The secretome of the free-living mycelium from the ectomycorrhizal basidiomycete *Laccaria bicolor*

Delphine VINCENT[1], Julien GIBON[2], Anne-Marie LOMENECH[3]; Stéphane CLAVEROL[3], Marc BONNEU[3], Marc-Henri LEBRUN[4], Francis MARTIN[2] and Christophe PLOMION[1].

[1]INRA UMR1202 BIOGECO, Bordeaux, France ; [2]INRA-UHP UMR1136 Interactions Arbres/Micro-Organismes, Nancy, France ; [3]Plate-forme Protéomique, Université Bordeaux 2, France ; [4]CNRS Bayercropscience UMR 2847, Lyon, France.

ANR-06-BLAN-0399 FUNGEFFECTOR

Abstract

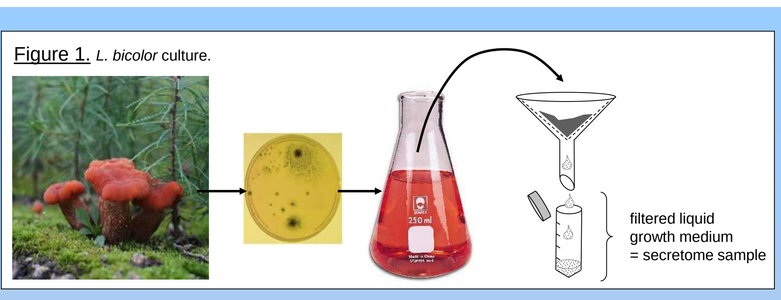
Fungal-plant interactions are widespread. For instance, most tree species develop symbiotic relationships with fungi to enhance nutrient absorption, whilst many crop diseases that result in massive yield losses are caused by fungi. Little is known about proteins, otherwise known as effectors, involved in these associations; secreted proteins (secretomes) are predicted to play a key role. We present the first secretome analysis of *Laccaria bicolor*, an ectomycorrhizal fungus symbiotically interacting with many tree species. Gel-based and gel-free proteomic approaches combined to mass spectrometry (MS) were exploited. The fungus was *in vitro* grown in liquid medium and secreted proteins were recovered from liquid medium filtrates. Lyophilized filtrates were processed using three proteomic techniques: two-dimensional electrophoresis (2-DE) followed by MS analyses, isoelectric focusing followed by MS analyses (IPG shotgun) and one-dimensional electrophoresis followed by MS analyses (1-DE shotgun). Many of the secreted proteins had unknown functions. Successful hits matched mainly to enzymes involved in cell wall modification or protein metabolism. This study paves the way for *in vivo* experiments and characterization of the fungal extracellular proteins involved in plant-fungus interaction.

Materials and Methods

Culture Conditions

Mycelium of *L. bicolor* strain S238N-H82 was grown under shaking in 1 L Erlenmeyer filled with 500 mL Pachlewski medium (Di Battista C. *et al.* Mycol Res 1996, 100:1315-24). After five weeks of growth at 25°C, the liquid medium containing the secreted proteins was filtered on paper and instantly frozen (Figure 1).

Figure 1. *L. bicolor* culture.



Sample processing

Liquid medium was further filtered (0.2 µm syringe filters), dialyzed (Spectra/Por 3 RC dialysis membranes, 3500 Da MWCO), lyophilized and resuspended in 0.5 mL of solution R (7M urea, 2M thiourea, 2% CHAPS, 1% DTT, 0.5% proteinase inhibitor mix, 0.5% pH 4-7/3-10 CAs). Protein content was assayed using 2-D Quant Kit (GE Healthcare).

Protein Separation

An outline of the 3 proteomic separation techniques used in this study is presented in Figure 2.

1/ IPG shotgun:

Secreted proteins (0.2 g) were trypsin-digested prior to IEF (18 cm pH 3.5-4.5 IPG DryStrip). The IPG strip was then cut into 40 fractions and peptides were eluted using C18 ZipTip columns prior to MS/MS analyses (Essader *et al*. Proteomics. 2005, 5:24-34).

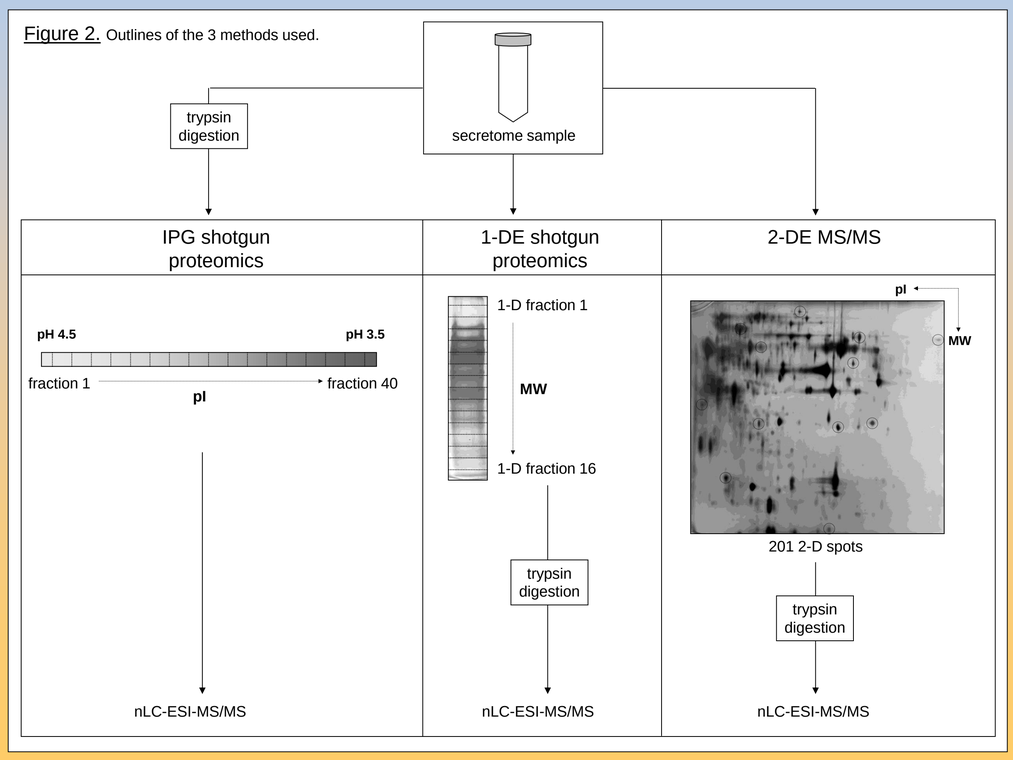
2/ 1-DE shotgun:

1-DE was performed by loading 0.2 g of secreted proteins. Each lane was cut into 16 fractions which were trypsin-digested prior to MS/MS analyses.

3/ 2-DE:

IEF was conducted using 24 cm IPG DryStrips of various pH ranges (3-11NL, 4-7 and 7-11NL) by loading 0.2 g of secreted proteins. The most abundant spots were manually excised from 2-D gels (134 spots from 3-11NL pattern, 46 spots from 4-7 patterns, and 21 spots from 7-11NL patterns) and trypsin-digested prior to MS/MS analyses.

Figure 2. Outlines of the 3 methods used.



Protein Identification

The peptide mixture was analyzed by on-line capillary HPLC coupled to a nanospray LCQ IT mass spectrometer (Thermo-Finnigan) for 2-D spots and nanospray LTQ IT mass spectrometer, for IPG strip and 1-D fractions.

The bioinformatics search was performed against protein sequences of *L. bicolor* publicly available at JGI (20616 entries, http://genome.jgi-psf.org/Lacbi1/Lacbi1.home.html. Martin *et al*. Nature 2008, 452:88-92). Data were searched by SEQUEST through Bioworks 3.3.1 interface.

Bioinformatics

Theoretical pIs and MWs were obtained by using online ExPASy compute pI/MW tool (http://www.expasy.org/tools/pi_tool.html).

SignalP algorithm (http://www.cbs.dtu.dk/services/SignalP/) was used online to predict targeted compartments.

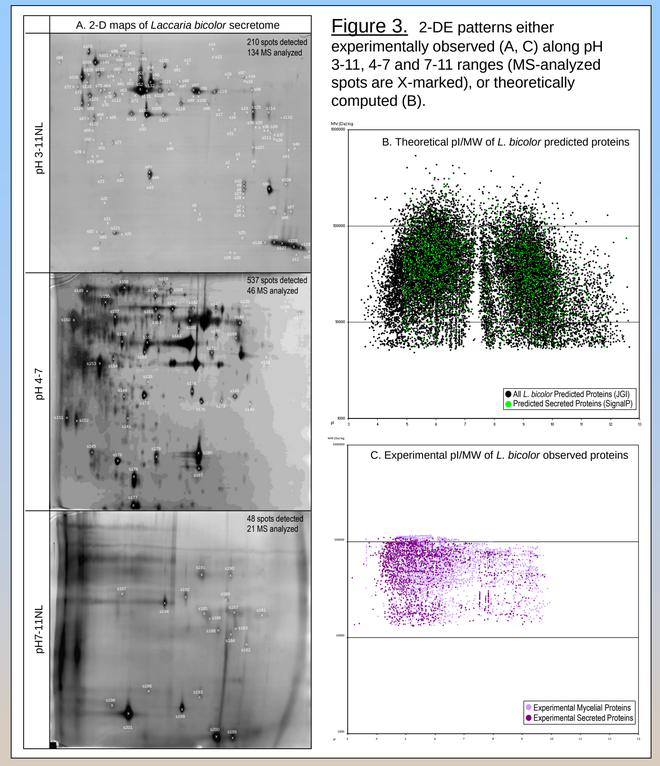
Protein sequences that could not be annotated by JGI were annotated using UniProtKB (http://www.uniprot.org/) or BLASTp algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) searching first SwissProt (SP, http://www.expasy.org) database, then the NCBI non redundant database (nr, http://blast.ncbi.nlm.nih.gov) when no annotation was retrieved using SP.

Carbohydrate-active enzymes or CAZYmes (http://www.cazy.org) were also searched.

Gene Ontology (GO, http://www.geneontology.org) terms were retrieved.

Results

Figure 3. 2-DE patterns either experimentally observed (A, C) along pH 3-11, 4-7 and 7-11 ranges (MS-analyzed spots are X-marked), or theoretically computed (B).



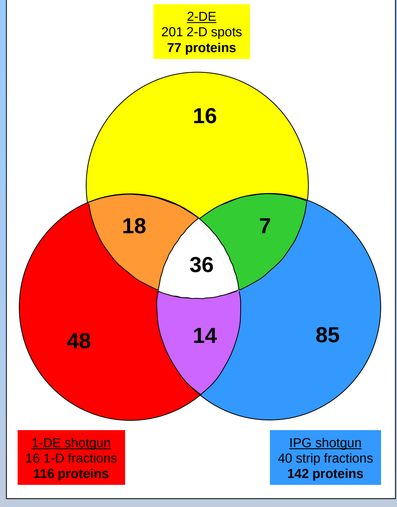
2-DE

210, 537 and 48 2-D spots were detected along 3-11NL, 4-7 and 7-11NL gradients, respectively (Figures 3A). Acidic proteins (pH 4-7, central panel) produced the best 2-D profile with the highest number of spots and satisfactory resolution, while alkaline patterns (pH 7-11NL, bottom panel) yielded very few poorly resolved spots.

Considering all the *L. bicolor* predicted proteins (20616 black dots in Figure 3B), we used SignalP algorithm to isolate the proteins predicted to be secreted and plotted them according to their theoretical pI/MW (3067 green dots in Figure 3B). It can be seen that as many acidic secreted proteins as alkaline secreted proteins were expected, which was highly inconsistent with 2-D patterns (brown dots on Figure 3C), displaying 11 times less basic proteins (537) than acidic ones (48).

MS analyses were successful for 161 spots (80%): 267 proteins identified in total, accounting for 77 unique accessions. 86 spots (43%) contained 1 protein, but up to 6 proteins could be identified in a single spot (i.e. s147). Accession jgi|Lacbi1|314722, a beta-N-acetylhexosaminidase, was identified in 47 distinct spots, as well as by shotgun proteomics.

Figure 4. Distribution of the 224 proteins identified by 2-DE, IPG or 1-D shotgun.



Comparison of the methods

Out of the 224 identified proteins, 36 were shared by all three methods, 7 proteins were common between IPG shotgun and 2-DE, 14 proteins were common between IPG shotgun and 1-DE shotgun, and 18 proteins were common between 1-DE shotgun and 2-DE (Figure 4).

Many proteins were identified using a given technique; 85 proteins were IPG shotgun-specific, 49 proteins were 1-DE shotgun-specific, and 16 proteins were 2-DE-specific.

IPG shotgun

524 proteins were identified in total, accounting for 142 unique accessions (Table 1). All 40 fractions yielded hits. On average, 13 proteins were identified per fraction, ranking from 1 (fractions 1, 38 and 40, corresponding to pH 4.50, 3.55 and 3.50, respectively) to 35 (fraction 2, pH 4.47). Peptides from the same proteins were found on average in 4 different fractions; 71 proteins were identified in single fractions and 2 proteins almost covered the whole length of the strip (accessions jgi|Lacbi1|292021, unknown protein, and jgi|Lacbi1|305896, 55 kDa immunogenic protein, were identified in 35 and 36 fractions, respectively).

1-DE shotgun

263 proteins were identified in total, accounting for 116 unique accessions (Table 1). All 16 fractions yielded hits. On average, 16 proteins were found per fraction, ranking from 4 (fraction 1, ~250kDa) to 52 (fraction16, ~10kDa). 70 proteins were identified in single fractions; 1 protein was found in 13 fractions (accessions jgi|Lacbi1|147000, a 88 kDa immunoreactive mannoprotein).

Table 1. Number of L. bicolor secreted proteins identified in each fraction of IPG strip or 1-D lane.

fraction	IPG shotgun	1-DE shotgun
1	1	4
2	35	8
3	32	10
4	27	17
5	27	22
6	25	19
7	18	22
8	4	13
9	10	21
10	5	17
11	7	11
12	7	11
13	8	9
14	10	13
15	15	14
16	18	52
17	23	
18	14	
19	16	
20	16	
21	17	
22	13	
23	16	
24	28	
25	32	
26	17	
27	11	
28	12	
29	6	
30	6	
31	5	
32	10	
33	9	
34	7	
35	4	
36	6	
37	3	
38	1	
39	2	
40	1	
total	524	263
min	1	4
max	35	52
mean	13	16
sd	9	11
unique	142	116

Prominent functional categories of L. bicolor *secretome*

A significant proportion of known proteins remained unclassified (Table 2) due to unknown process (22%), function (21%) or compartment (27%). Aside from unknown/unclassified proteins, *L. bicolor* secreted proteins were assigned to various categories: 16 biological processes (BP), 17 molecular functions (MF) and 9 cellular compartments (CC).

Some categories disappeared from one method to another and the proportions of the common categories varied. Overall, the most represented BP were “Protein modification, metabolism, transport and translation” (16%), “Glycolysis, tricarboxylic acid cycle, pentose-phosphate shunt and carbohydrate metabolism” (11%), “Cytoskeleton and cellular component organization and biogenesis” (8%), and “Cell wall organisation and biogenesis” (5%). The most frequent MF were “Protein binding” (18%) and “Hydrolase activities” (12%), notably due to the presence of many enzymes modifying cell wall components (14%, 31 CAZymes including 7 carbohydrate esterases, 22 glycoside hydrolases and 2 glycosyltransferases). GO predictions for CC were inaccurate because only 11% were predicted to be secreted (11 proteins in “Cell wall” and 14 proteins in “Extracellular” compartments).

Some of these technique-specific proteins participated to characteristic processes and bore unique functions. For instance, 1-DE shotgun allowed to identify proteins involved in “Cell wall organization and biogenesis” BP, “DNA binding” and “Phospholipase activity” MF, and “Cell wall” CC. Some 2-DE-specific proteins were specifically involved in the “Lipid metabolism” BP.

Table 2. Number of identified proteins per functional category according to Gene Ontology (GO) Biological Process (BP), Molecular Function (MF) or Cellular Component (CC) using 2-DE, 1-D shotgun, IPG shotgun or all three techniques (all). Bold numbers indicate categories specific to a technique. Underlined numbers indicate categories well represented by a particular technique. Italicized numbers indicate categories missing in a given technique.

	Functional classification	2-DE	1-D shotgun	IPG shotgun	all
	unknown protein	13	22	40	53
GO BP	unknown process	14	17	29	35
GO BP	protein modification, metabolism, transport and translation	8	22	20	35
GO BP	glycolysis, TCA cycle, pentose-P shunt and C metabolism	10	12	16	25
GO BP	cytoskeleton/cellular component organization and biogenesis	7	10	6	18
GO BP	cell wall organisation and biogenesis	7	**11**	4	11
GO BP	electron transport	5	6	4	9
GO BP	lipid metabolism	**6**	4	*0*	6
GO BP	response to stress	1	1	3	5
GO BP	signal transduction	*0*	2	3	4
GO BP	ion transport	1	*0*	3	4
GO BP	ATP biosynthesis	0	1	**3**	3
GO BP	cell adhesion	2	2	2	3
GO BP	endocytosis	1	1	**3**	3
GO BP	glycogen biosynthesis	*0*	1	2	3
GO BP	phosphate metabolism	2	2	1	3
GO BP	RNA processing, metabolism and transcription	0	1	2	3
GO BP	cell proliferation	0	**1**	**1**	1
GO MF	unknown function	12	15	25	31
GO MF	protein binding	11	23	24	40
GO MF	hydrolase activity	17	15	10	26
GO MF	oxidoreductase activity	6	5	5	10
GO MF	carbohydrate binding	4	7	3	8
GO MF	RNA binding	*0*	3	6	8
GO MF	transferase activity	3	3	**7**	7
GO MF	ATP binding	1	2	**7**	7
GO MF	dehydrogenase activity	*0*	5	2	6
GO MF	DNA binding	*0*	**5**	*0*	5
GO MF	electron carrier activity	3	4	3	5
GO MF	actin binding	1	1	2	4
GO MF	phospholipase activity	3	**4**	*0*	4
GO MF	ion binding	*0*	*0*	**3**	3
GO MF	antioxidant activity	1	*0*	1	2
GO MF	hydratase activity	1	1	**2**	2
GO MF	isomerase activity	*0*	0	**2**	2
GO MF	phosphatase activity	**1**	**1**	*0*	1
GO CC	unknown compartment	19	23	32	46
GO CC	cytoplasm	12	24	22	42
GO CC	intracellular	8	12	12	21
GO CC	nucleus	4	9	10	15
GO CC	extracellular	11	9	5	14
GO CC	cell wall	6	**11**	4	11
GO CC	membrane	4	3	7	10
GO CC	mitochodrion	*0*	2	5	7
GO CC	golgi apparatus	*0*	1	**3**	3
GO CC	endoplasmic reticulum	*0*	*0*	**2**	2

Conclusions

Multiple proteomic techniques were used to identify *Laccara bicolor* secreted proteins released in a liquid growth medium: IPG shotgun proteomics, 1-DE shotgun proteomics and 2-DE. IPG shotgun delivered the highest throughput, yet 1-DE shotgun and 2-DE allowed unravelling some unique proteins involved in specific functional categories. Such strategy greatly helped increasing the coverage of identified proteins with minimal overlap between techniques. Based on MS/MS analyses, *L. bicolor* secretome seems to primarily involve cell wall restructuring and protein metabolism.