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Spatial Differentiation in the Vegetative Mycelium of *Aspergillus niger*▽†

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Fungal mycelia are exposed to heterogenic substrates. The substrate in the central part of the colony has been (partly) degraded, whereas it is still unexplored at the periphery of the mycelium. We here assessed whether substrate heterogeneity is a main determinant of spatial gene expression in colonies of *Aspergillus niger*. This question was addressed by analyzing whole-genome gene expression in five concentric zones of 7-day-old maltose- and xylose-grown colonies. Expression profiles at the periphery and the center were clearly different. More than 25% of the active genes showed twofold differences in expression between the inner and outermost zones of the colony. Moreover, 9% of the genes were expressed in only one of the five concentric zones, showing that a considerable part of the genome is active in a restricted part of the colony only. Statistical analysis of expression profiles of colonies that had either been or not been transferred to fresh xylose-containing medium showed that differential expression in a colony is due to the heterogeneity of the medium (e.g., genes involved in secretion, genes encoding proteases, and genes involved in xylose metabolism) as well as to medium-independent mechanisms (e.g., genes involved in nitrate metabolism and genes involved in cell wall synthesis and modification). Thus, we conclude that the mycelia of 7-day-old colonies of *A. niger* are highly differentiated. This conclusion is also indicated by the fact that distinct zones of the colony grow and secrete proteins, even after transfer to fresh medium.

Filamentous fungi fulfill an essential role in global carbon cycling by degrading organic material. The organic material is colonized by a mycelium that consists of interconnected hyphae. These hyphae are exposed to a heterogenic substrate. The periphery of the colony is exposed to unexplored organic material, whereas its center is surrounded with a substrate that has been (partly) utilized. Little is known of how this heterogeneity affects cellular processes and gene expression in different parts of the mycelium. However, previous studies suggest that the vegetative colonizing mycelium is highly differentiated (11, 21, 24). Growth of *Aspergillus niger* and *Phanerochaete chrysosporium* mainly takes place in specific zones at the periphery and the center of the colony (11, 24). These zones also secrete proteins. For instance, glucoamylase is secreted at the periphery of an *A. niger* colony (24), whereas lignin peroxidase is secreted in the central growth zone of *P. chrysosporium* (11). Interestingly, the hyphae that release the peroxidase are morphologically distinct from the secreting hyphae at the periphery of the vegetative mycelium. The high degree of differentiation in fungal vegetative mycelia is also indicated by the fact that only a subset of growing hyphae at the periphery of an *A. niger* colony secrete glucoamylase (21, 24).

Previous studies have shown that certain genes are differentially expressed in central and peripheral zones of the vegetative mycelium (11, 18, 21, 25). In this study, a genome-wide expression analysis has been performed to assess spatial expression profiles in *A. niger* colonies. It is concluded that the vegetative mycelium of *A. niger* is highly differentiated and that both medium-dependent and medium-independent mechanisms are main determinants of spatial gene expression.

MATERIALS AND METHODS

Gene annotation. Annotation of the *A. niger* open reading frames (ORFs) has been described by Pel et al. (13). Tables S1 and S2 in the supplemental material present the ORF names of genes whose expressions have been described in this article.

Growth conditions. *A. niger* N402 (*cspA1*) (1) was grown at 30°C under constant light in water-saturated air. Colonies were grown as a sandwiched culture (24) in 9-cm petri dishes in a 0.2-mm thin layer of 1.25% agarose in between two perforated polycarbonate membranes (diameter, 76 mm; pore size, 0.1 μm; Osmonics, GE Water Technologies, Trevose, PA) placed on top of solidified (1.5% agar) minimal medium (7) with 25 mM maltose or xylose as a carbon source. Sandwiched cultures were inoculated with 1.5 μl of spore suspension (10^8 spores μl^{-1}) and were either transferred or not transferred after 6 days of growth to fresh solid medium or to liquid minimal medium contained in a ring plate (9). A ring plate consists of a polycarbonate disc (9 cm in diameter, 1.2 cm thick) with six ring-shaped wells. The inner two rings are collectively called ring 1 because of their small volume, and the outer ring is called ring 5. The wells are separated by 0.1 cm and are 0.5 cm deep and 0.5 cm wide.

Detection of growth, protein synthesis, and protein secretion. Growth, protein synthesis, and protein secretion were monitored as described previously (24). Sandwiched colonies were labeled with 185 kBq of [^{14}C]*N*-acetylglucosamine (specific activity, 2.04 gBq $mmol^{-1}$; Amersham Biosciences, United Kingdom) for 10 min to detect growth. Protein synthesis and secretion were monitored by labeling with 185 kBq of a mixture of ^{14}C-labeled amino acids (specific activity, 189 gBq $milliatom^{-1}$; Amersham Biosciences, United Kingdom) for 4 h. When colonies were labeled on solid medium, a protein binding polyvinylidene difluoride (PVDF) membrane was placed under the sandwiched culture to immobilize the secreted proteins. Label was adsorbed to a piece of rice paper and placed

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on top of the sandwiched culture. In cases of colonies placed on a ring plate, the label was applied directly in the medium or adsorbed to rice paper as described above. After being labeled, colonies were fixed with 4% formaldehyde. Fixed colonies and PVDF membranes were washed three times for 60 min with either 0.44 mM *N*-acetylglucosamine or 1% Casamino Acids, dried, and exposed to Kodak Biomax XAR film (Kodak Industrie, France). Labeled proteins in the culture medium of ring plates were separated on 10% sodium dodecyl sulfate (SDS)-phosphonoacetic acid gels and fixed with 45% methanol, 10% acetic acid. After the gel was enhanced with Amplify (Amersham Biosciences, United Kingdom), it was dried and exposed to Kodak Biomax XAR film (Kodak Industrie, France).

Analysis of macronutrients in the medium. Agar medium was collected from underneath each of the five zones of sandwiched cultures. Zone 1 consisted of the inner 1.5 cm of the colony, whereas zones 2 to 5 consisted of concentric rings with widths of 5 mm each. Samples were boiled for 5 min after addition of 2 volumes of water. Half of the sample was used to measure phosphate, nitrate, and sulfate and the other half to determine the sugar content. The sample was diluted 50 times in water to determine the phosphate, nitrate, and sulfate concentrations. Samples were filtered through a 0.45-μm-pore-size filter (Oxoid Ltd., London, United Kingdom) and then through a 0.22-μm-pore size polyether sulfone membrane (Millex-GP; Milipore, Cork, Ireland). Phosphate and nitrate levels were determined photospectrometrically with an AA3 autoanalyzer (Bran and Luebbe, Hamburg, Germany) according to the instructions of the manufacturer. Sulfate content was analyzed by high-pH anion-exchange chromatography fractionation using an AS14 column, with 1 mM $NaHCO_3$-3.5 mM Na_2CO_3 solution as an eluent at a flow rate of 1.20 ml $minute^{-1}$. Sugar concentration was measured after centrifuging 10-fold-diluted samples for 5 min at maximal speed and filtering the supernatant through a polyacrylamide Biogel P2 fine column (45- to 90-μm wet-particle size; Bio-Rad laboratories, CA). Fractions were screened qualitatively for sugar content by thin-layer chromatography using Silica Gel 60 high-performance thin-layer chromatography plates (VWR International, The Netherlands), with orcinol-sulfuric acid as the staining method. Sugar-containing fractions were analyzed by high-pH anion-exchange chromatography fractionation using a CarboPac PA-1 (250 by 4 mm) column, with a linear gradient resulting from 100 mM NaOH and 500 mM NaOAc in 100 mM NaOH as an eluent. The flow rate was 1.0 ml min^{-1}, and 1 mM isomaltose was used as the internal standard.

RNA isolation. Mycelium was ground using a microdismembrator (B.Braun GmBh, Melsungen, Germany), and RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer. The RNA was purified using a Nucleospin RNA cleanup kit (Macherey-Nagel GmBh, Düren, Germany). The concentration of RNA was measured at A_{260}. The quality of the RNA was analyzed with an Agilent 2100 bioanalyzer, using an RNA6000 LabChip kit (Agilent Technology, Palo Alto, CA).

Microarray analysis. Biotin-labeled antisense cRNA was generated by labeling 20 or 2 μg of total RNA with a BioArray high-yield RNA transcription labeling kit (ENZO) or an Affymetrix eukaryotic one-cycle target labeling and control reagent package, respectively. The quality of the cRNA was checked using the Agilent 2100 bioanalyzer. The labeled cRNA was hybridized to Affymetrix *A. niger* GeneChips (Affymetrix, Santa Clara, CA). The coding sequence of the annotated genome of CBS513.88 (13) was taken as the sequence template. Oligonucleotide probes were designed with 600-bp fragments, starting from the 3′ end of the gene. The probe sets consist of 12 pairs (match and mismatch) of 25-bp oligonucleotide probes, which are scattered across the chip.

Absolute values of expression were calculated from the scanned array by using Affymetrix GeneChip Operating System software after an automated process of washing and staining. Microarray Suite Affymetrix version 5.1 (Affymetrix Inc., Santa Clara, CA), Spotfire DecisionSite (Spotfire, Inc. Somerville, MA), GeneData Expressionist Analyst V Pro 2.0.18 (GeneData, Basel, Switzerland), and the R statistical environment (www.r-project.org) were used for data analyses. Arrays were hybridized with three independently obtained RNA samples of the peripheries of 7-day-old sandwiched cultures grown on maltose. Since the correlation between the samples was 0.982 and the average signal log ratio was found to be −0.044, it was decided that all other hybridizations would be done with biological duplicates.

Affymetrix DAT files were processed using the Affymetrix GeneChip Operating System. The CHP files were generated from CEL files by using Affymetrix Global scaling normalization to a target intensity value of 100 (TGT-100). Microarray data have been deposited at http://www.bio.uu.nl/microbiology/fung/PhD%20theses/AMLevin/index.html. Hierarchical cluster analysis (HCA) was performed with Spotfire, using the weighted-pair group method using average linkages, with correlation as a dissimilarity measure. Principal component analysis (PCA) was performed with the R program, with centering applied across samples and genes. For both HCA and PCA, data were $\log_2$ transformed after replacing all values under 10 with 10 to avoid extremely low log values. Genes that had no *P* assignment along any of the different samples were removed from the data set. Fisher's exact tests were performed with GeneData Expressionist, with only the groups with *P* values of ≤0.01 selected.

Statistical assessment of differences in expression between samples was performed with the R package maSigPro (3) and the R package Limma (Linear Models in Microarray Analysis) (17). In all analyses, a significance level of 0.05 was used and Benjamini multiple testing correction (16) was applied.

RESULTS

Localization of growth, protein synthesis, and protein secretion in nontransferred colonies in relation to the spatial availability of nutrients. Sandwiched colonies (see Materials and Methods) of *A. niger* were grown on minimal medium, using 25 mM maltose or xylose as the carbon source. After 3 days of growth, the diameter of each maltose-grown colony was 1.5 cm, after which the colony extended its diameter 1 cm per day. Growth on xylose was somewhat slower. The diameter of each xylose-grown colony was 5 cm after 7 days of growth instead of 5.5 cm, the value for maltose-grown colonies. Five concentric zones were arbitrarily distinguished in the sandwiched colony. Zone 1 is the innermost part of the colony, with a diameter of 1.5 cm. This zone represents the surface area of the colony after 3 days of growth. Zone 2 consists of the part of the colony between 1.5 and 2.5 cm, zone 3 between 2.5 and 3.5 cm, zone 4 between 3.5 and 4.5 cm, and zone 5 between 4.5 and 5.5 cm (Fig. 1). The outer borders of these zones constituted the edge of the colony at days 4 to 7, respectively.

The nitrogen, phosphate, and sulfur sources were not exhausted in the medium underlying the five zones of the colony after 7 days of growth (Table 1). In contrast, xylose had been depleted in the colony except in the outermost zone, where 3 mM (out of 25 mM) xylose was left. Similarly, maltose was exhausted in central zones 1 to 3, whereas 1 and 4 mM maltose, respectively, were detected in zones 4 and 5. Labeling with a mixture of ^{14}C-amino acids showed that proteins were formed throughout the mycelium (Fig. 1B). However, they were mainly secreted at the growing periphery of the colony (zone 5) (Fig. 1A and C). Growth also occurred within the innermost central zone (zone 1) (Fig. 1A), as was shown by labeling with [^{14}C]*N*-acetylglucosamine. These data and those published previously (24) show that cellular processes like growth and protein secretion can take place in the center of an *A. niger* colony, despite the absence of a carbon source in the underlying mycelium.

mRNA composition of central and peripheral zones of nontransferred colonies. RNA isolated from the different zones of 7-day-old sandwiched colonies were hybridized to whole-genome Affymetrix microarrays representing 14,420 unique *A. niger* ORFs (13). Approximately 47% of the genes were not expressed in any of the zones of xylose- or maltose-grown colonies, whereas 29.5% of the genes were expressed throughout the mycelium on both carbon sources (Table 2; see also Fig. S1 in the supplemental material). Of the genes expressed on xylose, 459 were not expressed in maltose-grown colonies. Of these genes, 16 were expressed throughout the xylose-grown colony (see Table S3 in the supplemental material). On the other hand, all genes expressed in maltose-grown colonies

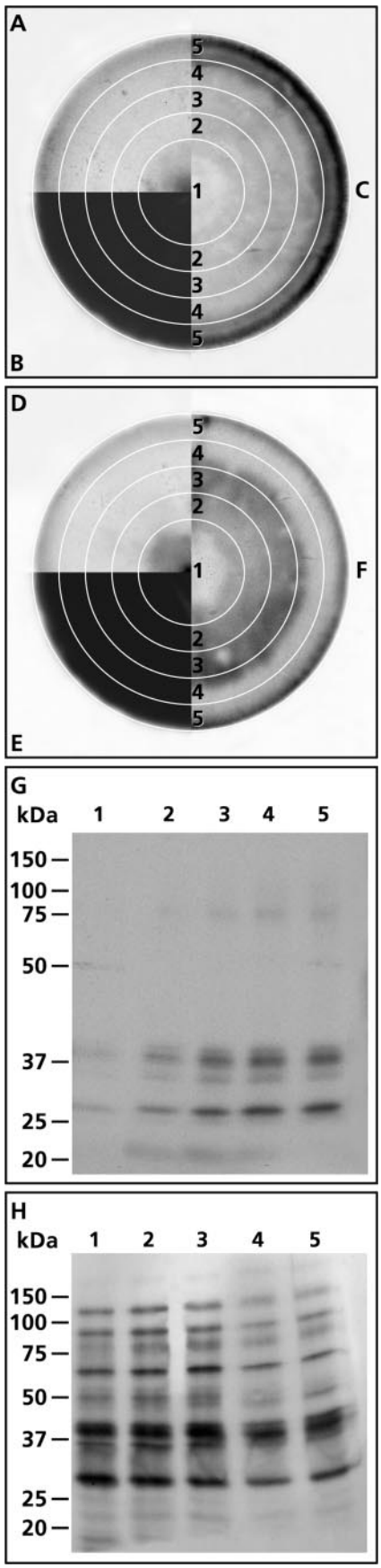

TABLE 1. Residual levels of nutrients in the media underlying the different zones of sandwiched colonies of *A. niger*[a]

Group and zone	Concn (mM) of indicated nutrient			
	Nitrate	Sulfate	Phosphate	Xylose or maltose
Xylose-grown colonies				
1	56.6 ± 21.1	1.49 ± 0.33	12.2 ± 2.9	ND
2	67.0 ± 7.3	1.58 ± 0.16	12.5 ± 2.9	ND
3	70.8 ± 5.5	1.86 ± 0.47	12.8 ± 3.6	ND
4	65.0 ± 7.3	1.59 ± 0.35	11.2 ± 2.3	ND
5	77.2 ± 6.1	1.81 ± 0.34	13.6 ± 2.1	3.33 ± 1.15
Maltose-grown colonies				
1	75.7 ± 2.5	2.6 ± 0.5	12.5 ± 0.2	ND
2	71.8 ± 4.3	2.2 ± 0.1	12.00 ± 0.4	ND
3	70.1 ± 2.1	2.3 ± 0.4	11.1 ± 0.9	ND
4	60.5 ± 15.2	1.8 ± 0.5	13.7 ± 1.7	0.33 ± 0.23
5	62.8 ± 18.8	1.9 ± 0.6	11.2. ± 3.6	4.3 ± 3.1

[a] Zones 1 and 5 represent the most central and peripheral parts of the colony, respectively. Minimal medium contains 71, 2, 11, and 25 mM of the N, S, P, and C sources, respectively. ND, not detected.

were also expressed in at least one zone in xylose-grown colonies (data not shown).

In the next step of the analysis, we focused on genes that were differentially expressed in either xylose- or maltose-grown colonies. About 14% of the total number of genes (i.e., about 30% of the active genes) was expressed in one to four zones of xylose- or maltose-grown colonies (Table 2; see also Fig. S1 in the supplemental material). Of these, almost 30% were expressed in a single zone (see Fig. S1 in the supplemental material). About 45% of the active genes were expressed in one to nine zones when the analysis was performed on the combination of the five zones of xylose- and maltose-grown colonies. Of these genes, 6% were expressed in a single zone only (Table 2; see also Fig. S1 in the supplemental material).

Spatial differences in gene expression were also shown by the number of genes whose expression levels were found to be at least two- or fourfold changed when the different zones within a colony were pairwise compared (Fig. 2B). In general, differences in expression increased with increasing distance between the zones. Peripheral zone 5 showed the highest number of genes that were up- or down-regulated in comparison to those in the other zones, and differences between zones 5 and 4 were more pronounced than those between any of the neighboring zones. The large number of genes that are differentially expressed between zones 1 and 5 of xylose- and maltose-grown

FIG. 1. Growth (A, D), protein synthesis (B, E), and protein secretion (C, F, G, H) in 7-day-old xylose-grown sandwiched colonies of *A. niger* with (D, E, F, H) and without (A, B, C, G) a 16-h transfer to fresh medium. Growth was monitored by labeling with ^{14}C-labeled *N*-acetylglucosamine, whereas protein synthesis and protein secretion were localized by incorporation of ^{14}C-labeled amino acids. Secreted proteins were detected by placing a protein binding PVDF membrane under the colony (C, F) or by SDS-polyacrylamide gel electrophoresis of the culture medium of a ring plate (G, H). Lane numbers indicate the concentric wells of the ring plate, lanes 1 and 5 representing the most central and peripheral zones, respectively.

TABLE 2. Percentages of genes that are not expressed, always expressed, and differentially expressed in zones of colonies grown on solid medium

Medium	% of genes		
	Not expressed	Always expressed	Differentially expressed
Maltose (zones 1 to 5)	51.4 ± 3.4	34.2 ± 5.7	14.4 ± 3.3
Xylose (zones 1 to 5)	52.5 ± 0.2	33.6 ± 1.4	13.9 ± 1.2
Total	46.5 ± 2.5	29.5 ± 3.0	24.0 ± 0.5

colonies (779 and 763 genes, respectively, that are at least twofold increased and 1,077 and 1,166 genes, respectively, that are at least twofold decreased) (Fig. 2B) shows that differential expression is not restricted to a small number of genes.

The gene expression data were analyzed by PCA to assess the extent to which the spatial expression in the colony is affected by the nature of the carbon source or by the position within the colony. This type of analysis results in a coordinate system, with the first and second (and third, etc.) coordinates representing the components causing the largest and second-largest (and third-largest, etc.) variance, respectively. PCA assigned 41% of the variability to the nature of the carbon source (Fig. 2A). The importance of the carbon source is illustrated by the fact that the zones of xylose-grown colonies and the zones of maltose-grown colonies cluster at the right and left parts of the representation, respectively. Fisher's exact test showed that the functional FunCat gene classes related to other proteolytic degradation and C-compound and carbohydrate utilization were significantly increased within the colony centers of maltose-grown colonies compared to those in xylose-grown colonies. On the other hand, the functional gene class related to C-compound and carbohydrate utilization as well as those related to degradation of foreign (exogenous) polysaccharides and other protein synthesis activities were decreased at the peripheries of the maltose-grown colonies (data not shown). Clearly, the differences in the gene expression levels of these functional gene classes represent a minor fraction of the total variation. Thus, most of the differentially expressed genes are scattered throughout other functional gene classes.

The position in the colony was shown to have an impact on gene expression similar to that of the carbon source. PCA showed that the inner three zones (zones 1 to 3) and to a lesser extent the outer two zones (zones 4 and 5) of the colony clustered (Fig. 2A). These results were in agreement with those for HCA (data not shown).

The fact that the 50 most periphery-specific and the 50 most center-specific genes encode predicted regulators, proteins involved in metabolism, stress response, and morphology, as well as extracellular enzymes, transporters, and other membrane proteins (see Table S2 in the supplemental material) suggested that differential expression is scattered over the functional gene classes. Indeed, Fisher's exact test showed only significant differences in the expression levels of gene classes related to metabolism and protein fate between the most peripheral and central zones of maltose-grown colonies (Table 3). When xylose was used as a carbon source, there were also differences in gene classes related to the cell cycle, cell communication, homeostasis of cations, cell fate, and control of cellular organization.

Localization of growth, protein synthesis, and protein secretion in transferred colonies. Six-day-old colonies were transferred to fresh medium for 16 h and subsequently pulse labeled with a mixture of ^{14}C-amino acids or [^{14}C]*N*-acetylglucosamine to assess whether the heterogeneity of the medium (Table 1) affected the spatial distribution of growth, protein synthesis, and protein secretion in a colony. Spatial growth and protein production in maltose- and xylose-grown colonies was not affected by transferring colonies to fresh medium (Fig. 1D and E). In contrast, secretion was observed not only at the periphery (zone 5) but also in central parts of the mycelium (zones 2 and 3) (Fig. 1F). These data show that central zones of the mycelium abundantly secrete proteins upon transfer to fresh medium, which apparently is not associated with an increase in growth in these parts of the colony. To exclude that this result was caused by the inabilities of these zones of the colony to import the chitin precursor, colonies were labeled with [^{14}C]*N*-acetylglucosamine for 10 min. In contrast to the experiments where growth was monitored, colonies were not fixed with formaldehyde before being washed with minimal medium. These experiments showed that the whole colony had the capacity to import the chitin precursor (data not shown).

To assess the complexity of the profile of secreted proteins, 6-day-old maltose- and xylose-grown sandwiched colonies were transferred to a ring plate (9). This polycarbonate disc with ring-shaped wells (see Materials and Methods) was filled with fresh medium. After 16 h, a ^{14}C-amino acid mixture was added to the medium contained in each of the concentric wells of the ring plate. SDS-polyacrylamide gel electrophoresis revealed that profiles of secreted labeled proteins were similar throughout the mycelium (Fig. 1H). In contrast, proteins were most abundantly secreted in the peripheral zones when the colony was transferred to a ring plate on top of which a colony had been grown for 6 days (Fig. 1G). It should be noted that the zones of the ring plate did not perfectly match the secretion zones, explaining why radioactive protein was observed in each ring of the colony.

mRNA composition in central and peripheral zones of the colony before and after transfer. We wondered about the extent to which differences in gene expression between peripheral and inner zones of 7-day-old xylose-grown colonies are due to the heterogeneity in the medium underlying the colony. To address this, mRNA composition was analyzed in peripheral zone 5 and central zone 3 of colonies that had either been or not been transferred for 16 h to fresh medium. Hybridization of the RNA to whole-genome microarrays of *A. niger* revealed that approximately 45% of the genes were not expressed in any of the cases, whereas 29% of the genes were expressed under all conditions and 2.6% of the genes were active in only a single zone of either transferred or nontransferred colonies (data not shown).

PCA of the expression profiles of zones 3 and 5 of transferred and nontransferred colonies attributed 53% of the data variability to the position in the colony (i.e., zone 3 versus zone 5, irrespective of transfer) (Fig. 3A). The second and third principal components (21% and 16% of the variability, respectively) were attributed to transfer within the zones and transfer independent of the zonal position (Fig. 3B). These data show

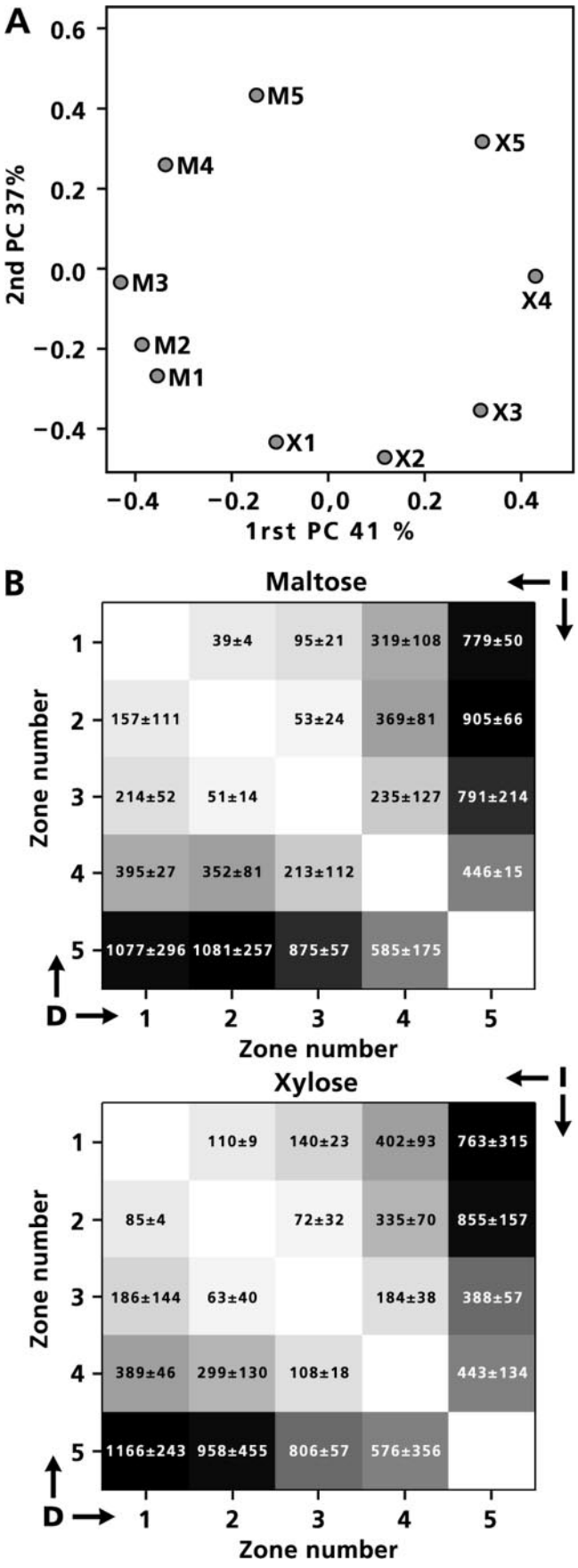

FIG. 2. Differential gene expression in five concentric zones of 7-day-old *A. niger* colonies grown on maltose (M) or xylose (X). Zones 1 and 5 represent the inner and outermost zones of the colony, respectively. Results for PCA (A) and count map (B) of differences in gene expression between the different zones of the colonies. Shade intensity in panel B is proportional to the number of genes with ≥2-fold increases (top right, marked I) or decreases (bottom left, marked D).

TABLE 3. Results for Fisher's exact test for the most peripheral and central zones of 7-day-old maltose- and xylose-grown sandwiched colonies of *A. niger*[a]

Category no. (description)	Result for:	
	M1 vs M5	X1 vs X5
01 (metabolism)		
01.01.10 (amino acid degradation [catabolism])	U	U
01.03.19 (nucleotide transport)	U	
01.05.01 (C-compound and carbohydrate utilization)	U	U
01.05.07 (C-compound and carbohydrate transport)	U	U
01.06.01 (lipid, fatty acid, and isoprenoid biosynthesis)	U	U
01.06.04 (lipid, fatty acid, and isoprenoid breakdown)	U	
03 (cell cycle and DNA processing)		
03.03.01 (mitotic cell cycle and cell cycle control)		U
06 (protein fate)		
06.07.03 (modifications by phosphorylation/ dephosphorylation)	U	U
006.07.05 (modification by ubiquitination/ deubiquitination)	U	U
06.13 (proteolytic degradation)		
06.13.04 (lysosomal and vacuolar degradation)	U	U
06.13.99 (other proteolytic degradation)		U
10 (cell communication/signal transduction)		U
14 (cell fate)		
14.01.03 (directional growth [morphogenesis])		U
30 (control of cellular organization)		U
34 (interaction with the cellular environment)		
34.01.01 (homeostasis of cations)		U

[a] M1 vs M5, maltose-grown colonies, zone 1 versus zone 5; X1 vs X5, xylose-grown colonies, zone 1 versus zone 5. U indicates up-regulation of functional FunCat categories.

that the position within the colony has a higher influence on gene expression than medium composition.

The number of genes that were two- and fourfold changed between zones 5 and 3 in transferred colonies was similar to that in nontransferred colonies (Table 4) and between two- and threefold higher than the number of genes differentially expressed between transfer and nontransfer samples of the same zone (Table 4). These data are in agreement with the distribution of variability shown by PCA. Fisher's exact test showed that expression levels of FunCat gene classes related to C-compound and carbohydrate metabolism, energy, cell rescue and defense, subcellular localization, and protein modification were decreased at the periphery upon transfer to fresh medium (Table 5) ($P \leq 0.01$). Changes were less pronounced in central zone 3. Genes related to subcellular localization were increased in this zone, whereas some groups involved in metabolism were decreased. A comparison between zones 3 and 5

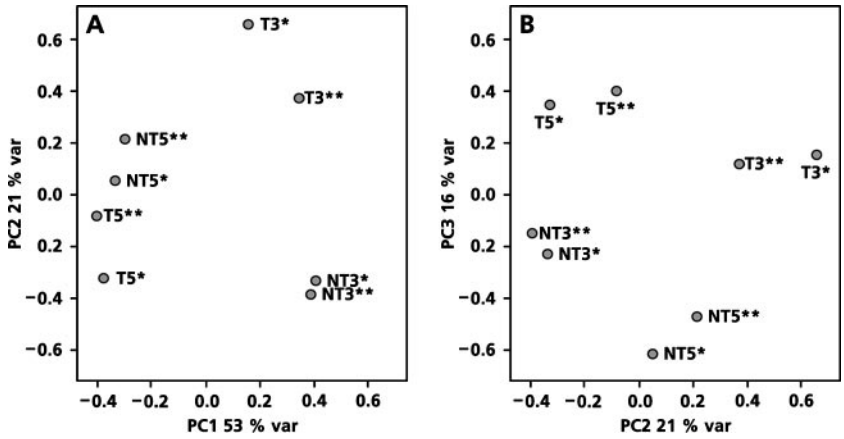

FIG. 3. PCA of a biological duplicate (* and **) of peripheral zone 5 and central zone 3 of 7-day-old xylose-grown *A. niger* colonies that had either been or not been transferred to fresh medium at day 6. (A) First and second components; (B) second and third components. T and NT indicate transferred and nontransferred colonies, respectively, and the numbers indicate the zones of the colonies.

with and without transfer was also made (Table 5). In nontransferred colonies, genes related to metabolism, protein fate, and transport facilitation were more highly expressed at the periphery, whereas the expression levels of genes involved in the cell cycle were reduced. Upon transfer, differences in expression in metabolic genes were reduced but became more pronounced for functional classes related to energy, protein fate, cell rescue and defense, cell fate, and subcellular localization. This finding is quite remarkable, considering that after transfer both zones were exposed to identical nutrient conditions.

Expression of specific gene groups in transferred and nontransferred colonies. Expression of genes related to growth (C, N, S, and P metabolism and cell wall synthesis and modification) and secretion in transferred and nontransferred colonies was assessed. Expression of proteases was also analyzed because they could be instrumental in the recycling of secreted proteins. Moreover, they affect production levels of secreted (heterologous) proteins.

AmyR- and XlnR-regulated genes. The transcriptional activators AmyR (14) and XlnR (20) each activate a specific gene set mainly encoding extracellular enzymes (5, 6, 19) when maltose and xylose are used as a carbon source, respectively. Both *amyR* and *xlnR* were most highly expressed at the peripheries of nontransferred xylose-grown colonies, but this was not observed when maltose was used as a carbon source (Fig. 4). The AmyR-regulated genes were most highly expressed at the peripheries of both maltose- and xylose-grown colonies, although expression levels were much lower on the latter carbon source (Fig. 4A and C). The expression profiles of XlnR-regulated genes on maltose were not uniform; some were higher at the periphery and some in the center, suggesting additional regulation of these genes apart from XlnR (Fig. 4B). However, expression of the XlnR-regulated genes followed the expression of *xlnR* when xylose was used as the carbon source. They showed generally 5- to 10-fold higher expression levels in the two outer zones of the colonies (Fig. 4D). After transfer to fresh medium, only *xyrA* was still significantly more highly expressed at the periphery (Fig. 5A). This finding shows that XlnR-related genes mainly react to the carbon source and not to the differentiation of the mycelium.

TABLE 4. Numbers of genes that are at least two- or fourfold increased or decreased between peripheral zone 5 and central zone 3 of 7-day-old xylose-grown sandwiched colonies

Ring comparison[a]	No. of genes increased		No. of genes decreased	
	Twofold	Fourfold	Twofold	Fourfold
5 vs 3	643	137	374	101
5T vs 3T	419	96	591	85
5 vs 5T	182	37	345	56
3 vs 3T	167	73	394	34

[a] T indicates 16-h transfer to fresh medium.

Nitrate, phosphate, and sulfate metabolism. The conversion of extracellular nitrate to intracellular ammonium involves three genes in *Aspergillus nidulans*: *crnA* (encoding a nitrate transporter), *niaD* (encoding nitrate reductase), and *niiA* (encoding nitrite reductase) (8). Surprisingly, although nitrate was present in similar concentrations across the medium (Table 1), the expressions of these genes were detected only at the peripheries of nontransferred maltose- and xylose-grown colonies (Fig. 6A and B). On the other hand, the nitrogen metabolic repressor *hNmrr* was most highly expressed in the central zones of the colony. Transfer to fresh xylose medium for 16 h hardly affected the spatial expressions of these genes (Fig. 5B). Taken together, these data show that spatial expression of genes involved in nitrate metabolism results from medium-independent mechanisms.

Phosphate and sulfate metabolism did not appear to be specific for the periphery of the colony either before or after transfer to fresh media (data not shown).

Cell wall synthesis and modification. The genome of *A. niger* contains 13 putative chitin synthases (*chsA* to *chsM*) and 14 putative chitinases (*chiA* to *chiN*) (13). These genes have highly variable expression levels and profiles. For both gene classes, genes with high and low expression levels were detected. While most genes have similar expression levels throughout nontransferred colonies, some are center specific (e.g., *chsL* and *chiE*) and others specific for the periphery (e.g.,

TABLE 5. Results for Fisher's exact test of peripheral zone 5 and central zone 3 of 7-day-old xylose grown sandwiched colonies of *A. niger* with or without transfer to fresh medium at day 6

Category no. (description)	Result for indicated zone comparison[a]			
	5 vs 3	5 vs T5	3 vs T3	T5 vs T3
01 (metabolism)				
01.01.01 (amino acid biosynthesis)	U			U
01.01.10 (amino acid degradation [catabolism])	U		U	U
01.02.01 (N and S utilization)	U			
01.05.01 (C-compound and carbohydrate utilization)	U	U	U	U
01.05.07 (C-compound and carbohydrate transport)	U	U	U	U
01.06.01 (lipid, fatty acid, and isoprenoid biosynthesis)	U			U
01.06.04 (lipid, fatty acid, and isoprenoid breakdown)	U			U
01.06.99 (other lipid, fatty acid, and isoprenoid metabolism)	U			
02 (energy)				
02.11.05 (accessory proteins of electron transport and membrane-associated energy conservation proteins)				U
02.11.99 (other electron transport and membrane-associated energy conservation proteins)		U		
02.13.03 (aerobic respiration)				U
03 (cell cycle and DNA processing)				
03.01.05 (DNA recombination and DNA repair)				D
03.03.01 (mitotic cell cycle and cell cycle control)	D			D
04 (transcription)				
04.05.01 (mRNA synthesis)				D
04.05.05 (mRNA processing)				D
06 (protein fate)				
06.07.99 (other protein modifications)		U		U
06.13 (proteolytic degradation)				U
06.13.04 (lysosomal and vacuolar degradation)	U			U
06.13.99 (other proteolytic degradation)	U			
11 (cell rescue, defense, and virulence)				
11.07.01 (detoxification involving cytochrome P450)		U		
11.07.99 (other detoxification)		U		U
11.10.07 (degradation of exogenous polysaccharides)		U		U
14 (cell fate)				
14.04.03 (fungal cell differentiation)				U
40 (subcellular localization)		U	D	D
67 (transport facilitation)	U			U

[a] T indicates transfer to fresh medium at day 6. U and D indicate up- and down-regulation of functional FunCat categories, respectively.

chsF and *chiF*) (Fig. 6C and D). The expression levels of two chitinase genes (*chiF* and *chiD*) and two chitin synthase genes (*chsM* and *chsF*) were significantly more highly expressed at the periphery after transfer, whereas none of the genes altered their expression levels in central zone 3 after transfer (Fig. 5C). This shows that differences in the expression levels of the genes encoding chitin synthases and chitinases are affected by medium-independent mechanisms. This is in agreement with the finding that the spatial incorporations of *N*-acetylglucosamine into chitin are similar in transferred and nontransferred colonies.

The *A. niger* genome contains four putative β-glucan synthases (*bgsA* to *bgsD*), two of which (*bgsB* and *bgsC*) are expressed throughout the colony in nontransferred colonies, whereas no expression was detected for the other two β-glucan synthase genes (data not shown). The expression profiles of the β-glucan synthases in zones 3 and 5 of xylose-grown colonies did not change upon transfer to fresh medium (Fig. 5C). Diverse expression profiles were observed for the five α-glucan synthases (*agsA* to *agsE*) (4). Gene *agsC* was periphery specific for both carbon sources (Fig. 6C and D), and its expression was not affected by a transfer to fresh medium (Fig. 5C). In contrast, expression of *agsE* was center specific in transferred and nontransferred xylose-grown colonies. From these data, it can be concluded that the differential expression of α-glucan synthases is not the result of the medium composition.

Five putative hydrophobin genes have been identified in the *A. niger* genome (13). *hfbE* (homologue of *Trichoderma reesei hfb1*) and *hfbD* (*Coprinus cinereus* COH1 homologue) were expressed at significant levels throughout nontransferred maltose- and xylose-grown colonies (Fig. 6E and F). The highest expression levels of these genes were found at the periphery and the center, respectively. On xylose, *hfbA* (*A. nidulans dewA* homologue) and *hfbB* (*A. nidulans dewB* homologue) were also

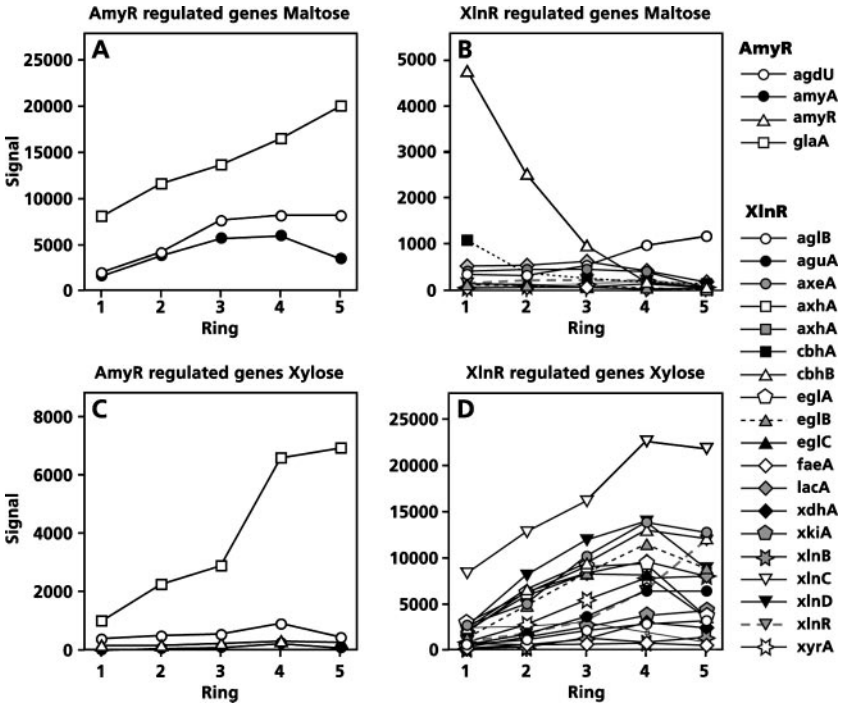

FIG. 4. Expression profiles of AmyR (A, C)- and XlnR (B, D)-regulated genes in colonies of *A. niger* grown for 7 days on maltose (A, B) or xylose (C, D). Zones 1 and 5 represent the center and the periphery of the colony, respectively. Only genes that showed significantly changed expression have been plotted. See Table S1 in the supplemental material for the corresponding ORF names.

expressed. The expression levels of these genes and of *hfbD* are center specific and peak in zone 2 (Fig. 6F). Upon transfer to fresh xylose medium, only one hydrophobin gene (similar to *HYP1* of *Aspergillus fumigatus*) changed its expression level significantly. Expression increased at the periphery but not in the center (Fig. 5C). Thus, differences in the expression levels of hydrophobin genes are not affected by the heterogeneity in the medium.

Protein secretion and proteases. Most genes of *A. niger* involved in protein secretion were expressed throughout xylose- and maltose-grown colonies. However, the expression levels of *cnx1*, *rab6*, *cypB*, *tigA*, *sec61a* and *-b*, and *hacA* were significantly higher at the periphery in both xylose- and maltose-grown colonies (Fig. 6G and H), whereas *pdiA* was more highly expressed at the periphery in maltose-grown colonies. After transfer of xylose-grown colonies to fresh xylose-containing medium, the expression levels of 9 out of these 12 genes increased in the central zone. As a result, only *cnx1*, *rab6*, and *sec61b* are still periphery specific after transfer (Fig. 5D). Thus, genes encoding proteins involved in secretion respond to a large extent to the composition of the medium.

Most genes encoding (putative) proteases (13) were evenly expressed in the mycelium. However, some genes were differentially expressed in nontransferred colonies (Fig. 6I and J), with the highest expression levels at the peripheries of maltose-grown colonies for *protA* (similar to the serine peptidase of *Oryza sativa*), *protC* (homologue to X-Pro dipeptidyl-peptidase IV of *Xanthomonas maltophilia*), *protD* (precursor of acid proteinase A), *protF* (homologue of *A. nidulans* carboxypeptidase I), *protG* (similar to aspartic protease pr1 of *Phaffia rhodozyma*), and *protI* (aspergillopepsin II). In contrast, *protE* (similar to the leucyl aminopeptidase *Ape2* of *Saccharomyces cerevisiae*) and *protH* (another homologue of carboxypeptidase I of *A. nidulans*) were more highly expressed in the center of the colony. In cases of xylose-grown colonies, *protH* and *protI* were shown to be center specific, whereas *protB*, *protD*, *protF*, and *protI* were more highly expressed at the periphery. After transfer of the xylose-grown colonies, the expression levels of the protease genes in the center are similar to those at the periphery before transfer (Fig. 5E). As a result, expression of *protE* and *protD* became center specific. Thus, it can be concluded that genes encoding proteins involved in protein degradation react to a large extent to the composition of the medium.

DISCUSSION

Fungal mycelia are exposed to heterogenic substrates. The substrate at the periphery of the colony is unexplored, whereas it is at least partially degraded in the center. The substrate heterogeneity is expected to affect local gene expression in the mycelium. This question was addressed by analyzing gene expression in five concentric zones of 7-day-old maltose- and xylose-grown colonies of *A. niger*. Clear differences were observed in the RNA profiles of peripheral and central zones of the colonies. These differences were shown to be due to the nature of the carbon source, the heterogeneity of the medium underlying the colony, and, to a large extent, medium-independent mechanisms. The last shows that colonies of *A. niger* are highly differentiated, which was also indicated by the fact that distinct zones of the colony grow and secrete proteins.

Approximately 50% of the genes of *A. niger* were shown to

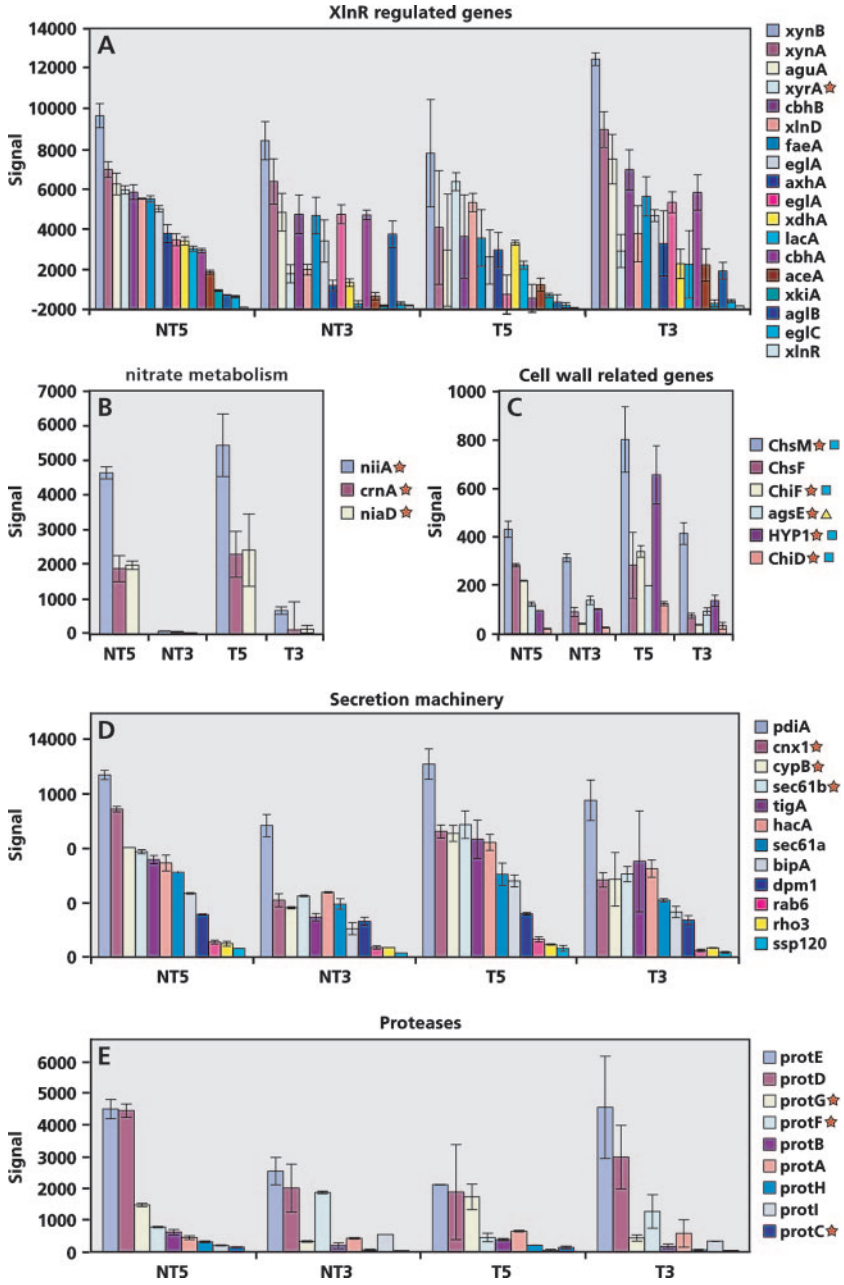

FIG. 5. Genes differentially expressed between the central zone 3 and peripheral zone 5 of 7-day-old xylose-grown colonies that were either transferred (T) or not transferred (NT) to fresh medium after 6 days of growth. Results are shown for XlnR-regulated genes (A); genes involved in nitrate utilization (B), cell wall formation and modification (C), and secretion (D); and genes encoding (putative) proteases (E). Genes labeled with a star are still differentially expressed between rings 3 and 5 after transfer to fresh medium. Triangles and squares indicate genes that have significant changes in expression in rings 3 and 5, respectively, after transfer to fresh medium. See Table S1 in the supplemental material for the corresponding ORF names.

be expressed on solid medium with either maltose or xylose as a carbon source. Of these genes, about 60% were expressed throughout the colony, whereas about 9% of the active genes were expressed in a single zone of a colony. PCA showed that 41% of the variability in gene expression between xylose- and maltose-grown colonies could be attributed to the difference in carbon source, whereas the position of a zone within the colony accounted for 37% of the difference in gene expression (see below). The extracellular enzymes and the metabolic pathways needed for utilization of maltose and xylose partly explain the differences in the expression profiles for both carbon sources. Interestingly, genes that are not directly related to the energy source are also affected (e.g., genes involved in cell wall formation and modification). The reason for this is not yet known.

Differences in gene expression were shown along the colony radius. The three central zones were most similar in their expression profiles. Only 4% of the expressed genes (~300 genes) were twofold up- or down-regulated between zones 1

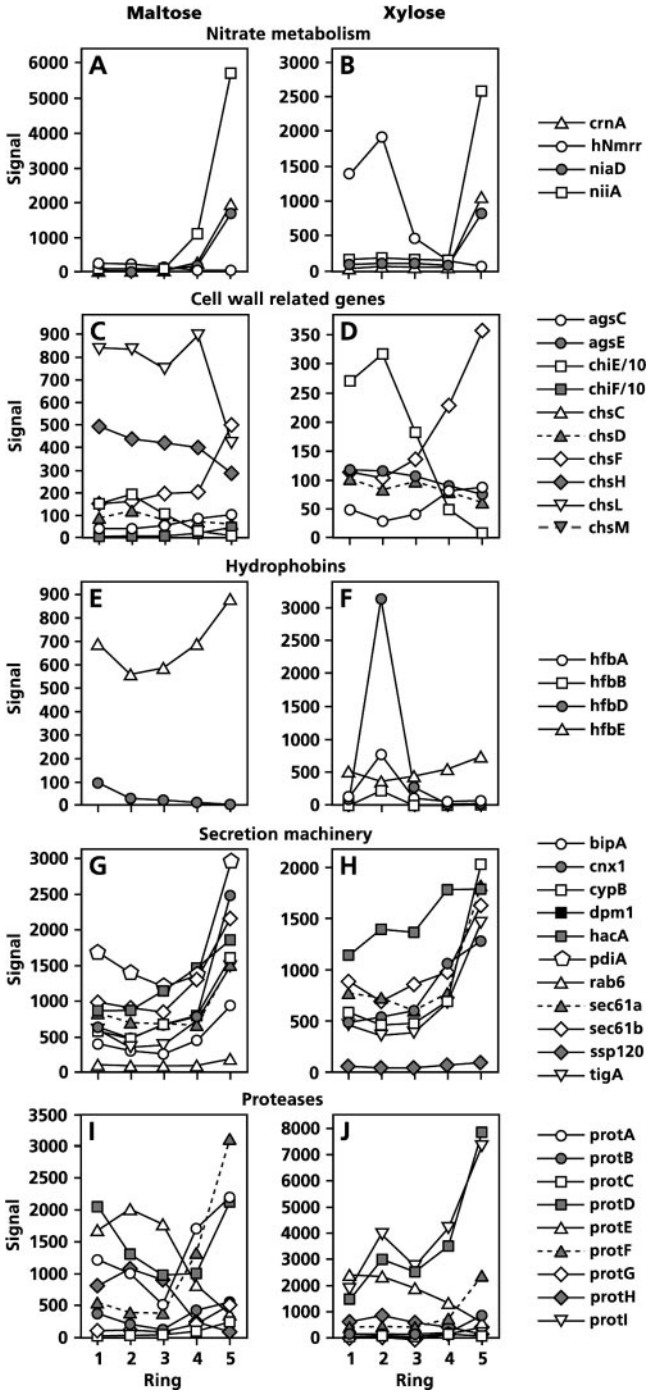

FIG. 6. Expression profiles of genes involved in nitrate metabolism (A, B), genes involved in cell wall synthesis and modification (C, D), genes encoding hydrophobins (E, F), genes encoding components of the secretion machinery (G, H), and genes encoding proteases (I, J). Zones 1 and 5 represent the most central and peripheral parts of the colony, respectively. Only significantly changed genes have been plotted. See Table S1 in the supplemental material for the corresponding ORF names.

and 3 of xylose- or maltose-grown colonies. The outer zone (zone 5) showed the most unique expression profile, with about 1,000 differentially expressed genes, compared to its neighboring zone. Zonal differences in gene expression were not restricted to specific gene groups. For instance, the 50 most periphery-specific and the 50 most center-specific genes included members of many different gene groups. These data contrast with a recent expression study performed with *Aspergillus oryzae* (10). Genes encoding extracellular functions were found to be active at the periphery only, whereas transporters were most active in the central part of the colony. This difference could be due to a different experimental approach. In the study of Masai et al. (10), expression was monitored with microarrays containing 3,000 cDNAs. These cDNAs were selected on the basis of an expressed-sequence-tag-sequencing project, which may well have created a bias in and between specific gene sets.

As mentioned above, 37% of the variability of gene expression between xylose- and maltose-grown colonies was attributed to the spatial position in the colony. In contrast to the N, S, and P sources, the C source was exhausted in the four inner zones of maltose- and xylose-grown colonies, whereas up to 40% of the C source was still available at the periphery. From this, it was hypothesized that the spatial component of differential gene expression was (partly) due to the heterogeneity of the medium underlying the colony. Indeed, by transferring colonies to a fresh medium, we here showed that genes induced by the xylanolytic regulator XlnR, genes encoding proteases, and genes encoding secretion machinery were up-regulated in the center when these colonies were transferred to fresh medium. However, the transfer did not result in a reduction in the number of genes whose expression levels were significantly different between the outer zone of the colony and central zone 3. About 1,200 genes were differentially expressed between these zones with and without transfer. The numbers of genes that were differentially expressed within a zone before and after transfer to a fresh medium were also similar. In both cases, more than 600 genes were found to have at least twofold-changed expression levels. These results indicate that developmental processes independent of the medium composition strongly affect the zonal gene expression. Indeed, PCA showed that 55% of the variability could be attributed to the position in the colony. The availability of the fresh medium accounted for about 37% of the variability (representing the second and third principal components combined). Among the genes whose spatial expression profiles were subject to medium-independent mechanisms are the genes involved in nitrate metabolism and genes involved in cell wall synthesis and modification.

Spatial differentiation was also reflected in the capacity to secrete. Nontransferred colonies mainly secreted proteins at the periphery. However, most genes encoding components of the secretory pathway were expressed throughout the colony. Yet, the expression levels of seven or eight genes of this category (*cnx1*, *rab6*, *cypB*, *tigA*, *sec61a* and *-b*, *hacA* on both xylose and maltose media, and *pdiA* on maltose medium only) were significantly higher at the periphery. These genes encode a protein involved in translocation across the endoplasmic reticulum membrane, the chaperone calnexin, two protein disulfide isomerases, a peptidyl-prolyl isomerase, a protein of the class of secretion-related GTPases and interacting proteins, and the regulator of the unfolded protein response. Could these expression levels explain why secretion is most pronounced at the periphery? It has been reported that secretion of manganese

peroxidase (MnP) can be increased four- to fivefold by overexpression of the endoplasmic reticulum chaperone calnexin (*cnx1*) (2), whereas overexpression of the heat shock protein *bipA* resulted in increased levels of unprocessed fusion protein (15). These examples show a correlation between production levels of proteins and the transcript levels of genes involved in the secretion pathway. They suggest that increases in the expression levels of up to eight genes may well account for the differences in zonal protein secretion in the colony.

Upon transfer to fresh medium, colonies secreted proteins not only at the periphery but also in central zones 2 and 3. The spatial secretion pattern correlated with increased expression levels of genes involved in secretion. Out of 12 genes, 9 showed higher expression levels in zone 3 upon transfer, which may explain the efficient secretion in this zone of the colony. Secretion in the central zones, as observed after transfer, did not correlate with increased incorporation of radioactively labeled *N*-acetylglucosamine. In agreement, genome-wide expression analysis did not indicate changes in the expression levels of genes related to cell wall synthesis. Moreover, lysis as a mechanism for the secretion in the central zone seems unlikely, since the secreted proteins contained radioactive amino acids that had been available in the medium during only the last 4 h of the cultivation.

The phenomenon in which abundant secretion did not correlate with apparent growth in central zone 3 upon transfer to fresh medium came as a surprise since it is generally accepted that only growing hyphae secrete proteins (24). The steady-state growth theory and the bulk flow theory predict such a correlation (22, 23). These theories describe that proteins are not able to diffuse through the cell wall of nongrowing hyphae, due to the small pores in this highly cross-linked structure. In growing hyphae, proteins would comigrate with the plastic cell wall polymers that are extruded at the hyphal apex. Taking this into account, how can we explain a "growth-independent" secretion? Possibly, pores in the cell walls are locally increased by the actions of cell wall-modifying enzymes. However, it should be noted that the expression levels of genes encoding such enzymes were not increased in the central zone after transfer to fresh medium.

Protein synthesis and growth occurred at both the periphery and the center of the colony (this study) (24). In agreement, genes involved in carbon, sulfate, and phosphate utilization were found to be expressed in the different zones of the mycelium. Likewise, chitin and glucan synthase genes as well as chitinase genes were active throughout the mycelium. Some of these genes were differentially expressed at either the periphery or the center, suggesting that the cell wall composition varies between different parts of the colony. It should be noted that growth was hardly, if at all, detected in zones 2, 3, and 4. The spatial growth pattern did not correlate with expression of genes involved in cell wall synthesis. Apparently, posttranscriptional regulatory processes are involved. This conclusion is in agreement with a study with *Paracoccidioides brasilensis* (12), where it was shown that transcript levels of chitin synthase genes did not correlate with chitin content in the cell wall during temperature-induced dimorphic transition.

In summary, we have shown that colonies of *A. niger* are highly differentiated. Differences in zonal gene expression are due to medium-dependent and medium-independent mechanisms. Future studies will aim at unraveling the molecular bases of these mechanisms. Moreover, we will address whether differentiation also occurs in liquid cultures, which is relevant for industrial fermentations.

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