cepacia* complex

Genomovar	% of isolates positive			
	Clinical (24)[a]		Environmental (95)	
	prnC	*emsR*	*prnC*	*emsR*
B. cepacia genomovar I	16.7 (4)	0	79.1 (75)	0
B. multivorans	0	0	0	0
B. cenocepacia genomovar Ⅲ-A	0	29.2 (7)	0	0
B. cenocepacia genomovar Ⅲ-B	0.8 (2)	16.7 (4)	7.4 (7)	11.6 (11)
B. stabilis	0	0	0	0
B. vietnamiensis	0	0	0	0

[a]Numbers of strains are in parentheses

References

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Flagellin glycosylation island in *Pseudomonas syringae*

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Keywords: glycosyltransferase, host specificity, plant pathology

Introduction

The deduced amino acid sequences of the flagellins of *Pseudomonas syringae* pv. *tabaci* and *P. syringae* pv. *glycinea* are identical; however, their abilities to induce a hypersensitive reaction are clearly different. The reason for the difference seems to depend on the posttranslational modification of the flagellins. To investigate the role of this posttranslational modification in the interactions between plants and bacterial pathogens, we isolated genes that are potentially involved in the posttranslational modification of flagellin (glycosylation island). We identified the genes involved in flagellin glycosylation and their deletion mutants were generated. Using these materials, we examined their influence on plant-microbe interactions and identified glycosylated amino acid residues.

Materials and Methods

The bacterial strains used in this study are *Pseudomonas syringae* pv. *glycinea* race 4 and *P. syringae* pv. *tabaci* Isolate 6605. For the inoculation test, bacteria were cultured in Luria-Bertani (LB) medium with 10 mM $MgCl_2$ at 25℃. For purification of flagellin proteins, bacteria that had been cultured overnight were incubated in minimal medium containing 10 mM mannitol and fructose as carbon sources for 24 h at 23℃. *Pseudomonas syringae* strains with each ORF in the glycosylation island deleted were generated based on homologous recombination by using the mobilizable cloning vector pK18*mobsacB* (5.7 kb, Km^r) (Takeuchi *et al.* 2003).

Results and Discussion

The sequence data revealed three ORFs, designated *orf1*, *orf2*, and *orf3*, between the flagellum structural genes *flgL* and *fliC* (Fig. 1). *orf1* and *orf2* showed significant homology to *orfN*, which was found in the glycosylation island of *P. aeruginosa* strain PAK; at the amino

acid level there was 32% homology in an 860-amino-acid region and there was 38% homology in a 577-amino-acid region, respectively. On the other hand, the *orf3* product showed homology to *orfC* in the glycosylation island of *P. aeruginosa* (29% homology), which was homologous to putative 3-oxoacyl-(acyl carrier protein) synthase III. To examine whether the genes that exhibited homology to the genes encoding putative glycosyltransferases are responsible for flagellin glycosylation in *P. syringae* pv. glycinea, we generated a defective mutant with a mutation in each ORF. SDS-PAGE analysis and glycodetection revealed that the deletion of *orf1* completely eliminated the ability to glycosylate flagellin proteins, whereas some glycosyl residues remained in the flagellin of the *orf2* mutant.

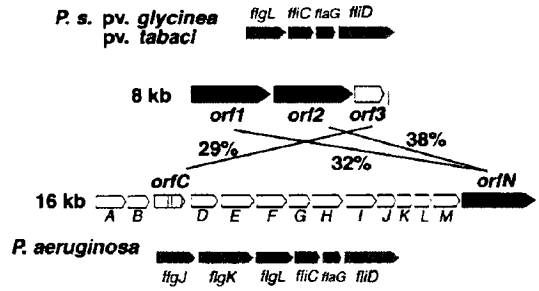

Fig. 1. Schematic representation of the ORFs in the region upstream of the fliC (flagellin) gene from the genomic sequence of *P. s.* pv. *glycinea* and pv. *tabaci* and their comparison with *P. aeruginosa* strain PAK.

To evaluate the effect of a defect in the genes in the glycosylation island on plants, we inoculated each mutant strain of *P. s.* pv. *glycinea* onto soybean (host) and tobacco (nonhost) leaves. When host soybean leaves were inoculated with each strain, the Δ*orf1* and Δ*orf2* mutants failed to cause prominent symptoms. In contrast, inoculation of the nonhost tobacco leaves with the *orf1* and *orf2* mutants resulted in lesion-like changes 10 days after inoculation, whereas inoculation with the wild-type strain did not have this effect. We also inoculated each mutant strain of *P. s.* pv. *tabaci* onto tobacco (host). These mutants, especially the Δ*orf1* mutant, failed to cause prominent symptoms.

To identify the glycosylated amino acid residues on flagellin from *P. s.* pv. *tabaci*, comparison of peptide fragments produced by the digestion of wild-type and Δ*orf1* mutant flagellins with asparatic N-peptidase was carried out by the reverse-phase column HPLC. The amino acids sequencing analyses of the peptides and alanine substitution experiments revealed that all glycan linked though serine residues at 143, 164, 176, 183, 193 and 201 (Fig. 2). These serines are located in the internal domain, which is expected to be surface-exposed part in flagella filament. MALDI-TOF MS analysis of flagellin proteins from Ser/Ala-substituted mutants revealed that the reduction of molecular weight was about 540 Da in each serine.

Fig. 2. Schematic representation of glycosylation sites in the flagellin amino acid sequence.

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Huanglongbing disease management in citrus in Indonesia—Current research

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Keywords: *Candidatus* Liberibacter asiaticus, *Diaphorina citri*, huanglongbing management.

Introduction

Huanglongbing, caused by *Candidatus* Liberibacter asiaticus, is a severe disease of citrus in Indonesia. In the past, oxytetracycline was used to control the disease, but this approach was not sustainable, as it gave only temporary control and left antibiotic residues on fruit. Eradication of diseased trees has been practiced in Indonesia since 1980s, but a lack of disease-free material, the high cost of replacing infected plants and an ineffective use of pesticides for control of the vector, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), have hampered attempts to build a sustainable citrus industry. Research is being conducted in Indonesia in collaboration with scientists from Australia, France, Japan and Viet Nam to improve the management of huanglongbing and its vector and to understand their biology.

Research Project

(1) Distribution and occurrence of *D. citri* and psyllid natural enemies. Surveys of the incidence of *D. citri* and its natural enemies are being conducted in several areas of the Indonesian archipelago including Java, Kalimantan, Sulawesi and Bali. Psyllid fauna and their natural enemies, including entomopathogens, predators, and primary and secondary parasitoids are being collected for identification their distributions will be related to geography (longitude, latitude, and altitude), climate, the nature and proximity of other vegetation and, where relevant, management practices.

(2) Distribution of known and potential alternative hosts of *D. citri* and L. asiaticus. Primary data on rutaceous plants are being obtained from: herbarium specimens

located at the Herbarium Bogoriense; the living collection at Indonesian Botanic Gardens; and recent surveys conducted by staff from the Bogor Botanic Gardens. Secondary data are also being obtained from published studies. These data will be related to geography (longitude, latitude, and altitude), ecosystems type (including climate, soil type, topography, and surrounding vegetation) and, where relevant, human activities. The presence of *D. citri* and the infection by L. asiaticus is also being recorded.

(3) The impact of temperature, altitude and genotype on the incidence of *D. citri* and disease severity. Six experimental fields at three different altitudes (50–100, 500–600, and 1000–1200 m above sea level) are being planted with Siem mandarin (*Citrus reticulata*) to determine the seasonal abundance of *D. citri* and disease development in relation to altitude, climate and management practices. Locations at the lowest altitude will also be planted with species of *Citrus* and their allies to determine their suitability as hosts for *D. citri* and L. asiaticus.

(4) The relationship between the feeding behaviour of *D. citri* and host plant phenology on disease transmission. Vector populations, tree phenology and rates of infection in 3–4-year-old orchards will be monitoring to determine the seasonal behaviour of adult psyllids including their movement, feeding activities and degree of aggregation between and during flushes. Glasshouse experiments will focus on the use a video camera and direct observations to describe adult behaviour on mature and immature foliage under a range of different environmental conditions.

(5) The impact of mineral and plant oil spray on mortality, feeding and oviposition behaviour of *D. citri* and the spread of huanglongbing. In the controlled environment glasshouses, the impact of horticultural and agricultural mineral oil (HMO and AMO) deposits on the feeding and oviposition behaviour of adult psyllids, nymph mortality of *D. citri* and the transmission of L. asiaticus are being observed. In field experiments, the impact of HMOs and AMOs on the ingress of psyllid adults into orchards and their subsequent aggregation and population growth in relation of timing of sprays and plant phenology will be studied.

Progress to date

- Huanglongbing is widely spread with different severities at different locations in the citrus centres throughout the Indonesian archipelago.
- The distribution of *D. citri* follows that of *Citrus* spp., *Murraya* spp. and *Bergera koenigii*. The psyllid has been found in lowland and upland (1200 m asl) citrus orchards.

- Citrus allies found in Indonesia include Aegle marmelos, Bergera koenigii, Clausena indica, Feroniella lucida, Limonia acidisima, Glycosmis pentaphylla, G citrifolia, Melicope spp., Murraya paniculata, M. exotica, Swinglea glutinosa and Triphasia trifolia.
- The natural enemies of *D. citri* found include two parasitoids (*Tamarixia radiata* and *Diaphorencyrtus aligarhensis*), several generalist insect predators and three species of entomopathogens (*Hirsutella citriformis*, *Paecilomyces fumosoroseus* and *Metarhizium* sp.).
- A study on the transmission of L. asiaticus to susceptible Siem mandarin found that bacterium spread unevenly in the leaf midribs, stem bark and root bark of host plants. In one infected plant, not every site assayed by PCR was found to be positive for L. asiaticus.
- Liberibacter asiaticus was found in the head, thorax and abdomen of *D. citri* suggesting that the bacterium is circulated around the psyllid's body.
- AMO deposits affected the feeding behaviour of *D. citri*. During a week of observations, psyllids prefer to feed on untreated plants compared to on plants treated with 0.25–0.50% oil. No psyllids were seen to feed on plants treated with higher oil concentrations.

Acknowledgements

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A preliminary study of a toxin associated with *Huanglongbing* disease on citrus

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Introduction

Huanglongbing (*HLB*), known as citrus vein phloem degeneration (CVPD) in Indonesia, is a very destructive disease caused by a phloem-limited, uncultivable bacterium, *Candidatus* Liberibacter asiaticus. There have been few studies on the characteristics of this pathogen nor on its interactions with its host plants. Symptoms of this disease include interveinal chlorosis or a general mottle on leaf laminas that may occur throughout infected trees or be confined to certain branches. Fruit produced by infected trees are small, malformed, bitter and may prematurely abscise. If they remain attached to the tree, the fruit do not color properly. Under poor management, most trees die from huanglongbing within 5 years of planting and may only yield effectively for 1 year. The uneven distribution of the bacterium within the host (Himawan unpublished, Garnier *et al.* 1983) together the symptomatology of the disease suggests that a toxin may be involved in disease development. Therefore, this research was conducted as a preliminary study to determine if toxic substance(s) occur in *HLB*-infected material.

Materials and Methods

Leaf midribs were removed from *HLB*-infected and healthy (both confirmed by PCR) Siem mandarins (*Citrus reticulata*). Fresh midribs were ground and extracted at room temperature with either phosphate buffered saline (PBS) or ethanol. These extracts were evaporated to dryness and then re-suspended in PBS to give the equivalent of 5 g midribs per mL. Aliquots of both extracts were diluted 2-, 5- and 10-fold in PBS. Further aliquots were heat-treated at 40, 60 and 100°C for 15 min. Control, diluted and heat-treated extracts were infiltrated into leaves of citrus of Siem mandarin and tobacco (*Nicotiana tabacum).* Any inhibition of bacterial cell growth was tested by placing 5 mm diameter filter paper discs soaked

for 30 min in various extracts on lawn growths of *Escherichia coli, Ralstonia solanacearum* and *Xanthomonas axonopodis* pv. *citri* on nutrient agar, CPG (10 g/L mannitol, 1 g/L casamino acid, 10g/L peptone, pH 7), and SPA (20 g/L sucrose, 5.0 g/L peptone 0.5 g/L dibasic potassium phosphate, 0.25 g/L magnesium sulphate, 15 g/L agar, pH 7.2) media.

Results and Discussion

The PBS and ethanolic extracts of *HLB*-infected midribs caused chlorosis in citrus leaves and hypersensitive, necrotic lesions in tobacco: no symptoms on citrus or tobacco were caused by extracts from healthy leaves. PBS and ethanolic extracts of *HLB*-infected midribs also caused zones of growth inhibition when tested against *E. coli, R. solanacearum*, and *X. axonopodis* (Table 1). The inhibition zone was greatest for *R. solanacearum* and least for *X. axonopodis*: no inhibition zones were produced by extracts from healthy midribs against all bacteria tested. Extracts made in PBS and ethanol retained their ability to induce chlorosis, to cause a hypersensitive reaction and to inhibit bacterial growth up to a 10-fold dilution. Heat treatment at 40° and 60°C did not affect the activity of extracts against *R. solanacearum*; however, the inhibitory effect against *X. axonopodis* was abolished by treatment at 60°C. The inhibitory activity against both species was lost by a heat treatment at 100°C (Table 2).

Table 1. Mean diameter (n = 4) of inhibition zones induced by filter discs soaked in undiluted, *HLB*-infected extracts of midribs.

Bacterial species	Mean of inhibition zones (mm)	
	PBS extraction	Ethanolic extraction
E. coli	9.75[a]	9.50[a]
R. solanacearum	17.50[b]	14.50[d]
X. axonopodis pv. *citri*	7.00[c]	6.50[c]

Means followed by different letters are significantly different at the 5% level according to Duncan's Multiple Range Test.

Table 2. Mean diameter (n=4) of inhibition zone induced by heat-treated PBS and ethanolic extracts of *HLB*-infected and healthy midribs.

Bacterial species	Extraction method	Temperature (°C)	Diameter of inhibition zone (mm)	
			HLB	Healthy
R. solanacearum	PBS	40	7.00	0.00
		60	7.00	0.00
		100	0.00	0.00
	Ethanol	40	6.50	0.00
		60	7.00	0.00
		100	0.00	0.00
X. axonopodis pv. *citri*	PBS	40	6.30	0.00
		60	0.00	0.00
		100	0.00	0.00
	Ethanol	40	6.00	0.00
		60	0.00	0.00
		100	0.00	0.00

Conclusion

Extracts of HLB-infected tissue caused a necrotic, hypersensitive reaction in tobacco, chlororsis of citrus leaf tissues and inhibited the growth of *E. coli, R. solanacearum*, and *X. axonopodis* pv. *citri*. The extracts remained active up to a 10-fold dilution and heat treatment for 15 min at 60°C abolished the inhibitory activity against *X. axonopodis* but not *R. solanacearum* whilst treatment at 100°C abolished the inhibitory activity against both organisms. Together, these data suggest L. asiaticus produces one or more toxic substances that are part of the disease syndrome.

Reference:

Garnier, M and Bove, J.M. (1983) Transmission of the organism associated with greening disease from sweet orange to periwinkle by dodder. Phytopathology 73:1358-1363.

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Phylogeny and taxonomy of bacterial nitrogen-fixing legume symbionts

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Phylogenetic Analysis

As of September 2004, bacteria that form nitrogen-fixing symbiotic associations with legumes have been confirmed in more than 49 species of 13 genera. Phylogenies of these taxa containing legume symbionts based on the comparative analysis of 16S rDNA sequences (Figure) show that they are not clustered in one lineage but are distributed in the classes *Alphaproteobacteria* and *Betaproteobacteria*, and dispersed over the following ten monophyletic groups, being intermingled with other taxa that do not contain legume symbionts (shown in parentheses below): Group 1, which comprises *Rhizobium* and *Allorhizobium* species containing legume symbionts (intermingled with *Agrobacterium* and *Blastobacter* species, which are nonsymbionts); Group 2, *Sinorhizobium* and *Ensifer* species (with unclassified nonsymbionts); Group 3, symbiotic *Ochrobactrum* strains (with nonsymbiotic *Ochrobactrum*, *Brucella*, and *Mycoplana* species); Group 4, *Mesorhizobium* species (with nonsymbiotic *Aminobacter* and *Pseudaminobacter* species and their relatives); Group 5, symbiotic *Bradyrhizobium* species and *Blastobacter denitrificans* (with nonsymbiotic *Bradyrhizobium*, *Agromonas*, *Nitrobacter*, *Afipia*, and *Rhodopseudomonas* species); Group 6, "*Methylobacterium nodulans*" (with nonsymbiotic *Methylobacterium* species); Group 7, *Azorhizobium* species (with nonsymbiotic *Ancylobacter*, *Xanthobacter* and *Aquabacter* species); Group 8, *Devosia neptuniae* (with nonsymbiotic *Devosia* species and unclassified nonsymbionts); Group 9, symbiotic *Burkholderia* species (with nonsymbiotic *Burkholderia* species); and Group 10, *Ralstonia taiwanensis* (with nonsymbiotic *Ralstonia* species).

Taxonomy

For Groups 6, 9, and 10, the present classification, in which "each monophyletic group comprises one genus wherein legume symbionts and nonsymbionts are intermingled with each other," is considered to be retained as is, because they are clearly separated from other genera at high bootstrap values and have already been sufficiently characterized based on polyphasic taxonomy. As for the remaining seven monophyletic groups, on the other hand, there are currently three options for emending their current classification (definitions and circumscriptions) at the generic level: A) the current classification shall be retained as is; B) all the genera within each monophyletic group shall be amalgamated into one single genus in conformity with the results of phylogenetic analysis; and C) each subordinate lineage in each monophyletic group shall be proposed as a genus. It is considered that research and discussions will be continuously conducted for emending the classification of these monophyletic groups based chiefly on Options B and C as preferable candidates.

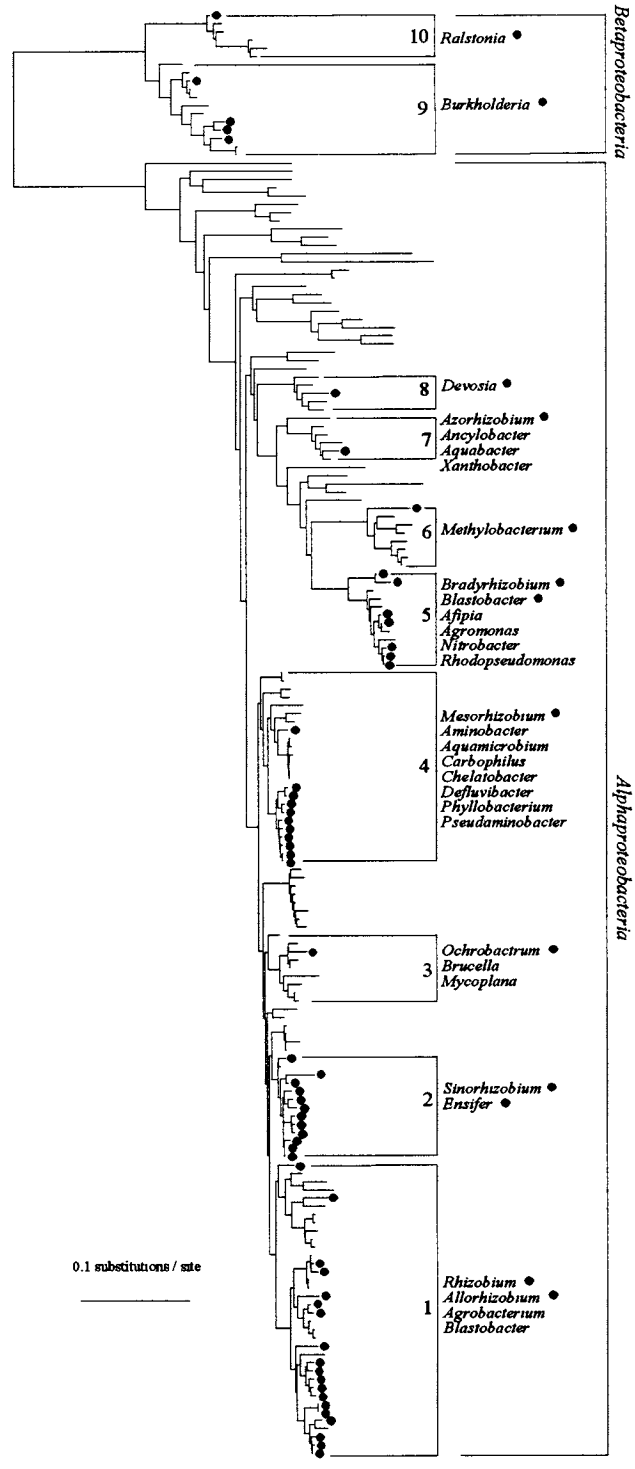

Figure. Phylogenetic relationships among legume symbionts and their relatives inferred based on the 16S rDNA sequence divergence.

The names of the genera contained in each monophyletic group are listed on the right. The phylogenetic positions of legume symbionts and the names of the genera which contain legume symbionts are indicated by the symbol ●.

Gene expression controlled by a conidiophore patterning regulator Acr1 in *Magnaporthe grisea*

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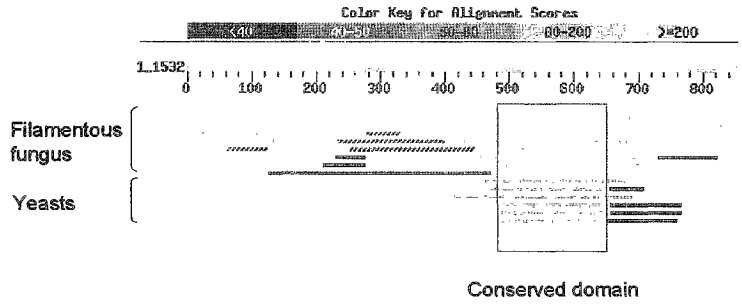
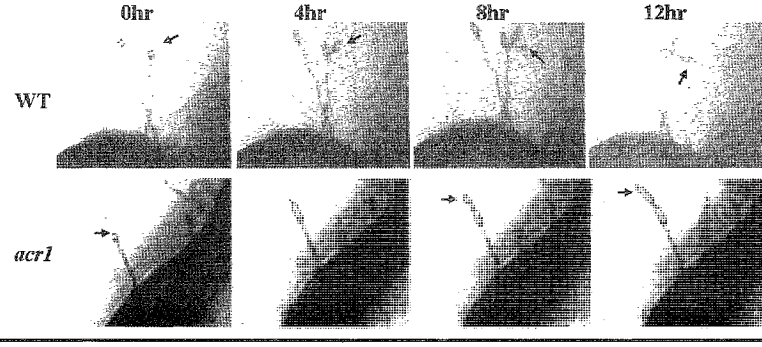
Keywords: conidiogenesis patterning, conserved domain, medA,

Rice blast disease, caused by the filamentous fungus *Magnaporthe oryzae*, is one of the most devastating diseases to global rice production. To understand the regulation mechanism of conidiation in *M. oryzae*, a morphological mutant, *acr1*, has been studied. Acr1 is a functional homolog of MedA, a conidiophore-developmental regulator in *Aspergillus nidulans*. Results of a protein-protein homology search (BlastX) indicated that the central domain in Acr1 is conserved among many fungus.

BlastX hits against Acr1 protein sequence



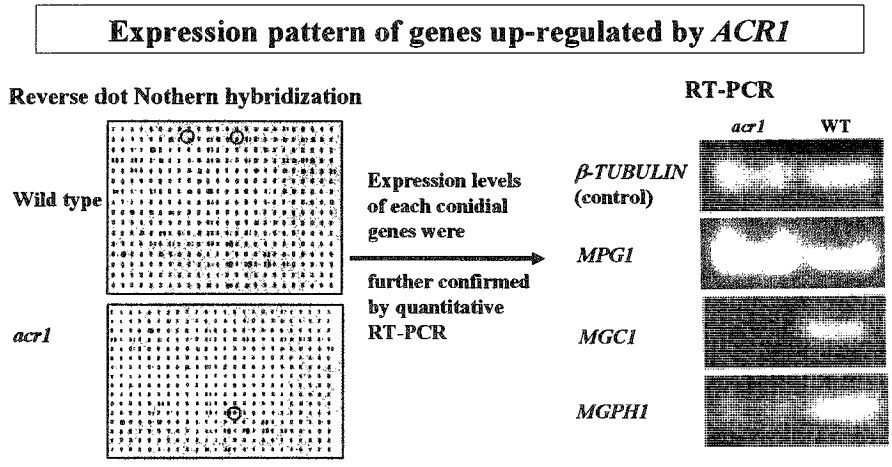
The loss-of-function Acr1 mutants produce chains of elongated conidia in a head-to-tail array, while the wild type conidia are produced in a sympodial array.



The sequence of conidia formation in the wild-type strain and the *acr1* mutant

In *acr1*, appressoria production is greatly reduced and the appressoria are defective in plant penetration. The reductions in the appressorium formation and penetration rates in the mutant are not complemented by the addition of exogenous cAMP.

To investigate the genes upregulated by Acr1, a nylon membrane arrayed with subtraction libraries constructed from conidial mRNA of *acr1* and its isogenic wild-type strain were subjected to a reverse Northern dot blot hybridization analysis. The results from this and subsequent RT-PCR analyses indicate that the expression of a G1-cyclin (*MGC1*) and glycogenphosphorylase (*MGPH1*) genes is positively regulated by Acr1.



Study on durability of resistance genes to blast disease (*Pyricularia grisea*) in the Mekong delta

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Keywords: durability, *Pyricularia grisea*, resistance genes,

Introduction

Rice blast caused by *Pyricularia grisea* Sacc. (Rossman *et al.* 1990), is one of major rice diseases in Mekong delta of Vietnam. Infected area increased from 1999 to 2003. During dry season 02-03, 189,000 ha was infected in a total of 1.6 M ha of direct seeded rice crop. Most of new releasing varieties were found to be susceptible to the disease. Identification of blast races using 129 blast isolates collected in many places of Vietnam (Noda *et al.* 1999) indicated 12 pathogenic groups based on their virulence to 12 Japanese differential rice varieties. It was also shown that none of isolates were virulent to differentials which carries genes *Pik-s, Pish, Pik, Piz, Pita-2, Piz-t and Pik-p*, therefore, this study intended to determine which resistance genes is more durable against blast over time and space. Study conducted using the same Kiyosawa varieties and 31 monogenic lines under natural condition of blast nursery (Ou 1965).

Materials and Methods

Blast nursery was laid out in 10 different places of Mekong delta which includes Can Tho (CT), An Giang (AG), Tra Vinh (TV), Soc Trang (ST), Tien Giang (TG), Kien Giang (KG), Ca Mau (CM), Ben Tre (BT), Long An (LA), Dong Thap (DT) on two different seasons: wet and dry season. Twelve Japanese Kiyosawa differential varieties and 31 monogenic lines provided by Dr Fukuta under IRRI-Japan collaborative research project. Resistance or subceptibility of varieties and lines which carry different blast resistance genes was scored based on 9 scale of IRRI (SES 1988): Score from 0-3 was resistance (R) , 4-6 was moderate susceptible (MS) and 7-9 was susceptible (S).

Results and Discussion

Results from blast nurseries conducted since 1980-2004 indicated that most of previously released varieties were susceptible to blast, some of them are still considered as durable resistance and cultivated in large scale for more than 15 years. Nowaday, resistant varieties are overcome by blast pathogen with average of 1-2 years after released (Table 1).

We found that in one location tested, reaction can be changed over time (8 seasons) from resistant to susceptible and vice versa. Some resistance genes could express resistance to blast for almost of times such as *Pi-km* (Kanto-51), *Piz; Pish* (Fukunishiki) *Pita-2, Pish* (PiNo.4), *Piz-t* (Toride 1), *Pik-p* (K60) (Table 2). When observation was conducted in 10 locations then reaction was also different, resistance genes found in almost 10 locations tested such as *Piz, Pish* (Fukunishiki), *Piz-t* (Toride 1), *Pik-p* (K60) and *Pik-m* (Tsuyuake, 10 % susceptible) (Table 3).

Some interchanges of resistance and susceptibility such as *Pi-s, Pish* (Shin 2, 40 % susceptible), *Pia* (Aichi Asahi, 70 % of susceptible), *Pii, Pik-s* (Ishikari Shiroke, 30 % of susceptible), *P-ik, Pi-sh* (Kusabue, 20 % suceptible), *Pi-ta* (Yashiro-mochi, 40 % susceptible), *Pita-2, Pish* (PiNo.4, 20 % susceptible), *Pi-b, Pi-sh* (BL1, 20 % susceptible), *Pi-t* (K59, 50 % susceptible). In this study some blast resistance genes have been identified to be more durability to blast pathogens such as *P ik-m* (Tsuyuake), *Piz, Pish* (Fukunishiki), *Piz-t* (Toride 1) and *Pik-p* (K60). However, using Kiyosawa's differential, some varieties carry two resistance genes Recently, reaction of 31 monogenic lines by natural infection of blast (multilocation test) and inoculation test with 540 isolates have been conducted (data not shown here). In near future gene pyramiding is going to be set up for blast resistance breeding program at CLRRI.

Table 1: Reaction of promising lines and varieties to blast (*Pyricularia grisea*) scored at OMon, Can tho from 1980-2004

No.	Varieties	Year released/ scale		Years after releasing *											
				90	91	92	93	94	95	96	97	98	99	00	02	04
1	IR 42	1980	5	+	+	+	+	+	+	+	+	+	+	+	+	+
2	IR 48	1980	1	-	-	-	-	-	-	-	-	+	+	+	+	+
3	IR 64	1984	1	-	-	-	-	-	-	-	-	-	-	+	+	+
4	OM 576	1986	4	-	+	+	+	+	+	+	+	+	+	+	+	+
5	IR 50404	1988	2	-	-	-	-	-	-	-	+	+	+	+	+	+
7	OM 1490	1992	1			(1)	-	-	-	-	-	-	+	+	+	+
8	OM 2037	1995	1						(1)	-	-	-	-	+	+	+
10	OM 2031	1997	1								(1)	-	-	-	+	+
15	VÑ 20	1997	7								+	+	+	+	+	+
15	KHAO 39	1998	9									+	+	+	+	+
11	OM 3536	2000	5											(5)	+	+
17	OMCS	2000	7											+	+	+
18	OM 4495	2000	7											+	+	+
20	OM 2717	2000	9											+	+	+
19	OM 3242	2001	9											+	+	+
12	OM 2517	2002	3												(3)	+
13	OM 2490	2002	3												(3)	+
14	OM 2519	2002	3												(3)	+
21	OM 2718	2002	7												(7)	+
22	OM 2492	2002	9												(9)	+

(*) infection level: (-) resistant ; (+) susceptible

Table 2: Reaction of Kiyosawa differential varieties with different resistance genes at Can Tho, from dry season 2000 to wet season 2003

Varieties	Resistance genes	Score of blast reaction over time at one location							
		Dry 00	Wet 00	Dry 01	Wet 01	Dry 02	Wet 02	Dry 03	Wet 03
1. Shin 2	*Pik-s, Pish*	S	S	S	R	R	S	S	S
2. Aichi Asahi	*Pi-a*	S	S	S	S	S	S	S	R
3. Ishikari Shiroke	*Pii-Pik-s*	S	S	R	R	R	S	R	R
4. Kanto 51	*Pi-km*	R	R	R	R	R	S	S	R
5. Kusabue	*Pi-k, Pish*	R	R	R	R	R	R	R	S
6. Fukurishikari	*Pi-z, Pish*	R	R	R	R	R	R	R	R
7. Yashiro-mochi	*Pi-ta*	S	S	R	S	R	R	S	R
8. Pi No.4	*Pita-2, Pish*	R	R	R	R	R	S	R	R
9. Toride 1	*Piz-t*	R	R	R	R	R	R	R	R
10. K60	*Pik-p*	R	R	R	R	R	R	R	R
11. BL1	*Pi-b, Pish*	S	S	S	R	S	S	S	R
12. K59	*Pit*	R	R	S	S	S	S	S	R

Table 3 : Reaction of Kiyosawa differential varieties with resistance genes at 10 different places in Mekong delta, wet season 2003

Varieties	Resistance* genes	Scores of varieties at different locations of Mekong delta **									
		CT	AG	TV	ST	TG	KG	CM	BT	LA	DT
1. Shin 2	*Pik-s, Pish*	S	S	S	S	R	R	S	R	S	S
2. Aichi Asahi	*Pi-a*	R	S	S	S	S	R	R	S	S	S
3. Ishikari Shiroke	*Pii-Pik-s*	R	S	S	S	S	R	R	R	S	R
4. Kanto 51	*Pi-km*	R	S	R	S	R	R	S	R	S	R
5. Kusabue	*Pi-k, Pish*	S	R	R	R	R	R	R	R	R	R
6. Fukunishiki	*Pi-z, Pish*	R	R	S	R	R	R	R	R	R	R
7. Yoshiro-mochi	*Pi-ta*	R	S	S	S	R	R	R	R	R	S
8. Pi No 4	*Pita-2, Pish*	R	S	S	R	R	R	R	S	R	R
9. Tonde 1	*Piz-t*	R	R	R	R	R	R	R	R	R	R
10. K60	*Pik-p*	R	R	R	R	R	R	R	R	R	R
11. BL1	*Pi-b, Pish*	R	R	R	R	R	R	R	S	R	S
12. K59	*Pit*	R	S	R	S	S	R	S	R	S	R

Note: * each variety may posseses two resistance genes

** CT= Can Tho, AG= An Giang, TV= Tra Vinh, ST= Soc Trang, TG= Tien Giang, KG= Kien Giang, CM= Ca Mau, BT= Ben Tre, LA= Long An, NT= Dong Thap

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Current status of asparagus diseases in Southeast Asia (SEA)

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Keywords: asparagus, diseases, Southeast Asia

Introduction

Asparagus (*Asparagus officinalis*) was introduced into SEA in 1950s (Anon. 1985) and quickly became preferred vegetable. Shoots are consumed fresh, except a few farms in Malang (Indonesia) and Malacca (Malaysia) that produced them for canning in 1980s. The canning factories have ceased operation due to insufficient supply of shoots. All plantations throughout SEA are facing serious problems, especially by diseases (Salleh *et al.* 1996).

Materials and Methods

A continuous survey was conducted on 24 farms in Malaysia, Indonesia, Thailand, and Brunei Darussalam for the last 15 years (Table 1). Plant parts showing abnormalities or disease symptoms were collected and the causal organisms isolated and identified (Salleh & Sulaiman 1984). Pathogenicity tests of selected fungal isolates representative of every species, except one rust species i.e. *Puccinia asparagi*, obtained from naturally diseased asparagus were carried out on healthy 10 month-old asparagus seedlings varieties Mary Washington (MW) and UC 157 in the greenhouse at the SBS, USM.

Table 1: Nineteen asparagus varieties planted in SEA

Asp. 83005	Mersterscha	Locullus	British Imperial	MW 500
UC 800	California	Poletum	Jainan	UC 309
Jersey Giant	Cas. 85057	Schwehinge	Darbone	Trio
Greenwich	UC 72	J 200	UC 157	

Results and Conclusion

Our survey showed that fungal diseases were more frequent and serious than those caused by bacteria and viruses. Nineteen introduced varieties from subtropical and temperate regions were recorded from the farms. The most destructive disease was crown and root rot (*Fusarium proliferatum)* (Salleh 1990), followed by wilts (*F. oxysporum* f. sp. *asparagi*) (Fig. 1), anthracnose (*Colletotrichum capsici*), brown rot (*Curvularia* spp.) (Salleh *et al.* 1996), Phomopsis blight (*Phomopsis asparagi*), stem canker (*Fusarium* spp.), Phytophthora rot (*Phytophthora megasperma*), rust (*Puccinia asparagi*), crown spot and shoot die-back (*Alternaria tenuissima*), gray mold (shoot blight) (*Botrytis cinerea*), purple spot (*Stemphylium vesicarium*), and Cercospora blight (*Cercospora asparagi*). The three most important viral diseases were mosaic, crinkles and witches broom. The most prevalent disease caused by bacteria was slimy rot (*Pseudomonas solanacearum*). Most of these diseases have not been reported from this region.

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Table 2: Asparagus sampling plots in SEA

Sampling Plots	Temperature (°C)[b] Day	Night	Rainfall (mm)[c]
[a] Bukit Temiang	32.1	21.6	272.4
Kampung Cikgu Ali	32.5	21	267.3
[a] Bertam	31	22.9	321
[a] Kubang Menerong	31.3	22.9	304.2
[a]Relau	31	23.4	241.6
[a] Sungai Ara	31	23.4	241.6
Kuala Berang	30.6	22.9	205.1
[a]Kota Bharu	31.6	23.2	313.9
Bacok	32.3	22.1	403.4
[a] Cameron Highlands	24.1	14.1	230.2
Kuantan	29.8	22.5	261.8
[a] Klang	32.6	22.8	240.9
Lorong	31.7	22.8	289.5
Merlimau	31.3	22	214.6
Johor Bharu	31.5	22.4	203.6
Semongok	33.6	21.5	349.3
[a] Tuaran	31.9	22.9	197.7
Kundasang	23.9	16.3	245
[a]Birau, Brunei DS	32.4	22.5	187.9
Medan, Sumatera	32	22	220
Berastagi, Sumatera	30	21	230
Ambarawa, Java	31.8	22	203
Malang, Java	31	21.1	223
Hatyai, southern Thailand	32	22	225

aFor experimental purposes
bDaily average of maximum (day) and minimum (night) temperatures
cMonthly average

Table 3: Diseases of asparagus

Disease	Pathogen
Fungi	
Crown & root rot	*F. proliferatum*
Wilts	*F. oxysporum* f. sp. *asparagi*
Anthracnose	*Colletotrichum capsici*
Brown rot	*Curvularia lunata*, *C. eragrostidis*, *C. pallesecens*, *C. brachyspora*
Phytopthora rot	*Phytophthora megasperma*
Stem rot	*Phomopsis asparagi*
Stem canker	*Fusarium proliferatum*
Purple spot	*Stemphylium vesicarium*
Leaf & twig necrosis	*Cercospora asparagi*
Rust	*Puccinia asparagi*
Crown rot & shoot die-back	*Alternaria alternata*, *A. tenuissima*, *A. brassicae*
Exserohilum spot	*Exserohilum* sp.
Viruses	
Mosaic	
Crinkles	
Witches broom	
Bacteria	
Slimy rot	*Pseudomonas solanacearum*

Taxonomic and phylogenic study on *Bipolaris* fungi from Japan and their productivity of ophiobolins

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Keywords: *Cochliobolus*, grasses, host non-specific toxin, leaf spot

Introduction

The genus *Bipolaris* (teleomorph: *Cochliobolus* and *Pseudocochliobolus*, synonym: *Helminthosporium*) is an important plant pathogenic fungus causing leaf spot and blight on many kinds of gramineous plants and crops. Recently, molecular phylogenetic analyses showed that *Bipolaris* is close to *Curvularia* and both formed a phylogenetic clade different from *Exserohilum* and *Drechslera,* the other '*Helminthosporium*' genera (Berbee *et al.* 1999). In this study, the Japanese isolates of *Bipolaris* were classified taxonomically and phylogenically with the productivity of ophiobolins, host non-specific toxin of sesterterpene.

Materials and Methods

Collection and phylogenic analysis: We collected 153 isolates of the genus mainly from southern area and examined them taxonomically under a light microscope. Whole genomic DNA was extracted from each isolate and used as a template for PCR. The ITS regions and 5.8 S rDNA were amplified with the PCR conditions using a primer pair of ITS1 and ITS4 and sequenced. Phylogenic analysis was made by NJ method.

Productivity of ophiobolins: CM (complete media) liquid cultures of the isolates were extracted with ethyl acetate. The extracted dried materials were extended on TLC with standard of ophiobolins by a 1:1 solvent of ethyl acetate and benzene. The Rf value and color of the spots of ophiobolins were determined by spraying vanilline in sulfic acid followed by heating.

Results and Discussion

We described a new species, *Co. heveicola* Tsukib. & W. H. Chung [=*B. heveae* (Petch) Arx], causing brown stripe on bermudagrass and Zoysia grass (Fig.-⑥, Tsukiboshi et al. 2004). Four groups of *Bipolaris* spp. were supposed to be new species based on their morphology and phylogeny (②③④⑧). *Cochliobolus peregianensis* was newly recorded in Japan causing eye spot, a new disease, of bermudagrass (⑦). The other four species were also

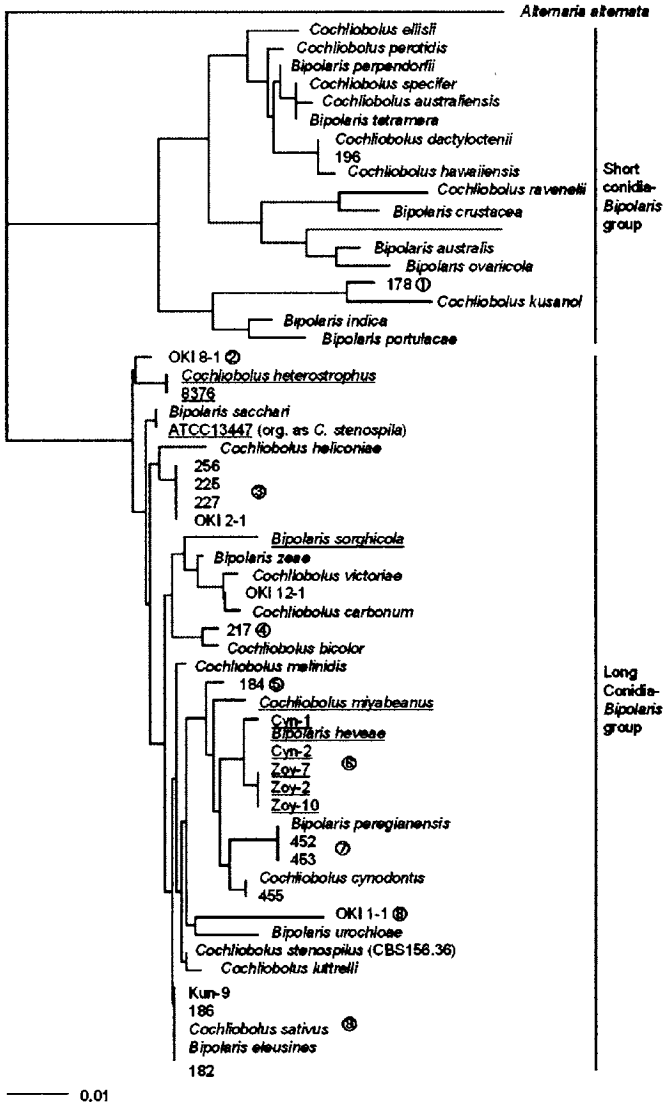

Fig. Phylogenic tree of *Bipolaris* fungi
The isolates with underlines produce ophiobolins.

thought to be new to Japan. rDNA-ITS regions of *Co. nodulosus* and *Co. setariae* at first sequenced (①,⑤). *Cochliobolus sativus* was found to cause a new leaf spot of orchardgrass (⑨). In results of phylogenic analysis, the *Bipolaris* isolates could be classified into the groups producing short or long conidia as reported before (Berbee *et al.* 1999). The groups were genetically distant to each other with 84% bootstrap value in a MP tree reflecting their difference in the morphology of teleomorph, *Cochliobolus* (long conidia) and *Pseudocochliobolus* (short conidia).

All the species producing ophiobolins belonged to the long-conidia *Bipolaris* group and were classified in close clades in the phylogenic tree. They produce curved and spindle-shaped conidia and were supposed to be genetically distant from *Bipolaris* producing straight and clavate conidia that never produced ophiobolins. The species of the short conidia group never produced ophiobolins.

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Studies on phytopathological, morphological and molecular variations of *Plectosporium tabacinum* in Japan

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Keywords: fungi, host specificity, identification, plant pathogen, taxonomy,

Introduction *Plectosporium tabacinum* (van Beyma) M.E. Palm, W. Gams & Nirenberg, which was validly described in 1995 (Palm *et al.* 1995), had been known as a common soil fungus at rhizospheres, and was isolated from lake sediment for the first time in Japan (Tubaki and Ito 1975). We obtained several isolates of the fungus pathogenic to plants in Japan, and found that it contained various strains phytopathologically, morphologically and molecularly, using the isolates. We present the details here.

Phytopathological variation Pumpkin (7 cultivars), garden ranunculus (1 cultivar) and lotus ginger (1 cultivar) were inoculated with 10 isolates (Table 1). Conidia of each isolate that had formed in culture on potato dextrose agar (PDA) at 25℃ in the dark for 2 weeks were suspended in sterilized distilled water at 5×10^5 conidia/ml to be sprayed onto 2–4 healthy plants per cultivar. Healthy plants sprayed with sterilized distilled water served as controls. All treated plants were covered with polyethylene bags and kept in a greenhouse at 22–25℃. The bags were removed after 2–3 days. Observation of symptoms and re-isolation of the fungus from the treated plants were performed. As a result, the respective isolates were virulent only to the original host plants, but the others caused no disease in the plants tested (Table 1). It was thought that the isolates of *P. tabacinum* had host specificity.

Table 1. Isolates used and their pathogenicity to pumpkin, garden ranunculus and lotus ginger.

Isolate	Collection locality	Isolation source	Plate 1	Pathogenicity to		
				pumpkin	garden ranunculus	lotus ginger
MAFF238627	Kagoshima, Japan	pumpkin [*Cucurbita* sp.]	①	+	−	−
MAFF238628	Ibaraki, Japan	pumpkin [*Cucurbita* sp.]		+	−	−
MAFF238629	Kagawa, Japan	garden ranunculus [*Ranunculus asiaticus*]	②	−	+	−
MAFF238632	Chiba, Japan	garden ranunculus [*Ranunculus asiaticus*]		−	+	−
MAFF238633	Chiba, Japan	anemone [*Anemone coronaria*]	③	−	−	−
MAFF238634	Ehime, Japan	sweet pepper [*Capsicum annuum* var. *grossum*]	④	−	−	−
MAFF238958	Tokyo, Japan	lotus ginger [*Crucuma alismatifolia*]	⑤	−	−	+
MAFF238960	Tokyo, Japan	lotus ginger [*Crucuma alismatifolia*]		−	−	+
MAFF238961	Tokyo, Japan	lotus ginger [*Crucuma alismatifolia*]		−	−	+
MAFF238962	Tokyo, Japan	lotus ginger [*Crucuma alismatifolia*]		−	−	+
MAFF238963	Chiba, Japan	lotus ginger [*Crucuma alismatifolia*]		−	−	+
MAFF238966	Miyazaki, Japan	radish [*Raphanus sativus*]	⑥	−	−	−
IFO9985	Ezypt	sweet violet [*Viola odorata*]		−	−	−
IFO30005	Hyogo, Japan	lake sediment		−	−	−

+: Virulent −: Virulence was not detected.

Morphological and molecular variations Morphological characters of *P. tabacinum* are as follows (Plate 2). Colonies in culture on PDA at 20℃ in the dark are flat with little aerial mycelium, smooth and cream to salmon or pale brown in color. Conidiophores are unbranched or occasionally branched, with conidiogenous cells often arising at right angles from vegetative hyphae. Conidiogenous cells are monophialides formed at the apices, or as short (adelophialides) or long blanches from vegetative hyphae, hyaline, smooth, cylindrical to obclavate, sometimes crooked or sinuous at the tips, often with single conidiogenous apertures, and occasionally with second apertures. Conidia are produced in colorless slime masses at the tips of the phialides, hyaline, smooth, oblong-ellipsoidal, usually asymmetrical to slightly curved, multiguttlate and most are 1-septate and with a few aseptate. Growth speed of mycelia and appearance rates of aseptate conidia on PDA at 20℃ in the dark varied from 2.7–4.7 mm/day and 0–28.6%, respectively, in 6–8 isolates. Sizes of septate and aseptate conidia that had formed on synthetic low nutrient agar (SNA) at 20℃ in the dark ranged from 4.0–12.0×1.0–5.5 μm and 2.5–8.5×1.0–3.5 μm, respectively, depending on the isolates. Ten isolates were also classified into 2 groups based on their sequence data of rDNA ITS regions, though their homologies were highest (>94%) with those of *P. tabacinum* registered in the DDBJ (Fig. 1). Thus, *P. tabacinum* was also found to contain various strains morphologically and molecularly.

Plate 1

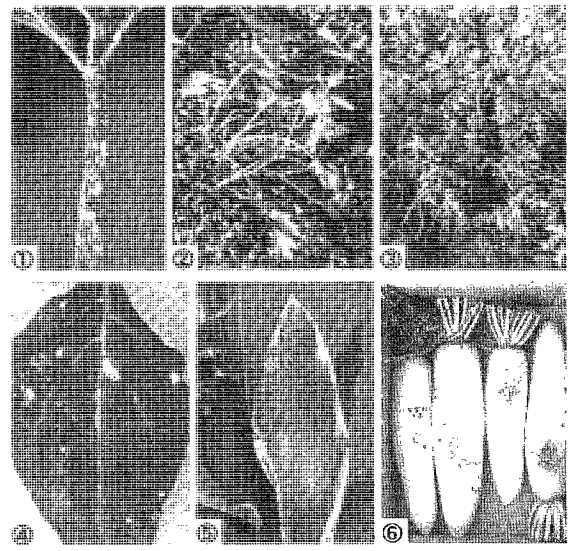
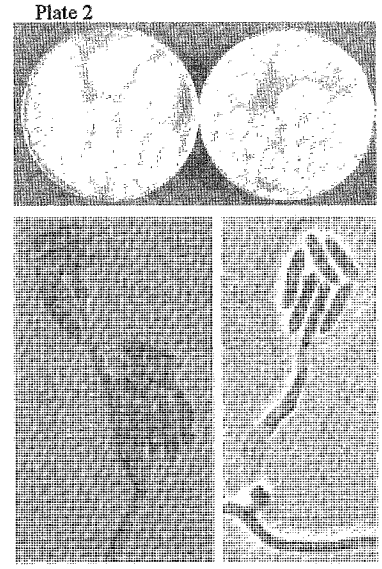


Plate 2



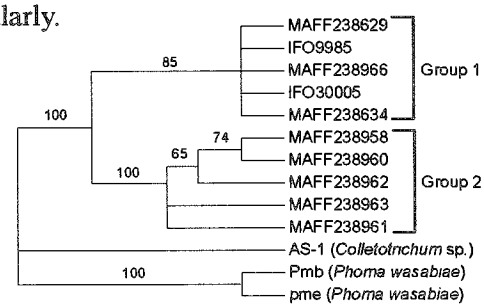


Fig 1. Grouping of *P. tabacinum* isolates based on neighbor-joining analysis for sequence of rDNA ITS regions ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATAT GC-3') were used as primers for PCR. Numbers above branches are bootstrap values (%)

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Morphology and molecular taxonomy of *Colletotrichum destructivum* (Teleomorph: *Glomerella glycines*) and related species

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Keywords: *Colletotrichum higginsianum, C. linicola, C. fuscum,* rDNA sequences

Introduction

Colletotrichum destructivum O'gara (Teleomorph: *Glomerella glycines* Lehman & Wolf), *C. higginsianum* Sacc., *C. linicola* Pethybr. & Lafferty and *C. fuscum* Laub. have similar morphological characters, e.g. conidia and appressoria, however their pathogenicity are different (Sutton 1980, 1992). We think those species as *C. destructivum sensu lato* on the basis of the morphological and molecular characterization.

Morphology

They produced conidia straight or slightly curved, fusiform to cylindrical, tapered to each end, 12.4-22.2 x 2.3-4.8 μm and formed appressoria clavate to obovate, dark brown, 6.5-12.9 x 4.1-7.9 μm on PCA slide culture at 25℃ under black light. Colonies on PDA, orange, pale orange, salmon pink, brown to sepia brown or rosy buff, with white felted aerial mycelia. Conidia formed on acervuli or on stromata covered with hyphae and with sparse setae.

Molecular taxonomy

The intraspecific DNA homologies of rDNA ITS2 and 28S rDNA domain2 sequences of *C. destructivum sensu lato* were 98.9 to 100%, but interspecifically 94.8 to 96.8% with *C. acutatum* Simmonds ex Simmonds, *C. coccodes* (Wallr.) S. Hughes and *G cingulata* (Stonem.) Spauld. & von Schrenk. In phylogenetic analysis using neighbor-joining method, the examined strains of *C. destructivum sensu lato* made a clade with 92% bootstrap value (Fig. 1). Thus *C. destructivum* and the related species are the same species.

Pathogenicity

Results of inoculation experiments showed host specificity of some of the isolates in the species. Although the isolates from crucifer and from flax were virulent only to their host plants, they didn't cause any diseases of other plants. *Colletotrichum destructivum* from legumes also attacked host plants and made small spots on the seedlings of crucifer and flax. Some isolates of the species were found to be host specific, and the others appeared to be omnivorous pathogens.

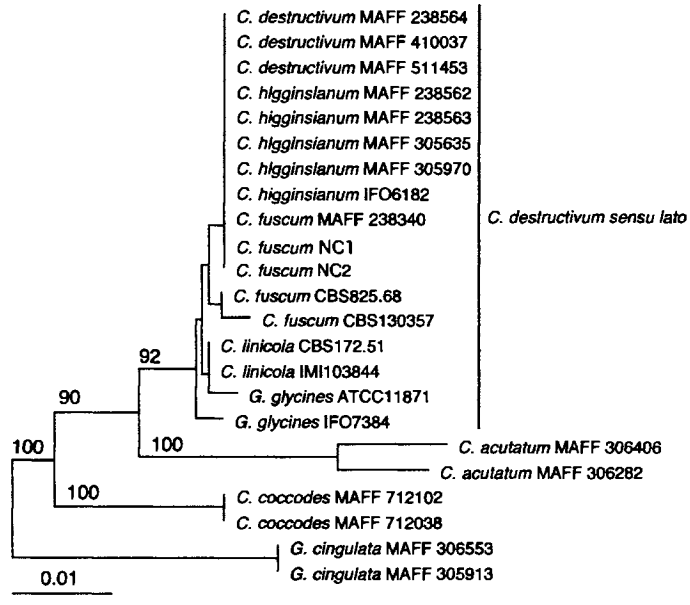

Fig. 1. Tree illustrating relatedness of *Colletotrichum* species, based on neighbor-joining analysis of the ITS2 and 28S rDNA D2 regions. Percentages of neighbor-joining analysis of 1000 bootstrapped data sets that support specific branches are indicated at the respective nodes. Bootstrap values greater than 90% are shown. Bar=distance corresponding to one base changes per 100 nucleotide positions.

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Pathogenicity of some *Colletotrichum* species to petals of *Antirrhinum majus*

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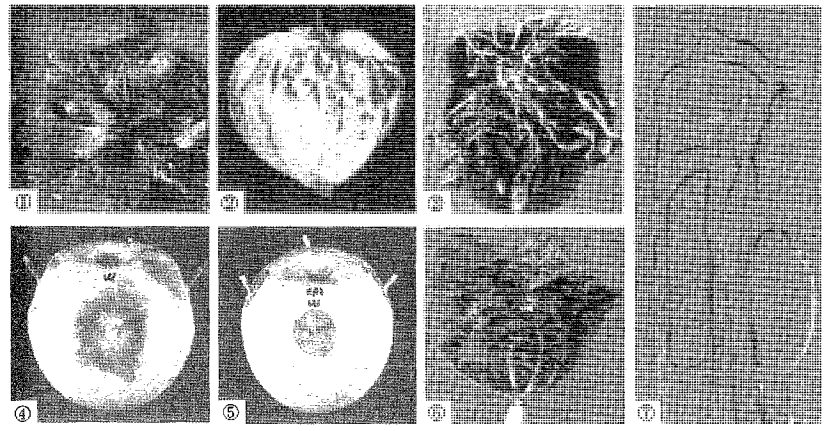
Keywords: anthracnose, flower disease, fungi, host range, plant pathogen,

Introduction Anthracnose of snapdragon (*Antirrhinum majus*, scrophulariaceous plants) was reported to be caused by *Colletotrichum gloeosporioides* (Nanbu, 1916; Kobayashi et al., 1992). However, no details on the identification and pathogenicity of the causal fungus as well as its symptoms have been described, and no isolates of the pathogen have been preserved. We reported previously that *C. destructivum* was virulent only to petals of the plant (Tomioka *et al.* 2000; Moriwaki *et al.* 2002, 2003). In this study, the pathogenicity of *C. gloeosporioides*, *C. acutatum*, *C. dematium* and *C. circinans* to the plant was investigated.

Materials and Methods Snapdragon was inoculated with each fungal isolate listed in Table 1. Conidial suspension ($1\times10^{4-6}$ conidia/ml) was sprayed onto healthy plants. Healthy plants sprayed with sterilized distilled water were served as controls. All plants were kept and observed in a green house at 24–28℃. Re-isolation of the fungi from the treated plants was also performed. The experiments were replicated twice for plants at both the seedling and the flowering stages.

Table 1 *Colletotrichum* isolates used.

Species	Isolate	Collection locality	Isolation source	Photo
C. destructivum	MAFF238453	Miyagi, Japan	dahurian patrinia [*Patrinia scabiosaefolia*]	①
	MAFF238560	Saga, Japan	lantern plant [*Physalis alkekengi* var. *francheti*]	②
	MAFF238340	Kagawa, Japan	nemesia [*Nemesia strumosa*]	③
C. gloeosporioides	MAFF238043	Ibaraki, Japan	apple [*Malus pumila*]	④
C. acutatum	MAFF306546	Ehime, Japan	apple [*Malus pumila*]	⑤
C. dematum	MAFF238704	Miyazaki, Japan	radish [*Raphanus sativus*]	⑥
C. circinans	MAFF238640	Kagawa, Japan	onion [*Allium cepa*]	⑦



Results Not only *C. destructivum* but also *C. gloeosporioides*, *C. acutatum*, *C. dematium* and *C. circinans* were found to be pathogenic to snapdragon. When healthy plants at the flowering stage were inoculated with the isolates, they developed brown blotches on their petals only (Fig. 1). Water-soaked, irregularly shaped blotches and/or necrotic spots 0.5–1 mm in diameter appeared on petals 3–4 days after inoculation (Fig. 2). The lesions gradually enlarged and coalesced, resulting in early blight of whole petals by 10 days after inoculation. Each fungus was re-isolated from the diseased petals, but not from stems and leaves of the plants. Control plants had no symptoms. Neither fungus was re-isolated from the control plants, demonstrating that the isolates were pathogenic to snapdragon. No isolate produced symptoms on the plants at the seedling stage. Neither fungus was re-isolated from the plants.

Fig. 1 Petal blight of snapdragon caused by inoculation with the *Colletotrichum* isolates

Discussion In the first report for snapdragon anthracnose, lesions that appeared on leaves and/or stems were noted to result in early death of the diseased plants (Nanbu 1916). Symptoms identified here differ from those described in the report. But, this study supports the presence of the disease by *C. gloeosporioides*, and indicates that not only *C. gloeosporioides* and *C. destructivum* but also *C. acutatum*, *C. dematium* and *C. circinans* can correspond to its pathogen. *Colletotrichum* species are broadly classified into the following two groups: one produces curved conidia, and the other forms straight conidia. *Colletotrichum dematium* and *C. circinans* are the former, and *C. gloeosporioides*, *C. destructivum* and *C. acutatum* are the latter. *Colletotrichum* may be generally virulent to snapdragon and other scrophulariaceous plants, which are important as ornamental plants in agriculture. It is interesting whether the virulence of other *Colletotrichum* species occur only in petals or not.

Fig. 2 Lesions on a petal of snapdragon inoculated with *C. gloeosporioides*.

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Virulence of *Fusicoccum aesculi*, *Phomopsis phomoides*, *Fusarium lateritium* and *Stemphylium lycopersici* to sweet pepper fruits

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Keywords: fungi, identification, new disease, plant pathogen, postharvest disease

Introduction Four postharvest diseases of sweet pepper (*Capcicum annuum* var. *grossum*) were frequently found in Kagawa Prefecture, Japan, in 1999–2001. We repeatedly isolated anamorphic fungi from the diseased fruits, identified them as *Fusicoccum aesculi*, *Phomopsis phomoides*, *Fusarium lateritium* and *Stemphylium lycopersici* and demonstrated their pathogenicity to sweet pepper. We present the details here.

Symptoms Diseases caused by *F. aesculi*, *P. phomoides* and *S. lycopersici* were found on ripe fruits (Plates 1, 3), and disease by *F. lateritium* was found on non-ripe fruits (Plate 2). The diseases by *F. aesculi* and *P. phomoides* were recognized on fruits diseased by mixed infection of the two fungi. In all four of the diseases, water-soaked lesions or necrotic lesions appeared initially. They gradually enlarged and softened, resulting in entire rot of the affected fruits. In the fruits diseased by *F. aesculi* and *P. phomoides*, their pycnidia appeared to exude conidial masses. In those by *F. lateritium* and *S. lycopersici*, their conidiophores and conidia appeared. The

Plate 1

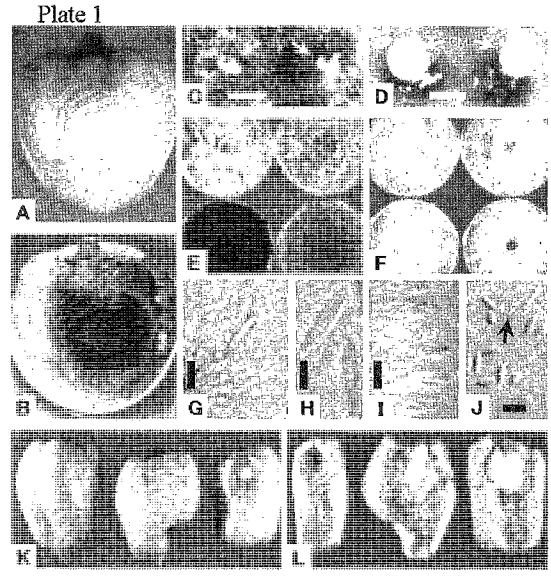
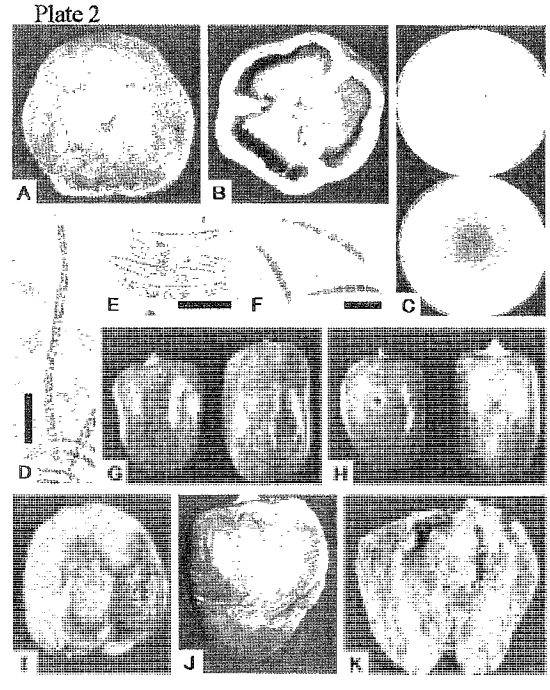


Plate 2



isolates of each fungus produced conidia, which were as same as those on the lesions, on potato dextrose agar (PDA) or synthetic low nutrient agar (SNA) at 25℃ under a black light (BLB), and were identified as the fungal species based on conidiogenesis.

Pathogenicity A conidial suspension of each isolate ($1\times10^{4-6}$ conidia/ml) was dripped onto the surface of healthy fruits, or injected into healthy fruits. Healthy fruits treated with sterilized distilled water served as controls. All fruits were kept in moist condition at 24–28℃. The conidial suspension was also sprayed onto healthy seedlings. Healthy seedlings sprayed with sterilized distilled water served as controls. All seedlings were kept in a green house at 24–30℃. As a result, the natural symptoms were reproduced by inoculation with each isolate. The reproduction by the isolates of *F. aesculi*, *P. phomoides* and *S. lycopersici* was successful on ripe fruits but not on non-ripe fruits. That of *F. lateritium* was successful on both ripe and non-ripe fruits. Control fruits had no symptom. Each fungus was re-isolated from the diseased fruits, but not from the control fruits, demonstrating that the isolate was pathogenic to sweet pepper fruits. No isolate produced any symptom on seedlings, and neither fungus was re-isolated from them.

Disease name Sweet pepper is a new host for every species of *F. aesculi*, *P. phomoides* and *F. lateritium*. The disease by *S. lycopersici* had not been also reported previously, though leaf spot of the plant caused by the fungus has been noted (Saito *et al.* 1968, 1970). We coined the term "fruit rot" to refer to all four of the diseases.

Plate 3

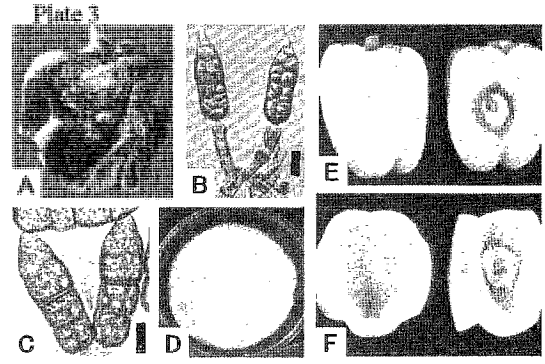


Plate 1

A–D Natural symptoms and signs of sweet pepper fruit rot caused by mixed-infection of *F. aesculi* and *P. phomoides*
A. Fruit rot in an early stage. B. Fruit rot with numerous pycnidia of *P. phomoides* in a late stage. C Pycnidia of *F. aesculi* and their conidial masses (CM: conidial masses, bar: 400 μm) D Pycnidia of *P. phomoides* and their conidial masses (CM: conidial masses, bar: 400 μm)

E–J Morphological and cultural characters of the pathogens of sweet pepper fruit rot, *F. aesculi* and *P. phomoides*.
E. Colonies of *F. aesculi* isolate PF1 produced on PDA at 25°C for 10 days (left: in the dark, right: under black light; upper: surface view; lower: reverse view) F. Colonies of *P. phomoides* isolate PP1 produced on PDA at 25°C for 10 days (left: in the dark, right: under black light; upper: surface view; lower: reverse view). G Conidiogenous cells and conidia of *F. aesculi* isolate PF1 produced on PDA at 25°C under black light for 10 days (bar: 20 μm). H. Conidia of *F. aesculi* isolate PF1 produced on PDA at 25°C under black light for 10 days (bar: 10 μm) I Conidiogenous cells and conidia of *P. phomoides* isolate PP1 produced on PDA at 25°C under black light for 10 days (bar: 20 μm) J. Conidia of *P. phomoides* isolate PP1 produced on PDA at 25°C under black light for 10 days (arrow: a β conidium) (bar: 10 μm)

K, L. Symptoms reproduced 8 days after inoculation of sweet pepper fruits with *F. aesculi* isolate PF1 and *P. phomoides* isolate PP1 (left to right: control, isolate PF1 and isolate PP1)
K. Outside symptoms. L. Inside symptoms

Plate 2

A, B. Natural symptoms and signs of sweet pepper fruit rot caused by *Fusarium lateritium*
A. Outside symptoms and signs (white mycelia on epicarp). B. Inside symptoms and signs (white mycelia around seeds).

C–F. Morphological and cultural characters of the pathogen of sweet pepper fruit rot, *F. lateritium* isolate SF1.
C. Colonies produced on PDA at 23–28°C in the shading natural light for 5 days (upper: surface view, lower: reverse view). D. A conidiophore and a young conidium produced on SNA at 25°C in the dark for 4 days (bar: 10 μm). E. Conidia produced on SNA at 25°C in the dark for a week (bar: 10 μm) F. Cotton blue-stained conidia produced on SNA at 25°C in the dark for a week (bar: 10 μm).

G–K. Symptoms reproduced 8–14 days after inoculation of sweet pepper fruits with *F. lateritium* isolate SF1 (G–I: 8 days after inoculation, J, K: 14 days after inoculation).
G Outside (unwounded side) symptoms of unripe fruits (cv. High Green) (left: control)
H. Outside (wounded side) symptoms of unripe fruits (cv. High Green) (left: control)
I. Outside (unwounded side) symptoms of a ripe fruit (cv. Spirits). J. Outside (unwounded side) symptoms and signs (white mycelia) of an unripe fruit (cv. High Green).
K. Inside symptoms of an unripe fruit (cv. High Green)

Plate 3

A. Natural symptoms and signs of sweet pepper fruit rot caused by *Stemphylium lycopersici*

B–D Morphological and cultural characters of the pathogen of sweet pepper fruit rot, *S. lycopersici* isolate SS1.
B. Conidiophores and young conidia produced on a lesion (bar: 10 μm) C Mature conidia formed on a lesion (bar: 10 μm) D A colony produced on PDA at 25°C under black light for 8 days.

E, F Symptoms reproduced 11 days after inoculation of sweet pepper fruits with *S. lycopersici* isolate SS1 (left: control)
E Outside symptoms F. Inside symptoms

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Saccharomyces cerevisiae genome-wide mutant screen for antifungal activities of yeasts, *Williopsis mrakii, Kluyveromyces lactis* and *S. cerevisiae*

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Keywords: chitin, glucan, killer protein, RNA polymerase

Introduction

Antagonistic interactions between yeasts by secreted proteinaceous toxins appear to be quite high in natural habitat. To know the molecular mechanisms of yeast killer proteins, we screened a collection of *S. cerevisiae* mutants, individually deleted for 4901 yeast genes, for altered sensitivity against purified killer proteins of *W. mrakii* (WmKT) and *K. lactis* (KlKP). The sequenced and analyzed *S. cerevisiae* genome has enabled to construct a collection of mutant strains deficient in each gene by targeted gene disruption (Winzeler *et al.* 1999). Such a collection promotes the discovery of not only cellular roles of genes, but also the mechanism of toxin action. To know the characteristic of each killer action, we compared the results of sensitivity to WmKT and KlKP with the results of *S. cerevisiae* K1 killer protein (Pagé *et al.* 2003), respectively.

Materials and Methods

Purification of killer proteins: *K. lactis* killer protein was concentrated and purified from 1.3 L of YPD culture broth of *K. lactis* IFO1267 to 13ml by hydroxyapatite column chromatography as described in (Sugisaki *et al.* 1984). Killer protein of *W. mrakii* was purified from 50 ml of YNB culture broth to 3.5 ml by Macro Prep High S support column (BIORAD).

Analysis of sensitivity to killer protein: Approximately 1 x 10^3 yeast mutant cells were inoculated in 60 μl of 1M sorbitol-YPD liquid medium with diluted KlKP and WmKT in round bottomed 96 well microplates, respectively. The growth of mutants at 30°C was obsreved for 3 days.

Results and Discussion

The yeast cell wall is made principally of four components: mannoproteins, chitin,

β-1,3-glucan and β-1,6-glucan. Cell wall receptors of K1 and KlKP are reported as β -1,6-glucan and chitin, respectively. And WmKT was reported to inhibit cell wall β -1,3-glucan synthase of *S. cerevisiae* (Kimura *el al.* 1997). Killer spectrum of WmKT on the gene disruptants of N-glycosylation of olygosaccharide and its transfer to protein make the yeast cells resistant to K1 and WmKT, respectively. However, these mutants are sensitive to KlKP. As glucan mutants accumulate chitin, the increase in sensitivity of the mutants may be attributed to the increase of chitin. On the other side, deletion of the gene for RNA polymerase II machinery makes yeast cells resistant to HMK, KlKP and K1. Therefore, the specific cell wall structure and transcription machinery could easily be one of the potential mechanisms involved in antifungal actions, which is obtained during the course of its evolution.

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Isolation and characterization of bacteria in fermented vegetables sold in northern part of Vietnam

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Keywords: bacteriocin, coliform bacteria, lactic acid bacteria

Introduction

Fermented vegetables are an integral part of the diet of people in many countries. The organic acids or other metabolites produced by lactic acid bacteria during fermentation process give the raw vegetables desirable taste, flavor or texture. In addition, fermentation enriches food substances biologically with vitamins, proteins, essential amino acids and fatty acids. The organic acids or bacteriocins produced by long period fermentation may suppress the growth of pathogenic or spoilage bacteria.

The microfloras of Sauerkrauts or Japanese fermented vegetables are influenced by salt concentration. In high salt concentration, the growth of bacteria tends to be repressed by low water activity and high ionic activity in general. In the suitable concentration, salt will suppress the growth of undesirable bacteria without affecting that of lactic acid bacteria. However, not many reports related to the fermentation bacteria in the South-East Asian lightly fermented (salted) vegetables are published. So we investigated about the chemical property or microflora of lightly fermented vegetables in the northern part of Vietnam.

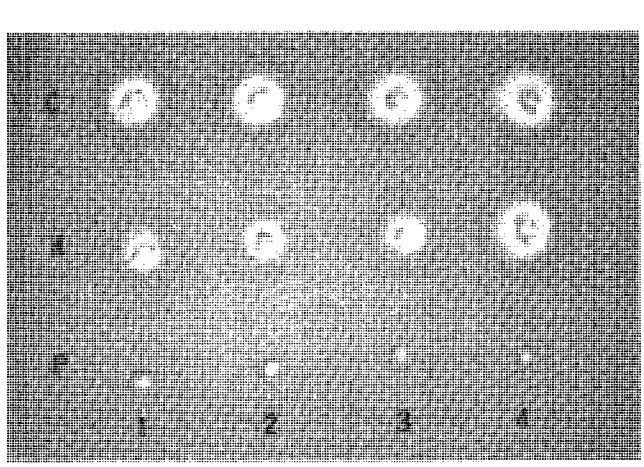
Materials and Methods

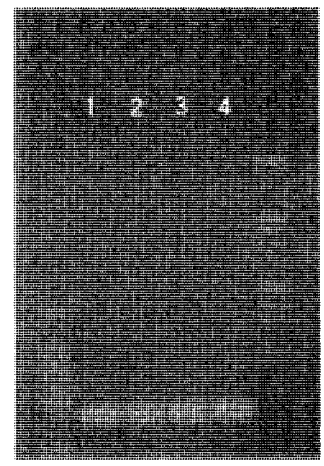
The lightly fermented vegetables sold in Hanoi city were collected in March 2003. Each ten grams of samples were stomached with 90 grams of phosphate buffered saline (PBS). In case of the measurement of pH or salt concentration, we used distilled water for stomaching instead of using PBS. One milliliter of solution was decimally serially diluted and used for enumeration according to the standard method.

The coliform bacteria grown in the desoxicolate agar plate were purified and identified by api20 kit followed by biochemical tests. The lactic acid bacteria (LAB) were similarly identified by using api50 kit. Bacteriocin production by LAB was assayed by agar well diffusion assay using *Lactobacillus plantrum* or *Listeria monocytogenes* as an indicator strain. The *nis*A genes in the bacteriocin producing strains were confirmed by PCR method.

Results and Discussion

Salt concentrations, pH and micro flora of 5 kinds of 37 samples were analyzed. The salt concentrations tend to be lower than that of Japanese lightly fermented vegetables and some samples did not ferment enough to increase the acidity. The aerobic plate count varied between 5 to 8 log CFU/g. Most of them were lactic acid bacteria (LAB), including *Lactobacillus*, *Leuconostoc*, *Lactococcus* and *Pediococcus*. Two to five log CFU/g of coliform bacteria also detected; all of identified strains were similar to the known natural flora on fresh vegetables, however. Bacteriocin producing strains effective for *L. monocytogenes* were screened from the isolated 200 LABs. Three *Lactococcus lactis* subsp. *lactis* strains producing nisin were obtained. The DNA sequence of *nis*A gene (structural gene of nisin A) of each strain was completely the same as that of previously reported one. One of the isolated *Bacillus subtilis* strain producing polyglutamic acid was found to produce antimicrobial compound(s) against Gram-positive *L. monocytogenes*.





(1) Nisin A producing strain, (2) Nisin Z producing strain, (3) NFRI7426, (4) NFRI7427
(C) Control, (H) Heat treatment, (P) Protease treatment

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A new method to obtain plasmid variants from *Lactococcus lactis*

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Keywords: incompatibility, plasmid curing

Introduction

Lactococcal strains generally carry a number of theta (θ)-replicating plasmids which are essential for fermentation or remain cryptic. Plasmid curing technique was performed to investigate the diverse properties of plasmids, and the treatment was currently performed by culturing with mutagenic chemical as acridine orange, culturing in unbuffered medium, exposure of cells to elevated growth temperatures, regeneration of bacterial protoplast or composite those methods. However, plasmids can not be chosen for excluding by these current methods. Moreover, there is a great risk of mutation that spoils property good for fermentation by the aforesaid treatments for plasmid curing. In this poster we present a new method to selectively exclude a residentθ-plasmid from *Lactococcus lactis* without producing any genomic damages and to obtain the plasmid variants as starters for products.

Materials and Methods

Bacterial strains, plasmids and culture: *E. coli* XL1-Blue (Stratagene) and a plasmid vector pBluescript II were used for plasmid construction. *Lactococcus lactis* ssp. *lactis* DRC1, N7, 527 and 712 were used for preparation of nativeθ-plasmids and for bacterial hosts of transformation. *E. coli* was grown on LB agar or LB medium at 37°C, supplemented with 50 μg ampicillin (Ap) ml^{-1} when required. *Lactococcus lactis* were grown in TYG agar or TYG medium (1% tryptone, 0.5% yeast extract, 0.5% sodium chloride, 1% glucose and 1% sodium succinate; pH 6.8) at 30°C, supplemented with 5 μg erythromycin (Em) ml^{-1} when required.

An outline of strategy for the selective exclusion of resident θ-plasmids, which are major in lactococci, was as follows: (1) Construction of universal receptor vector pDB1 for PCR fragment FV(X) containing variable region of native θ-plasmid replicon. (2) Construction of artificial replicons pCV(X)s being incompatible to a resident plasmid in lactococci by cloning with FV(X)s into pDB1. (3) Transformation of *L. lactis* strain with pCV(X) carrying erythromycin resistant (Emr)-gene. (4) Isolation of Emr-transformant. (5) Continuous cultivation in TYG-Em medium = Selective exclusion of the resident plasmid. (6) Isolation of

derivative excluded the resident plasmid. (7) Continuous cultivation in TYG medium without Em to exclude the pCV(X). (8) Isolation of Em-sensitive variant containing no exogenous DNA.

The construction of pCV(X)s and plasmid exclusion with pCV(X)s are described in detail in Results and Discussion. Transformation of *L. lactis* with a pCV(X) was performed by electroporation. Emr-transformants were selected on TYG-Em agar.

Results and Discussion

Three sets of oligonucleotide primers P1-P2, P3-P4 and P5-P6 were designed to amplify three parts (FC1, FC2 and FV(X)) of major lactococcal θ-plasmid replicons. The upstream part (FC1) and the downstream part (FC2) were amplified by PCR from a lactococcal θ-plasmid pDR1-1, which were from *L. lactis* DRC1. The FC1 and FC2 contained the conserved region of θ-replicon. The middle part (FV(X)) that contained a variable region of each replicon was amplified with P5-P6. The FC1, FC2 and an Emr-gene were cloned one after another into *Ban* III-*Eco* RI site, *Pst* I-*Xba* I site and *Sac* I site of pBluescript II, generating pDB1, which was used as a receptor vector for FV(X)s. Templates for PCR amplification of FV(X)s were purified by electrophoresis from total plasmids in the above lactococci. The FV(X)s could be inserted into *Nru* I-*Xho* I site of pDB1, generating artificial hybrid replicons pCV(X)s. Eight kinds of pCV(X)s could be constructed with FV(X)s, which were amplified from resident plasmids in 4 lactococci. After transformation of *L. lactis* wild-type strains with those pCV(X)s, four kinds of transformants (DRC1 carrying pCV1 and pCV5, N7 carrying pCVc8 and 712 carrying pCVm6) were isolated. The Emr-isolates were subcultured in TYG-Em media for selective exclusion of the resident plasmid, which were incompatible to co-existing pCV(X). Presence of the resident plasmid was examined by PCR analysis. After 100 generations, the variants losing the resident plasmid were appeared at 20 to 50% of a rate. The selectively plasmid exclusions of wild-type lactococci were ultimately succeeded with above 4 pCV(x)s (pCV1, pCV5, pCVc8 and pCVm6) in this manipulation. Finally, in order to exclude the pCV(X), the plasmid variants with pCV(X) were subcultured in TYG media. After 100 generations, each pCV(X) was completely excluded because the artificial replicon was instable without selective pressure. The plasmid variants produced by this method are able to use for starters for food-products because they contain no exogenous DNAs.

Effect of plasmids of *Lactococcus* strains on cytokine production from murine macrophage cells

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Keywords: probiotics, plasmid-curing, immunomodulatory activity

Introduction

Probiotics are commonly defined as viable microorganisms that exhibit a beneficial effect on the health of the host when they are ingested (Lee and Salminen 1995). One of the effective probiotic properties is their ability to stimulate host immunity. However, only limited information is available on the probiotics factors involved in the immunomodulatory functions of such microorganisms (Kitazawa *et al.* 1992; De Ambrosini *et al.* 1996). Since lactococci harbor various plasmids utilized for food industry, the immunological roles on the host are to be focused on. In the present study, the effects of a plasmid in lactococci on the cytokine response of a murine immunocompetent cell line are examined.

Materials and Methods

The lactococcal strains used in this study were grown in M17 broth (Difco) supplemented with 0.5% glucose. The cultures were heated at 100°C for 50 min and freeze dried. A murine macrophage cell line (J774.1) was obtained from ATCC (American Type Culture Collection). The cells were routinely grown at 37°C in a 5% CO_2-95% air atmosphere in RPMI-1640 supplemented with 10% inactivated (30 min, 56°C) fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 5 $\times 10^{-5}$ M 2-mercaptoethanol. For the assay, J774.1 cells were seeded at 5 x 10^5/ml cells into 24-well tissue culture plates in 1 ml/well and incubated for 48 hours. Then the medium was refreshed and the cells were further incubated with or without 100μl of additional stimuli such as lactococci or their cell wall fractions (10μg/RPMI medium) for 24 hours. *E. coli* LPS (Sigma, 1μg/ml) was used as a positive control for the stimulation of J774.1 cells. After the culture, the supernatants were harvested and stored at -80°C for cytokine assay by ELISA. Each experiment was conducted over two passages of the cells.

Results and Discussion

The plasmid-cured variant of *Lactoocccus lactis* subsp. *lactis* biovar *diacetylactis* DRC1 (strain DRC1021) stimulated IL-12p40 and IL-6 production by J774.1 cells nearly two times more than the wild-type strain. In contrast, plasmid-cured variant of *L. lactis* subsp. *lactis* biovar *diacetylactis* N7 did not show such kind of effects.

The introduction of pDRSE5 into strains DRC1021 and N7 reduced the immunostimulatory activity to 1/8 and 1/4, respectively, of those by the wild types in terms of the cytokine production by J774 cells. Thus, the insertion or curing of some plasmids such as pDRSE5 or inherent plasmids in lactococci could apparently modulate the ability of host strains to stimulate cytokine production by macrophage cells. Moreover, similar results in IL-6 production were obtained by the stimulation with the cell wall fractions prepared from strains DRC1 and DRC1021, while not the case for IL-12 production. It was reported that some stress rearranged the plasmid composition in bacteria and changed the properties of the bacterial cells (Kim *et al.* 2001). These results suggest that the plasmids would affect the immunostimulatory activity of lactococci via possible changes in the properties of the hosts' cellular components including their cell wall. Although it is not clear at present how the plasmid changes chemical and/or structural composition of cell wall, the present study strongly suggests the potentials of plasmid-curing and the insertion to create immunologically functional probiotics.

Acknowledgement

The authors thank to the late Dr. Yasuhito Fujita for providing plasmid variants.

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Effects of a probiotic strain on the cellular immune response to food antigens in mice

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Keywords: allergy, IgE, β-lactoglobulin, ovalbumin, probiotics

Introduction

Probiotic bacteria, such as lactic acid bacteria, have been known to modulate the host immune responses. However, the mechanisms by which the probiotics exert their immunomodulatory effects are not fully elucidated. Our previous studies showed that oral administration of *Lactococcus lactis* subsp. *lactis* G50 (G50) could suppress the food antigen-specific IgE antibody response in mice (Kimoto *et al.* 2004). In the present study, we examined the effects of G50 feeding on the cellular immune response in mice to the potent allergens, ovalbumin (OA) and β-lactoglobulin (LG).

Materials and Methods

Mice: Female BALB/c mice were purchased from Charles River Japan. The mice were used at 6 - 8 weeks of age.

Preparation of lactic acid bacteria and its feeding: G50 was grown in M17 broth supplemented with 0.5% glucose for 18 hr at 30℃. The bacteria (0.2 mg/ml) in saline or saline alone were fed to mice once a day for 7 days by gastric intubation with an animal feeding needle.

Cell cultures: The spleen cells were harvested from the mice fed G50 or saline alone. The cells were seeded into 24-well plates (4 x 10^6 cells/well) and stimulated with 100 μg/ml of LG or OA. The culture medium was RPMI-1640 containing 50 μM 2-mercaptoethanol, 10 mM HEPES, 10 U/ml of penicillin, 100 μg/ml of streptomycin and 10% fetal calf serum. After 72 hr of culture at 37℃ in a 5% CO_2 atmosphere, the culture supernatants were collected and cytokine productions were measured by commercial ELISA kits. To examine the suppressive effects of G50-feeding on the antibody response, the spleen cells (2 x 10^6 cells /well) from mice immunized with LG or OA and the spleen cells (2 x 10^6 cells /well) from mice fed lactic acid

bacteria or saline alone were co-cultured either directly in a 24-well plate or separately using culture inserts with 0.45 μm filters. The cells were cultured in the presence of the antigens for 72 hr and additional 72 hr in the absence of the antigens. The antigen-specific antibodies in the supernatant were detected by ELISA.

Results and Discussion

The spleen cells from mice fed G50 or saline were stimulated with OA or LG *in vitro* and cytokines (IL-4, IL-6, IL-10, TNF-α and IFN-γ) were measured in the culture supernatants. The IFN-γ production by the OA- or LG-stimulated spleen cells from mice fed G50 was significantly higher than those of control mice (Fig. 1). The cells from mice fed G50 also showed a slight increase in the production of IL-6 and TNF-α. The IL-4 and IL-10 secretions were not detected. The antigen-specific antibody production *in vitro* by the spleen cells from OA- or LG-immunized mice was suppressed in the case of the co-culturing with the cells from mice fed G50 (Table 1). Moreover, the suppressive effect was reduced in the separate co-culture system with culture inserts. On the other hand, the levels of IgG2a antibodies (Th1-dependent) were increased in the co-culture with the cells from mice fed G50 (Table 1). Then, the IFN-γ secretion in the supernatant was increased, while the IL-13 secretion was slightly decreased (Table 1). These findings suggest that the cells stimulated with G50 through the gut play immunosuppressive roles in the response to food antigens via Th1-type cytokine production. The probiotic strain such as G50 can be used as innovative tools to treat or prevent allergy by suppression of Th2-dominating immune responses.

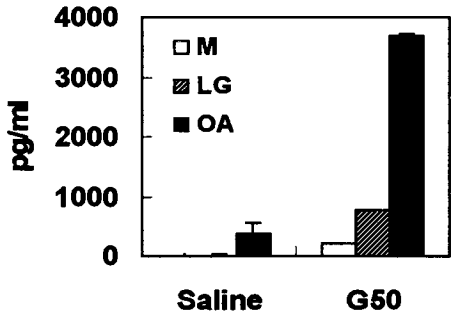

Fig. 1. IFN-γ production in the culture supernatants of the spleen cells from the mice fed G50.

Table.1 Effects of G50-feeding on the *in vitro* OA-specific antibody response and cytokine production

	Saline	G50
Antibody (O.D. 450nm)		
IgG1	1.414±0.021	1.062±0.052
IgG2a	0.155±0.005	0.432±0.013
IgE	0.494±0.028	0.357±0.006
Cytokine (pg/ml)		
IFN-γ	1637±74	2291±84
IL-13	242±5	202±3

References

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4. TRAINING COURSE

Training Course
"Identification and preservation of plant pathogens"

16 October 2004 in NIAS Genebank

Schedule

9 : 00～ 9 : 20	Orientation
9 : 20～ 9 : 55	Introduction of MAFF Genebank (The whole system and the plants section)
9 : 55～10 : 05	Taking of a group photo
10 : 05～10 : 25	Introduction of MAFF Genebank (continued) (The microorganisms section)
10 : 25～10 : 40	Break and Q&A
10 : 40～12 : 00	Practice DNA sequencing for molecular identification of plant pathogens
12 : 00～13 : 00	Lunch
13 : 00～16 : 30	Practice
13 : 00～14 : 00	Preservation of plant pathogenic fungi
14 : 00～15 : 00	Preservation of plant pathogenic bacteria
15 : 00～15 : 20	Break
15 : 20～16 : 30	DNA sequencing for molecular identification of plant pathogens (continued)
16 : 30～17 : 00	Q&A

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