Comparative Genomics Using Microarrays Reveals Divergence and Loss of Virulence-Associated Genes in Host-Specific Strains of the Insect Pathogen *Metarhizium anisopliae*\(^V\)†

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Many strains of *Metarhizium anisopliae* have broad host ranges, but others are specialists and adapted to particular hosts. Patterns of gene duplication, divergence, and deletion in three generalist and three specialist strains were investigated by heterologous hybridization of genomic DNA to genes from the generalist strain Ma2575. As expected, major life processes are highly conserved, presumably due to purifying selection. However, up to 7% of Ma2575 genes were highly divergent or absent in specialist strains. Many of these sequences are conserved in other fungal species, suggesting that there has been rapid evolution and loss in specialist *Metarhizium* genomes. Some poorly hybridizing genes in specialists were functionally coordinated, indicative of reductive evolution. These included several involved in toxin biosynthesis and sugar metabolism in root exudates, suggesting that specialists are losing genes required to live in alternative hosts or as saprophages. Several components of mobile genetic elements were also highly divergent or lost in specialists. Exceptionally, the genome of the specialist cricket pathogen Ma443 contained extra insertion elements that might play a role in generating evolutionary novelty. This study throws light on the abundance of orphans in genomes, as 15% of orphan sequences were found to be rapidly evolving in the Ma2575 lineage.

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different pathogenic strategies to be understood from a broad perspective.

Patterns of gene duplication, divergence, and deletion can be specifically determined by heterologous hybridization of total genomic DNA to microarrays (11, 20, 27). Heterologous hybridization has provided a fast and powerful tool facilitating the merging of functional genomics with physiology, ecology, and evolution (7, 31, 38) in species of yeast (22, 27), fish (9, 24), mammals (23, 25), and plants (1, 15). We have already verified that an array of Ma2575 ESTs can be used for heterologous hybridization with cDNAs. Thus, Ma2575 arrays were used to probe the causes of sectorization (production of nonsporulating cultures) in two commercial strains of *M. anisopliae* var. *anisopliae*. Probes from both strains cross-reacted strongly with the arrays, although with different expression profiles (46). We also used Ma2575 arrays to identify hundreds of genes differentially regulated by Ma324 in response to host or nonhost cuticles (45). Although only 8% of paralogous Ma2575 genes have greater than 80% identity, we expected cross-hybridization would potentially overestimate the overlap in genes expressed by different strains. However, individual genes within gene families were distinguished, revealing processes unique to Ma324 (45). In this study we exploit the fact that heterologous cDNA can provide information on physiological processes to allow us to gain a mechanistic perspective on the different life-styles that exist in insect-fungus interactions.

**MATERIALS AND METHODS**

Fungal strains and growth conditions. This study employed six strains of *M. anisopliae* that represent the range of phylogenetic variation and evolutionary distance within the species and that differ in host ranges and in responses to chemical and physical stimuli (40). All fungal strains were obtained from the U.S. Department of Agriculture Entomopathogenic Fungus Collection in Ithaca, NY. The three generalist strains are *all M. anisopliae* var. *anisopliae*. Strain numbers are Ma2575, Ma549, and Ma820. Besides their original hosts, they can at least infect caterpillars (*Manduca sexta*) and crickets (*Acheta domestica*) in the lab and usually more insects, for example, Ma2575 was isolated from the pecan weevil and also infests locusts (10). Generalist strains can germinate in many nutrients and produce appressoria against a hard hydrophobic surface (plastic petri dish) in yeast extract medium (40). The three specialized strains infect only a few species. They show little or no germination in yeast extract or glucose medium, e.g., *M. anisopliae* var. *acridum* Ma324 (specific for acridids), *M. anisopliae* var. *majus* Ma297 (specific for scarab beetles), and *M. anisopliae* var. *anisopliae* Ma443 (specific for Gryllids) (40). Fungal strains were routinely grown at 27°C on potato dextrose agar. For preparation of genomic DNA, fungal spores were cultured in Sabouraud dextrose broth at 27°C.

Genomic DNA preparation and construction of microarray. Fungal mycelia from 48-h Sabouraud dextrose broth cultures were collected by filtration and washed with sterile distilled water three times. The high-molecular-mass total genomic DNA of each strain was prepared as previously described (47). The construction of the cDNA microarrays used in this study has been previously described (46). This array harvests PCR-amplified fragments from the unique cDNA clones from *M. anisopliae* var. *anisopliae* Ma2575 and a few genes from *M. anisopliae* var. *acridum* Ma324 absent from the libraries of Ma2575. In total, 1,748 amplified clones were printed in triplicates on the slides. Additional background control was provided by 8 randomly distributed spots of 3 g of total genomic DNA was fragmented by restriction endonuclease digestion with RsaI and MseI (New England Biolabs). The digested genomic DNAs were concentrated with Microcon YM-30 filters (Millipore) and genomic DNAs were labeled with green Cy3 dUTP-tagged or red Cy5 dUTP-tagged nucleotides as described previously (27).

Three microarray slides were used per comparison (cDNAs were replicated in triplicate on each slide). Each strain’s DNA was cohybridized with Ma2575 DNA in dye-swapping replicate experiments and the relative hybridization efficiencies (fluorescence ratios) of their DNA for Ma2575 genes were compared. We used standard microarray hybridization conditions for this work (46) that provides a stringency at which 90% matching over 60 bases should suffice to form a stable hybrid (48).

**CGH data analysis.** The intensity of the spots on the cDNA slides was calculated using the Spotfinder version 2.2.4 program from the Microarray software suite TM4 from JCVI (http://www.tm4.org/spotfinder.html) and applying local background subtraction for each spot. The spotted microarray data preprocess- ing platform Ginkgo was used for comparative genome hybridization (CGH) analysis (http://plgrc.jcvi.org/index.php/bioinformatics/ginkgo.html). Intensity-dependent variation was normalized using the histogram mode centering algorithm with means and standard deviations as parameters. A dye consistency check was performed after normalization to eliminate systematic errors. This flip-dye analysis used a standard deviation criterion of threefold to filter spots with inconsistent values. Consolidation of the triplicate intensity values of channels A and B (1,756 unigenes, with channel A referring to the reference Ma2575) was also performed using the in-slide replicate algorithm of this platform.

**Southern blot analysis.** Genomic DNA samples (10 μg) were digested overnight at 37°C using 100 units of EcoRI, EcoRV, BamHI, BglII, or XbaI (New England Biolabs). Using standard protocols, digested samples were electrophoresed in 0.8% Tris-acetate-EDTA–agarose gels and blotted onto Hybond N+ membrane. According to probe was immunologically detected by anti-DIG antibody conjugated with alkaline phosphatase (Roche), according to the manufacturer’s instructions.

**Microarray data accession number.** The microarray data obtained in this study have been deposited in the NCBI Gene Expression Omnibus database under accession number GSE18486.

**RESULTS**

**CGH.** Fragmented genomic DNA from six *M. anisopliae* strains (Ma297, Ma324, Ma443, Ma549, Ma820, and Ma2575) was cohybridized with DNA from strain Ma2575 to microarrays of Ma2575 ESTs. The resulting hybridization patterns were used to organize the strains into a hierarchical tree that placed *M. anisopliae* var. *acridum* strain Ma324 into a relative outgroup position (Fig. 1). This is consistent with phylogenies of *Metaherbizum* spp. (12). Normalized average intensity ratios were used as the main parameter to compare the hybridization with three replicate slides.

To investigate the relationship between the strength of hybridization of homologous gene pairs and their degree of nucleotide identity, we used 30 corresponding cDNA sequences from *M. anisopliae* strains Ma324 and Ma2575 identified by
Freimoser et al. (17) to plot the percent nucleotide identity against log₂ ratios calculated from cohybridization data (Fig. 2). The percentage of nucleotide sequence identity ranged from 87.7% to 99.5%. Linear regression analysis was employed to predict the best-fit line, demonstrating a moderately good correlation ($r^2 = 0.66; P < 0.0001$) between nucleotide sequence identity and the log₂ ratio. It is likely that the correlation was not closer to 1, because hybridization is also influenced by the GC/AT ratio and the distribution of highly conserved regions in sequences (48).

**Categorization of conserved and divergent genes.** Greater than 90% of all the arrayed genes showed decreasing efficiency of hybridization with increasing distance of the two strains as determined by the normalized intensity ratio averages (Table 1). However, individual genes evolve at different rates. Using the cohybridization of strains Ma324 and Ma2575 as a point of reference, we arbitrarily divided genes into three groups according to their normalized intensity ratios. The high-similarity group 1, with $I_B/I_A$ ratios of ≥0.5, corresponds to ≥95% nucleotide sequence similarity; the moderate-similarity group 2 with $I_B/I_A$ ratios of 0.25 to 0.5 corresponds to 60 to 95% similarity; the group 3 sequences with $I_B/I_A$ ratios of <0.25 possess low homologies or are absent in Ma324.

Previously, ESTs of *M. anisopliae* were subdivided into broad functional categories (17). Functional categories where >90% of the genes fall into group 1, i.e., have high nucleotide identity, include amino acid metabolism, nucleotide metabolism, cell cycle/division and growth, N, P, and S metabolism, translation and posttranslational modification, regulatory function, DNA replication, recombination and repair, energy metabolism, transcription, and RNA processing and degradation. Thus, as expected, major life processes are highly conserved, presumably due to purifying selection.

A total of 118 (6.7%) of Ma2575 genes were predicted to be highly divergent or absent ($I_B/I_A < 0.25$) in Ma324, indicative of functional differences (Table 2; Fig. 3). The absence of homologous sequences in strain Ma324 was confirmed by Southern hybridization analysis with probes comprising the complete *M. anisopliae* strain Ma2575 open reading frame (ORF) of 26 genes (Fig. 4, 5, and 6). The genes were chosen as a representative sampling of transposable elements, cell metabolism, stress response and defense, cell structure, and signaling functions. In every case, the Southern blotting data validated the results from microarrays.

Thirty-five out of 237 Ma2575 orphan sequences (i.e., with no homologs in databases) only hybridized to Ma2575 DNA. Low-stringency Southern hybridization confirmed the absence of CN808668 in the genome of Ma324 (Fig. 5E). Several other sequences with homologs in phylogenetically distant organisms but none in published fungal genomes hybridized only to Ma2575 DNA, e.g., EST CN809303, which is similar (E = 2 × 10⁻⁷) to a bacterium (*Rubrobacter xylanophilus*) short chain dehydrogenase/reductase. Its absence in Ma324 and Ma443 was confirmed by Southern analysis (Fig. 5A). However, se-

### Table 1. Normalized average $I_B/I_A$ ratios for the different strain combinations*.

| Cohybridization     | Avg in-slide $I_B/I_A$ ratio |
|---------------------|----------------------------|
| Ma2575 vs Ma2575    | 0.94                       |
| Ma280 vs Ma2575     | 0.69                       |
| Ma549 vs Ma2575     | 0.72                       |
| Ma443 vs Ma2575     | 0.62                       |
| Ma297 vs Ma2575     | 0.55                       |
| Ma324 vs Ma2575     | 0.41                       |

* $I_A$ (from channel A) refers to the signal for the reference strain, *Metarhizium anisopliae* var. *anisopliae* Ma2575, and $I_B$ is the normalized signal value of the indicated test strain (Ma820, Ma549, Ma443, Ma297, or Ma324).

### Table 2. Functional categories of *M. anisopliae* var. *anisopliae* Ma2575 genes predicted to be of low nucleotide sequence similarity or absent in *M. anisopliae* var. *acridum* Ma324.

| Functional category          | No. of genes | % of total |
|------------------------------|--------------|------------|
| Hypothetical proteins or orphans | 55           | 46.61      |
| Cell metabolism              | 19           | 16.10      |
| Lipid metabolism             | 7            | 5.93       |
| Carbohydrate metabolism      | 7            | 5.93       |
| Amino acid metabolism        | 3            | 2.54       |
| Nucleotide metabolism        | 2            | 1.69       |
| Cell structure and function  | 13           | 11.02      |
| Cell wall protein            | 7            | 5.93       |
| Transport proteins           | 3            | 2.54       |
| Signaling                    | 3            | 2.54       |
| Transposable elements        | 10           | 8.47       |
| Stress response and defense  | 10           | 8.47       |
| Stress response              | 4            | 3.39       |
| Detoxification               | 3            | 2.54       |
| Secondary metabolites        | 3            | 2.54       |
| RNA metabolism               | 4            | 3.39       |
| Protein metabolism           | 4            | 3.39       |
| Ribosomal proteins           | 1            | 0.85       |
| Proteolysis                  | 2            | 1.69       |
| Protein modification/targeting | 1           | 0.85       |
| Energy metabolism            | 3            | 2.54       |
| Total                        | 118          | 100.00     |

![Graph](image)

**FIG. 2.** Standard curve used to determine the relationship between nucleotide sequence similarity for genes of *Metarhizium anisopliae* strains Ma2575 and Ma324 and normalized fluorescence ratios. Sequences for 30 *M. anisopliae* genes of known similarity were included (17). The means of the log₂ ratio values for each gene were plotted against the percent similarity.
quences from Ma2575 that are highly conserved in other fungal species could also show rapid evolution and loss in *Metarhizium* genomes. Thus, the strain Ma2575 EST AJ273858, which is similar (4/11003 10/11002 42) to the ankyrin repeat protein (stress response protein) of *Aspergillus fumigatus*, was highly divergent in most strains. Low-stringency Southern hybridization confirmed the absence of this sequence in Ma443 and Ma324 (Fig. 5B).

Ten genes encoding proteins involve in the stress response and defense were found to hybridize poorly with *M. anisopliae* var. *acridum* Ma324 genomic DNA. Four of these had high similarity to genes encoding proteins involved in the stress response. Aside from the ankyrin repeat protein (AJ273858), these included CN809313, similar (6/11003 10/11002 14) to an ABC transporter of *Emericella nidulans*, which is involved in contact-dependent secretion, virulence, and resistance to antifungal compounds (43), CN808854, similar (8/11003 10/121) to an *Aspergillus fumigatus* flavin-binding monooxygenase-like protein, and CN808382, similar (1/11003 49) to the short chain dehydrogenase CN809303 (involved in carbohydrate metabolism) (Fig. 5). Except for CN809303, the Ma2575 genes were very similar to sequences in *A. fumigatus* (Table 3). The EST AJ274133, which encodes an esterase STE1 that increases virulence when expressed in *Beauveria bassiana* (34), was also absent in Ma324 (Fig. 5A).

Other poorly conserved genes in Ma324 (e.g., those with IB/IA ratios of <0.25) have previously been associated with *M. anisopliae* Ma2575 virulence. These include a set of functionally coordinated genes comprising four nonribosomal peptide synthases and a polyketide synthase (CN808619) likely involved in toxin generation (17, 45), which were divergent or missing in Ma443 and Ma324 as confirmed by Southern blot analysis. We confirmed that the absence of these sequences was a characteristic of *M. anisopliae* var. *acridum* by including genomic DNA from *M. anisopliae* var. *acridum* strain 3612 in the Southern blot assays (Fig. 4). The cytochrome P450 (CN809217) was very similar (E = 6 × 10⁻⁶⁸) to an enzyme involved in secondary metabolite biosynthesis in *Fusarium sporotrichioides* (6). Other key divergent or missing genes in specialists included a chymotrypsin (AJ273081) produced by *Saccharomyces cerevisiae*. Of the others, three were involved in detoxification, including AJ273280, which is similar (1 × 10⁻¹⁴) to an ABC transporter of *Emericella nidulans*, which is involved in contact-dependent secretion, virulence, and resistance to antifungal compounds (43), and two others that were confirmed to be absent in Ma324 and Ma443 genomic DNA (Fig. 5).

Nineteen genes encoding proteins involved in lipid, carbohydrate, and amino acid metabolism were also divergent. Southern analysis confirmed that both Ma324 and Ma443 lacked genes encoding extracellular 3-ketosteroid 1-dehydrogenase (CN809570), squalene-hopene cyclase (CN808855), and cytochrome P450 monoxygenase (CN808662), all of which are involved in lipid metabolism, and carbohydrate glycosyl hydrolase (CN808813) and short chain dehydrogenase CN809303 (involved in carbohydrate metabolism) (Fig. 5A). The latter two were confirmed to be absent in Ma324 and Ma443 genomic DNA (Fig. 5).
Ma2575 during penetration of host cuticle (36) and absent from published fungal genomes (21). Southern blot analysis confirmed its absence in both Ma443 and Ma324 (Fig. 5D), suggesting that this gene is highly expendable and multiple lineages have independently lost it. An aflatoxin biosynthesis ketoreductase (CN808727) involved in the biosynthesis of aflatoxins by \textit{Aspergillus parasiticus} (44) was absent in Ma324 (Fig. 5). EST AJ273858, a Ma2575 homolog (7.9/10 of yeast aminopeptidase yscIII, had diverged beyond hybridization in Ma443 but was present in Ma324. The yeast enzyme is a vacuolar \textit{Y}-aminopeptidase with a viable null mutant that cannot hydrolyze Lys-Ala (19).

A Ma2575 hydrophobin (AJ274156) involved in cell wall structure and nonspecific adhesion to hydrophobic cuticle surfaces (40) showed only 38.9% sequence identity to its counterpart in Ma324 (BQ143508) according to CLUSTAL W alignment analysis. Several other cell wall proteins were also highly divergent, including glycosphatidylinositol-anchored cell wall beta-1,3-endoglucanase (AJ273279), which is important for cell wall stability (32), mixed-linked glucanase precursor MLG1 (CN808527), cell wall synthesis protein (CN808518), cell wall protein (CN808796), putative endochitinase CHI2 (CN808888), and an extensin-like protein (CN808213).

Ten of the transposable elements in Ma2575, including Restless-like transposase (AJ274202), polyprotein (AJ272783 and AJ274240), transposases (AJ274329, AJ272685, AJ273458, CN808708, and CN808808), reverse transcriptase (CN809546), and Gag-like polyprotein (AJ274338), either lack homologs in the other strains or they are highly divergent (Fig. 6). All were absent in Ma324. However, a transposase (CN808708) and Gag-like polyprotein (AJ274338) that each hybridized to four bands with Ma2575 DNA were highly redundant in Ma443 (Fig. 6A). These findings are in agreement with the array data and suggest that these sequences had multiplied in the Ma443 genome.

Thirty Ma324 sequences were also arrayed with the Ma2575 sequences and employed in dye swap experiments. These sequences included CN808542, similar (1 × 10^{-33}) to an endoglucanase B from \textit{Aspergillus kawachi}, and
The central strategy of this work was to examine pathogen genome evolution and host range usage by confining the comparisons within a single species while exploring adaptive radiation within this species as far as possible. *M. anisopliae* is a particularly good model system for studying evolutionary processes because it consists of strains that in terms of developmental processes are almost indistinguishable from each other but that differ dramatically in host range and possibly saprophytic competence. Given that specialization has occurred many times in *M. anisopliae*, this organism provides an unusual opportunity to study a species containing a large number of independently evolved models of adaptation and response. These comparisons provide a novel perspective on the evolution and strategies of highly specialized fungi. As a radiating species, its natural molecular variation offers the chance of finding processes of both adaptive change and phylogenetic differentiation still in operation, even in intermediate states.

We used PCR-amplified cDNA, as their longer sequences are better than short oligonucleotides for heterologous gene expression studies. The Ma2575 sequences we arrayed are full length, or nearly so, and most are 600 to 800 bp long. For genes encoding secreted products (hydrophobins, subtilisins, chitinases, etc.), nucleotide identities between orthologs in Ma324 and Ma2575 range from 93% to 98%, with the most closely matching sequences at functional domains (3, 17, 37). The findings presented in this study reinforced these data, showing that ~6.7% of Ma2575 genes were absent or highly divergent in Ma324. We may have underestimated the level of functionally divergent genes in different lineages, because our ability to detect divergence or inactivation of genes relies on sequence divergence or deletion of the locus preventing hybridization with the probe on the microarray. However, mitigating this consideration is that a small amount of sequence divergence may change hybridization intensity. Also, for genes facilitating an opportunistic life-style, we are confident that the arrays included loci required for living in multiple habitats. That is because the libraries were created from a strain with a broad host range and the arrayed genes included those expressed when the organism lives saprophytically (46). It is likely, given the reduced diet breadth of specialized lineages (40), that these genes will be represented in any strains undergoing degradation. Thus, our experiments have a high probability of providing a picture of the features characterizing specialized and generalized fungal pathogen populations.

The evidence for divergence of genes in Ma324 suggests a potential role for specific gene loss in the emergence of some of the differences between strains in diet breadth and host range. Although Ma324 is the most divergent strain from Ma2575, other specialists also show reduced diet breadth and loss of toxins (41). Degenerative changes occur in bacteria that are obligate pathogens because some genes are no longer needed or possibly because specialization reduces effective population size, which increases fixation of deleterious mutations (28). However, gene loss has also been proposed as an important force driving the evolution of recently evolved novel lineages (29).

The most obvious categories of genes expected to undergo degradation or deletion in a specialized pathogen will be those
### TABLE 3. *M. anisopliae* var. *anisopliae* Ma2575 genes predicted to be of low similarity (subgroup 3) in the genome of *M. anisopliae* var. *acridum* Ma324 based on CGH analysis

| Category and gene identifier | Putative or known function | Best match | Closest relationship | E-value |
|-----------------------------|----------------------------|------------|----------------------|---------|
| Transposable elements       |                            |            |                      |         |
| CN808708                    | Transposase                | AAB63315   | Botryotinia fuckeliana| 7.0E-146|
| AJ274338                    | Gag-like polyprotein       | CA96388    | Fusarium poae        | 6.0E-07 |
| AJ274202                    | Restless-like transposase  | AAK16925   | Nectria haematococca | 200E-13 |
| AJ272685                    | Transposase                | AAV28708   | Fusarium oxysporum   | 5.0E-13 |
| AJ273458                    | Transposase                | AAV28708   | Fusarium oxysporum   | 2.0E-09 |
| CN809546                    | Reverse transcriptase      | ABC24970   | Monascus pilosus     | 4.0E-34 |
| AJ272783                    | Polyprotein                | CAB91877   | Phaeosphaeria nodorum| 5.0E-165|
| AJ273429                    | Transposase                | AAV28708   | Fusarium oxysporum   | 6.0E-33 |
| AJ274240                    | Polyprotein                | CAB91877   | Phaeosphaeria nodorum| 1.0E-22 |
| CN808808                    | Transposase-like protein   | BAA32244   | Fusarium oxysporum   | 7.0E-39 |
| Stress response and defense |                            |            |                      |         |
| Stress response             |                            |            |                      |         |
| AJ273858                    | Ankyrin repeat protein     | EDP48589   | Aspergillus fumigatus | 4.0E-42 |
| CN809313                    | Ice nucleation protein     | AAQ14297   | Gibberella acuminata | 4.0E-78 |
| CN808429                    | Integral membrane protein, Mpv17/PMP22 family | XP_748225 | Aspergillus fumigatus | 3.0E-49 |
| CN808677                    | Cell wall integrity and stress response component 4; Wsc4p | XP_011835 | Saccharomyces cerevisiae | 2.0E-10 |
| Detoxification              |                            |            |                      |         |
| CN808854                    | Flavin-binding monoxygenase-like protein | XP747888 | Aspergillus fumigatus | 8.0E-121|
| CN808382                    | Pyridine nucleotide-disulfide oxidoreductase | XP001272526 | Aspergillus clavatuse | 1.0E-161|
| AJ273280                    | ABC transporter            | AAF29805   | Emericella nidulans  | 1.0E-14 |
| Secondary metabolites       |                            |            |                      |         |
| CN809217                    | Cytochrome P450             | AAK33073   | Fusarium sporotrichioides | 3.0E-138|
| CN808619                    | Polyketide synthase        | AAR90254   | Botryotinia fuckeliana | 1.0E-66 |
| CN808727                    | Aflatoxin biosynthesis ketoreductase NOR-1 | Q00278 | Aspergillus parasiticus | 1.0E-24 |
| Cell structure and function |                            |            |                      |         |
| Cell wall protein           | Related to mixed-linked glucanase precursor MLG1 | CAV8654 | Neurospora crassa | 1.0E-37 |
| CN808796                    | Cell wall protein          | BAD01559   | Aspergillus kawachii | 8.0E-13 |
| CN808518                    | Cell wall synthesis protein | BAC82484 | Penicillium chrysogenum | 1.0E-57 |
| CN808888                    | Putative endochitinase CHI2 | CAC07216 | Metarhizium anisopliae | 1.0E-137|
| AJ273279                    | GPI-anchored cell wall beta-1,3-endoglucanase EglC | XP00139526 | Pyrenophora tritici-repentis | 3.0E-70 |
| CN808213                    | Extensin-like; with SH3 Src homology domain | NP594444 | Schizosaccharomyces pombe | 1.0E-15 |
| AJ274156                    | Hydrophobin-like protein ssgA precursor | AAA33418 | Metarhizium anisopliae | 3.0E-50 |
| Transport proteins          |                            |            |                      |         |
| CN809357                    | Transporter, putative      | CAE47906   | Aspergillus fumigatus | 2.0E-32 |
| CN808929                    | Related to NCE102 protein, nonclassical export membrane protein | CAD37009 | Neurospora crassa | 9.0E-25 |
| CN808769                    | Adaptn-car-binding coat-associated protein | DAA01434 | Mus musculus | 5.0E-16 |
| Signaling                   |                            |            |                      |         |
| AJ273356                    | Putative mitogen-activated protein kinase kinase 2 | CAC07966 | Leishmania mexicana | 2.0E-17 |
| AJ273657                    | SRPK1-like kinase          | NP_013943  | Saccharomyces cerevisiae | 7.0E-12 |
| CN808754                    | Serine kinase SRPK2        | AAC05299   | Homo sapiens         | 4.0E-21 |
| Cell metabolism             |                            |            |                      |         |
| Lipid metabolism            |                            |            |                      |         |
| AJ274133                    | Esterase; STE1             | CAB63910   | Metarhizium anisopliae | 3.9E-99 |
| CN808855                    | Squalene-hopene cyclase    | XP751356   | Aspergillus fumigatus | 1.0E-74 |
| CN808662                    | Cytochrome P450 monoxygenase | AAF26280 | Aspergillus parasiticus | 8.0E-09 |
| CN808333                    | Putative P450 monoxygenase | AAO73449 | Fusarium sporotrichioides | 1.0E-173|
| CN809570                    | Putative 3-ketosteroid-delta-1-dehydrogenase | NP_822771 | Streptomyces avermitilis | 3.0E-15 |
| CN809534                    | Phosphatidylinositol phospholipase | CAB92911 | Candida albicans | 8.0E-23 |
| CN808853                    | Lanoster synthase-related protein | XP_326612 | Fusarium oxysporum | 2.0E-22 |

Continued on following page
| Category and gene identifier | Putative or known function | Best match | Closest relationship | E-value |
|-----------------------------|---------------------------|------------|----------------------|---------|
| Carbohydrate metabolism     |                           |            |                      |         |
| CN808775                    | Homocitrate synthase, mitochondrial precursor | Q12726 | Yarrowia lipolytica | 2.0E-76 |
| CN808813                    | Glycosyl hydrolase         | XP001273136 | Aspergillus clavatus | 1.0E-70 |
| CN809153                    | Probable homoaconitase precursor | CAD71225 | Neurospora crassa | 3.0E-84 |
| CN809303                    | Short chain dehydrogenase/reductase family protein-like | BAD09200 | Orzyza sativa | 2.0E-09 |
| CN809316                    | n-Alkane-inducible cytochrome P450 gene (ALK1) essential for n-decane assimilation | BAA31433 | Yarrowia lipolytica | 2.0E-22 |
| CN808940                    | CGI-49 protein, saccharopine dehydrogenase | AAD34044 | Homo sapiens | 1.0E-15 |
| CN808435                    | Glucosidase                | AAO34674 | Gibberella zeae | 7.0E-17 |
| Amino acid metabolism       |                           |            |                      |         |
| AJ274200                    | Proline oxidase            | CAC18796 | Emericella nidulans | 2.0E-42 |
| CN808802                    | Glycine/ D-amino acid oxidases | ZP_00096788 | Novosphingobium aromaticivorans | 4.0E-14 |
| CN808928                    | Glutamine synthetase (glutamate-ammonia ligase) | EAA69962 | Gibberella zeae PH-1 | 1.0E-119 |
| Nucleotide metabolism       |                           |            |                      |         |
| CN809258                    | Similar to rRNA intron-encoded homing endonuclease | XP_372959 | Homo sapiens | 1.0E-17 |
| CN808663                    | Adenine phosphoribosyltransferase | AAA68956 | Mastomys hildebrantii | 4.0E-06 |
| Energy metabolism           |                           |            |                      |         |
| CN808777                    | Putative oxidoreductase    | AAL58884 | Aspergillus nidulans | 4.0E-32 |
| CN809440                    | Cytochrome p450 (E-class), putative | CAF32039 | Aspergillus fumigatus | 5.0E-07 |
| A1274160                    | Probable short chain dehydrogenase | AAG05038 | Pseudomonas aeruginosa | 1.0E-13 |
| Protein metabolism          |                           |            |                      |         |
| Ribosomal protein           |                           |            |                      |         |
| CN809545                    | 40S ribosomal protein S0   | Q01291 | Neurospora crassa | 1.0E-110 |
| Proteolysis                 |                           |            |                      |         |
| AJ273081                    | Chymotrypsin               | CAB44651 | Metarhizium anisopliae | 2.0E-51 |
| CN809169                    | Glutathione S-transferase I | NP_588298 | Schizosaccharomyces pombe | 2.0E-42 |
| Protein modification/        |                           |            |                      |         |
| targeting                   |                           |            |                      |         |
| CN809424                    | Transferase family protein | XP_001266554 | Neosartorya fischeri | 2.0E-39 |
| RNA metabolism              |                           |            |                      |         |
| CN809525                    | Probable transcription factor | T37601 | Schizosaccharomyces pombe | 7.0E-09 |
| CN809374                    | Flavocytochrome b558 (NADPH oxidase gp91phox) | BAA95154 | Tursiops truncatus | 1.0E-07 |
| CN808682                    | TPR (transcriptional repressor)-containing protein Mgq1 | AAK58576 | Ustilago maydis | 1.0E-107 |
| CN809621                    | Antisilencing protein, causes depression of silent loci when overexpressed; Asf1p | NP_012420 | Saccharomyces cerevisiae | 4.0E-42 |
| Hypothetical proteins or    |                           |            |                      |         |
| orphans                     |                           |            |                      |         |
| CN808665                    | Hypothetical protein FG02705 | EAA67953 | Gibberella zeae PH-1 | 5.0E-17 |
| CN808811                    | Hypothetical protein FG02893 | EAA72393 | Gibberella zeae PH-1 | 2.0E-64 |
| CN808833                    | Hypothetical protein MG04736 | EAA50977 | Magnaporthe grisea | 6.0E-53 |
| CN809268                    | Hypothetical protein FG10959 | EAA74243 | Gibberella zeae PH-1 | 2.0E-07 |
| CN809246                    | Hypothetical protein FG11155 | EAA75365 | Gibberella zeae PH-1 | 1.0E-19 |
| CN809389                    | Predicted protein          | EAA51141 | Magnaporthe grisea | 3.0E-72 |
| AJ274045                    | Hypothetical protein FG06966 | EAA76426 | Gibberella zeae PH-1 | 1.0E-43 |
| CN809166                    | Hypothetical protein MG03337 | EAA51742 | Magnaporthe grisea | 2.0E-22 |
| CN808764                    | Hypothetical protein FG09539 | EAA76655 | Gibberella zeae PH-1 | 9.0E-12 |
| CN808135                    | Hypothetical protein MG09337.4 | EAA51320 | Magnaporthe grisea | 2.0E-58 |
| AJ273482                    | Predicted protein          | EAA68277 | Gibberella zeae PH-1 | 4.0E-16 |
| AJ272778                    | Hypothetical protein AN2582.2 | EAA64687 | Aspergillus nidulans | 5.0E-22 |
| CN809260                    | Hypothetical protein       | XP84749 | Gibberella zeae | 2.0E-11 |
| CN808381                    | Hypothetical protein MG06755.4 | EAA55098 | Magnaporthe grisea | 6.0E-16 |

Continued on following page
required primarily to live in alternative hosts or as a saprophyte. One of the most intensely studied virulence attributes of *M. anisopliae* is the ability to secrete destruxins encoded by peptidyl synthases (33). The selective divergence or loss of toxin-encoding genes in the specialists suggests they confer considerable selectable functions for Ma2575 but either provide no benefits to Ma324 or are detrimental. It is probably significant that specialist strains kill their host slowly (8). Presumably, strains that are not specifically adapted to subvert/avoid/overcome the immune response of a particular insect are best served by achieving a rapid kill with toxins, whereas an adapted strain may optimize utilization of host nutrients and production of infectious propagules by growing within the living host. Production and transmission of propagules only occurs after host death, so if hosts are in the growing phase during infection, the reduced virulence resulting from loss of toxins may also allow for increased reproduction per host by allowing the pathogen to exploit the extra host tissue generated by the additional host growth (14). Consistent with this, genetic engineering to increase the speed of kill by *M. anisopliae* resulted in reduced sporulation on cadavers (30). Active toxins may therefore have placed a specific pathogen at a selective disadvantage that could drive inactivation of the gene. Loss-of-function mutations are presumably a one-way street and will be deleterious to a specialized strain if it returns to its ancestral habitat. It could also constrain opportunistic host switching.

From the likely function of genes that have been degraded (or silenced), we can assess their conceivable effects on host specialization, virulence, and/or expendability for virulence. However, for a given gene, this is only a hypothesis and needs separate experimental support. A direct approach is through examination of genotype-phenotype correlations. Consistent with our array results, Ma2575 has several peptide synthases and produces destruxins A, B, and E, while strain Ma297 only produces destruxin A (2). Likewise, destruxins do not play a role in the pathogenesis of Ma324 (35). Specificity, or lack of it, has always been an issue with *M. anisopliae* (26). Instances where generalist strains are closely related to specialists suggest that host range can be substantially altered in a short period of evolutionary time (40). It is possible therefore that

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**TABLE 3—Continued**

| Category and gene identifier | Putative or known function | Best match | Closest relationship | E-value |
|-----------------------------|----------------------------|------------|---------------------|--------|
| CN809340                    | Hypothetical protein FG02946 | EAA71001   | Gibberella zeae PH-1 | 3.0E-29 |
| CN809111                    | Putative membrane protein   | CAB65565   | Streptomyces coelicolor | 1.0E-09 |
| CN808278                    | Hypothetical protein FG00492 | EAA68724   | Gibberella zeae PH-1 | 4.0E-12 |
| CN808208                    | Hypothetical protein FG09877 | EAA70103   | Gibberella zeae PH-1 | 3.0E-07 |
| CN809490                    | Hypothetical protein AN9016.2 | EAA64348   | Aspergillus nidulans  | 2.0E-73 |
| CN809544                    | Hypothetical protein AN3935.2 | EAA59244   | Aspergillus nidulans  | 4.0E-09 |
| CN809615                    | Orphan                      |            |                     |        |
| AJ274328                    | Orphan                      |            |                     |        |
| CN808763                    | Orphan                      |            |                     |        |
| CN808668                    | Orphan                      |            |                     |        |
| CN808749                    | Orphan                      |            |                     |        |
| CN808778                    | Orphan                      |            |                     |        |
| CN808897                    | Orphan                      |            |                     |        |
| CN808997                    | Orphan                      |            |                     |        |
| CN809648                    | Orphan                      |            |                     |        |
| CN809229                    | Orphan                      |            |                     |        |
| AJ272938                    | Orphan                      |            |                     |        |
| CN808540                    | Orphan                      |            |                     |        |
| CN808973                    | Orphan                      |            |                     |        |
| CN809674                    | Orphan                      |            |                     |        |
| CN809028                    | Orphan                      |            |                     |        |
| CN808529                    | Orphan                      |            |                     |        |
| AJ273884                    | Orphan                      |            |                     |        |
| CN809072                    | Orphan                      |            |                     |        |
| CN809454                    | Orphan                      |            |                     |        |
| CN809898                    | Orphan                      |            |                     |        |
| CN808807                    | Orphan                      |            |                     |        |
| CN809563                    | Orphan                      |            |                     |        |
| CN809452                    | Orphan                      |            |                     |        |
| CN808412                    | Orphan                      |            |                     |        |
| CN808880                    | Orphan                      |            |                     |        |
| CN809004                    | Orphan                      |            |                     |        |
| AJ273764                    | Orphan                      |            |                     |        |
| AJ272837                    | Orphan                      |            |                     |        |
| AJ272822                    | Orphan                      |            |                     |        |
| AJ274006                    | Orphan                      |            |                     |        |
| AJ273965                    | Orphan                      |            |                     |        |
| CN809633                    | Orphan                      |            |                     |        |
| CN808693                    | Orphan                      |            |                     |        |
| CN809144                    | Orphan                      |            |                     |        |
| AJ273455                    | Orphan                      |            |                     |        |

* Gene identifiers refer to the accession number of the EST in the NCBI database.
some imported specialized biocontrol agents may have the potential to switch hosts in response to selection pressures in new habitats. What we lack is a means to predict when such evolution is, and is not, expected. An understanding of genetic changes that have enabled evolution of major host range changes in the past could help predict future evolutionary changes. For example, genomic degradation could reasonably be expected to reduce specialists future adaptive options. Confirmation of gene loss would therefore impact risk assessment issues.

This study throws light on the “mysterious abundance” of orphans in genomes (39). Only 35 out of 237 (15%) arrayed “Metarhizium-specific sequences” were in the highly divergent group 3. This suggests that 85% arose de novo in a common ancestor of Ma2575 and Ma324, presumably after it had already diverged from other pyrenomycetous fungi, given that those other fungi lack the sequences. Heterologous probes from different strains showed reduced signal strength for the same 35 orphans as Ma324 probes, suggesting that these have lost sequence similarities because of rapid evolution in Ma2575. They are unlikely to be pseudogenes, as they were identified from cDNAs, confirming active expression of mRNA (45).

Several Ma2575 retrotransposon genes were not detected in other strains, indicating that since their divergence Ma2575 may have acquired different mobile genetic elements. Conversely, there was an expansion in the number of a subset of insertion elements in Ma443 that hybridized to respective sequences from Ma2575. This has obvious implications for strain stability that are of importance when considering the commercial development of a strain and the possibility of alterations in virulence and host range.

This study shows that comparing multiple strains will provide an excellent framework for the analysis of pathogenesis and host specificity. Genetic variation is a powerful tool to study adaptation, and we expect future studies to address a number of basic yet poorly understood questions that span much of molecular evolution, including the following: what roles do changes in gene complement or expression profiles play in generating intraspecific differences? How do these differences correlate with metabolic and biosynthetic adaptations to specific hosts? What are the relative rates of different kinds of mutations and do these vary between strains? What are the mechanisms by which novel pathogens emerge with either wide or narrow host ranges? Are the same genes involved in the evolution and maintenance of specialization in different strains? What variables drive the functional divergence of gene variants between strains, e.g., does gene novelty correlate with life-style or lineage?

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