

Proteomics as a Tool to Study Microbial Interactions

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Abstract: In microbial ecology, researchers have started to use a great variety of methods initially developed by molecular biologists. Mostly, these studies have dealt with microbial diversity in specific environments. Recently, new questions have been raised, e.g. what changes occur within a microbial community during competition or after a change in the surrounding environment? For this, molecular tools such as mRNA differential display, microarrays and proteomics can be employed. In this review, the use of proteomics for studies of microbial interactions is discussed. One aspect of competition between microbes can be simulated by treatment of one microbe with antibiotics produced by a competing microbe. A more complicated approach involves co-cultivation of the competitors. In order to reveal species-specific protein patterns, it is advisable to maintain the organisms separated. In a somewhat reversed experimental design, the target gene for an antibiotic is disrupted, and changes in the mutant proteome are subsequently screened for. Generally, a proteomic study will reveal proteins with both expected and surprising changes in abundance upon competition, but also previously unknown proteins are likely to be identified. It is obvious that most antibiotics can trigger secondary responses, which will result in a change of abundance of several proteins. However, an approach based on proteomics alone may not be sufficient to obtain a complete data set for describing microbial interactions. Therefore, further studies are necessary for proteins whose quantitative profile changes, e.g. by generating knockout strains for phenotypic analysis. Despite some inherent limitations, proteomics is a useful method, and an important complement to other approaches for studies of microbial interactions.

Key Words: Proteomics, proteome analysis, microbial interactions, microorganisms, fungi, yeasts, bacteria, antibiotics.

INTRODUCTION

In the ecosystems various microorganisms occupy the same habitat and coexist. Microbial interactions differ and can be, e.g. mutual, parasitic and competitive. These events can be studied at different levels, ranging from the whole ecosystem to the gene expression in a single organism. At the ecosystem level, the main concern is to describe variations in the surrounding environment and the content of species present. To study changes in gene expression and protein synthesis in single organisms, it is convenient to simplify the ecosystem. Thus, the microbe of interest can be co-cultured with an interacting organism in a defined and reproducible lab-scale condition. Upon encountering other microorganisms, the protein synthesis in a single microbe will change in order to adapt to the new environment, i.e., the proteome in the target organism will change. Alterations in the proteome are not unique to a competitive situation but may also apply to, e.g. mutualistic interactions. Changes in the complement of proteins present in an organism can be assessed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Thus by applying proteomic techniques, different protein patterns from a specific organism exposed to different environments can be compared. The main goal of such studies is to identify proteins whose abundance is altered when an organism encounters other microbes (or, in special cases, microbe-produced com-

pounds). With a focus on fungi, this review summarizes the proteomic work that has been ongoing in this field. Potential problems and benefits of proteomics approaches in studies of microbial interactions will also be discussed.

WHY STUDY MICROBIAL INTERACTIONS?

Effects and Targets for Antibiotics

Most studies concerning the competition between different microbes have been aimed at elucidating the synthesis of antibiotic secondary metabolites. The predominant hypothesis is that these secondary metabolites are synthesised to give the producing organism a competitive advantage by killing or inhibiting growth of other microbes (Maplestone *et al.*, 1992). According to that proposal, the biosynthetic genes for a specific antibiotic are usually located in the same gene cluster as the corresponding resistance genes, thus relating synthesis of the antibiotic to competitive advantage (Stone and Williams, 1992). Alternative hypotheses have been proposed, e.g. the reduction of abnormally high concentrations of intermediate metabolites during growth arrest. One argument states that the concentrations of secondary metabolites in the field are not high enough to stop growth of other microbes (Gottlieb, 1976). Almost all antibiotics used today are of microbial origin. In medicine, an increasing problem with pathogenic microbes becoming increasingly resistant against the most commonly used antibiotics is being experienced (Cowen, 2001; Lipsitch, 2001). It is important to understand the mechanistic effects of an antibiotic and the pathways and probabilities for development of resistance.

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With this knowledge, it might be possible to employ combinatorial chemistry and structural biology to develop derivatives of the antibiotic that circumvent or even prevent rapid mutation to resistance of the pathogen (Hughes, 2003). Certainly, with an increased effort to study different microbes, new antibiotics will be discovered. Most published proteomics studies dealing with microbial interactions aim at an understanding of the responses of certain microbes towards a specific antibiotic. Although ecological questions were not the primary objective, interesting general data concerning microbial interactions can be obtained from these studies. Likewise, more general studies of microbial stress responses may be of great interest for investigations aimed at specific drug responses.

Secondary Effects when Using Microbes for Preservation and Biocontrol

Fungal infection of crops intended for food and feed is a serious agricultural problem. Much effort is going on to replace or decrease the use of fungicides by fungal antagonistic microbes, e.g. *Pseudomonas* species (Gerhardson, 2002). When food and feed are stored, some microbes such as lactic acid bacteria (Lindgren and Dobrogosz, 1990), and the yeasts *Candida sake* (Vinas *et al.*, 1998) and *Pichia anomala* (Druevofors *et al.*, 2002; Jijakli and Lepoivre, 1998) can be used to protect the food from toxic fungi such as *Penicillium* and *Botrytis*. Here it is essential not only that fungal growth is decreased, but also to know if the production/accumulation of toxic compounds produced is decreased. It is noteworthy that fungal-antagonistic microbes, which diminish fungal spoilage of food and feed may also function against human pathogenic fungi or *vice versa*.

Microbial Interactions in Fundamental Ecology

In times with rising threats and an increased concern about the environment, it is important to approach an understanding of how organisms interact. Although microbes are small in size, they are present in abundance, are ubiquitous, and play decisive roles in all aspects of ecology. Fungi together with algae or cyanobacteria can live in mutual dependence and form a symbiotic organism, the lichen. By studying lichens, one can address co-evolution and survival under extreme conditions. Another interesting aspect of lichens is that their symbiosis can include both mutualism and predation (Honegger, 1991). Fungi and plants can form mycorrhiza; the fungus increases the effective root surface of the plants and facilitates uptake of nutrients. In return, the plant provides the fungus with carbohydrates. It is known that bacteria are involved in this symbiosis as well (Garbaye, 1994). Since formation of mycorrhiza is crucial for normal growth of many plants, knowledge of the nature of this symbiosis, including all the organisms involved, is of great interest.

MOLECULAR BIOLOGY IN ECOLOGICAL RESEARCH

Microbial Diversity

Today, the most widely used molecular method in ecology research is the polymerase chain reaction (PCR). With the possibility to amplify DNA rapidly from microbes

of a microbial community under study, it is now often possible to identify the complement of species present, even in cases where the majority cannot be cultured. The same method can also be used to screen for genes encoding specific enzymatic activities of biochemical potential. In combination with PCR, various fingerprinting methods can be used to obtain information about the species composition. The diversity within microbial communities and methods used to describe it has been reviewed recently (Kent and Triplett, 2002; Torsvik and Ovreas, 2002).

Quantitative Molecular Studies

Even if a species, or a gene, is present in a community it is not necessarily biologically active. By investigating gene expression it is possible to monitor active (living) organisms and genes dynamically. These approaches can be carried out on natural communities from field studies, or on single species in the laboratory. Changes in an expression of a single gene as the relative abundance of mRNA transcripts can be determined using fluorescent *in situ* hybridisation (FISH), Northern blots or by different variants of reverse transcription PCR (Freeman *et al.*, 1999). With such a method, e.g. mRNA differential display (RNA-DD; Liang and Pardee, 1992) or microarrays (Brown and Botstein, 1999; Duggan *et al.*, 1999) it is possible to detect total gene expression changes in a single microbe. Thus, it is possible to identify genes involved in a certain response, e.g. a microbial competition. For these experiments it is important to choose a suitable model system for the competing organisms. The presence of specific mRNA transcripts can also be visualised in the field using FISH (Moter and Gobel, 2000). The model systems used to prepare mRNA for an expression study are in all aspects equivalent to the models used in proteomics. Both approaches have their advantages and limitations. One important difference is the stability; while mRNAs are relatively short-lived molecules, proteins, in general, tend to be more stable. Therefore, short-term changes in the expression / synthesis may perhaps be most conveniently studied at the mRNA level. On the other hand, since regulation often occurs also at post-transcriptional levels, mRNA levels may be misleading in some instances, and a determination of the final gene product, the protein, may be more instructive for general metabolic potential.

EXPERIMENTAL DESIGN

The most important concern when using proteomics to study microbial interactions is the choice of a system that faithfully mimics the situation of interest. For example, if one wants to elucidate effects on the proteome profile when a microbe is subjected to one specific hostile antibiotic, then the target organism can be cultivated in the presence and absence of the antibiotic. Subsequently, the proteome profiles obtained under two different conditions can be compared. It is important to distinguish between short-term and long-term effects caused by the antibiotic. As mentioned above, studies of immediate responses in the microbe may be provided best by determination of mRNA levels, since, in general, the proteome at that point of time has been synthesised prior to the treatment. It is possible to circumvent this problem by adding radioactively labelled amino acids to the culture at the same time as the antibiotic. With this approach,

only proteins that were synthesised after addition of the antibiotic will be visualised using autoradiography. If working with a fast growing microbe (even after treatment with the antibiotic), this step may not be necessary since only a minor fraction of the proteins were synthesised prior to the treatment. It is not always possible to use a specific antibiotic instead of growing two microorganisms together, and therefore the competing microbes may have to be co-cultured. Also, in a competition usually more than one antibiotic compound is produced. In addition, there are substances produced that are less toxic but still have antagonistic effects, e.g. several bacteriocins produced by lactic acid bacteria (LAB) (reviewed in Riley and Wertz, 2002). These substances may not be soluble at concentrations high enough to cause a severe effect at the protein level in the target organism. However, it has been shown that an organism can change the expression of several genes after encountering only subinhibitory concentrations of several different antibiotics (Goh *et al.*, 2002). One useful alternative approach, if the specific target for an antibiotic is known, is to disrupt the gene encoding the target product. If the observed phenotype is identical to the morphology caused by the antibiotic, then proteomics can be applied on the mutant and the profile compared with that of the wild-type strain. By combining several different approaches on the same system, it is possible to discriminate responses to a specific antibiotic from the more complicated scenario in co-cultures, or more so in complex small ecosystems. In the latter case not only changes in protein abundance due to the antibiotic, but also responses to the nutritional competition will be measured. An alternative is to compare the protein pattern from different strains of the same bacteria, e.g. to find proteins that are unique or absent in strains that are resistant to a specific antibiotic. This approach is widely used to study the protein patterns in bacteria, e.g. in *Lactobacillus sanfranciscensis* (De Angelis *et al.*, 2001), *Staphylococcus aureus* (Cordwell *et al.*, 2002) and *Streptococcus pneumoniae* (Cash *et al.*, 1999). However, the disadvantage of comparing different strains is that strain characteristics not linked to the resistance will be monitored as well.

EXPERIMENTAL PROCEDURES AND PROBLEMS

Preparation and Separation of the Protein Extract

The main limitation of proteomics using 2D-PAGE is that, on each gel, only a fraction of the proteins will be displayed, i.e. the prominent and successfully extracted proteins, within the experimental parameters used. However, more proteins could be made visible/detectable if the parameters are slightly altered. Thus, it is always possible to change the pI intervals in the first dimension and the gel concentration in the second. The method for protein extraction can also be adjusted. One way to improve resolution is to start by separating a specific organelle and then separating its protein components by 2D-PAGE. Accordingly, both cell wall-(Pardo *et al.*, 2000) and plasma membrane-(Navarre *et al.*, 2002) proteins from *S. cerevisiae* have been successfully analyzed on 2D-PAGE. If the number of different proteins is reduced in a preparation, even proteins present in minor quantities can be displayed on the gel by increasing the amount of loaded proteins. The sensitivity of protein detection can also be improved by

testing different staining methods. In my experience, working with parallel silver-stained gels and radiolabelled proteins, the latter provided the best resolution and the highest reproducibility. To conclude, the field of proteomics is expanding rapidly, and technical improvements will further facilitate extraction, separation and visualisation of proteins (Harry *et al.*, 2000). It is possible that in the future all proteins in the proteome can be analysed using 2D-PAGE. However, the total number of gels needed to cover the entire proteome might be an obstacle.

How to Interpret the Results?

Naturally, the use of proteomics alone does not provide comprehensive information about how microbes interact in ecosystems. It is convenient to work with a model organism, preferentially one for which the entire genomic sequence is available. Otherwise, it is difficult and time-consuming to identify the protein and to deduce its complete amino acid sequence. Without a sequenced genome, or a large number of known expressed sequence tags (EST) from a specific microbe (or from a close relative), it may not be recommended to perform proteomics on that organism.

Proteomic studies usually identify a number of previously described proteins that have their abundance changed under one specific condition but often no logical explanations can be proposed as to why these proteins should be involved in the actual response. Obviously, the mechanisms that lead to synthesis of specific proteins are complicated events and it is practically impossible to predict secondary effects that alter the level of synthesis. Additional experiments may provide some answers. To learn more about an unknown protein, perhaps the most straightforward approach is to disrupt the encoding gene and study the phenotypical consequences. Therefore, proteomics is best performed with model organisms with well-developed molecular techniques, including a functional transformation system. Figure (1) summarises an experimental outline from the ecosystem of interest to the final analysis of the proteins involved in the interaction.

PROTEOMIC STUDIES IN MICROBIAL INTERACTIONS

Responses in Filamentous Fungi

In our laboratory, we are screening for yeasts and bacteria with a potential use in biocontrol against filamentous fungi. In one study, it was found *Streptomyces halstedii* strain that produces the macrolide antibiotics bafilomycin B1 and bafilomycin C1 (Frändberg and Schnürer, 1998; Frändberg *et al.*, 2000). The bafilomycins and the related concanamycins are known inhibitors of vacuolar ATPases (Bowman *et al.*, 1988; Dröse and Altendorf, 1997). These substances inhibit growth, and in filamentous fungi the morphology is severely altered. Slow growth and hyperbranching hyphae result in an extremely reduced radial growth. It has been shown in *Neurospora crassa* (Bowman *et al.*, 2000) and *Aspergillus nidulans* (Melin, Schnürer and Wagner, manuscript submitted) that the V-ATPase is the main target for the antibiotics. The function of the V-ATPases is to acidify various organelles, e.g. lysosomes in animals and vacuoles in fungi and plants (Forgac, 1989).

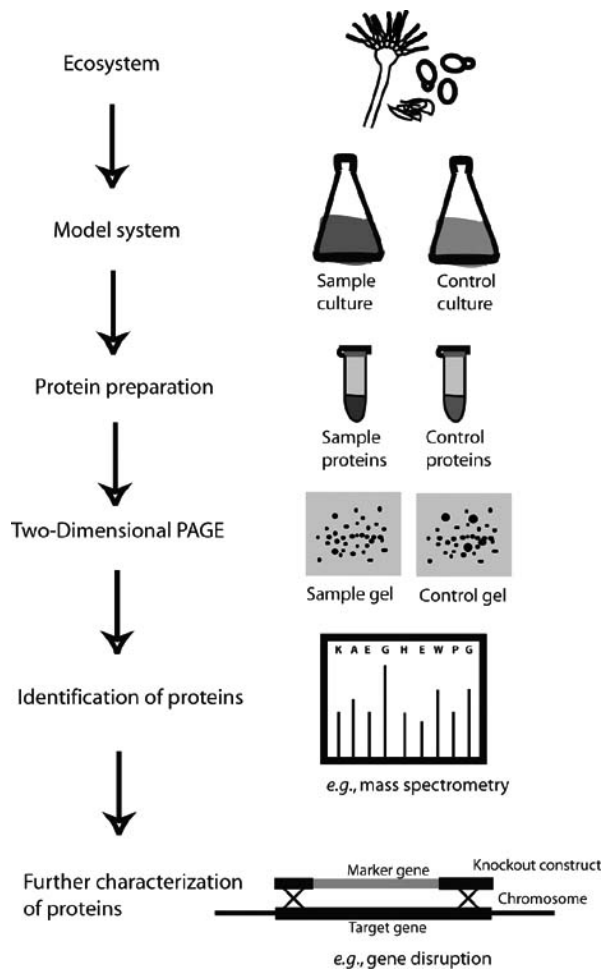


Fig. (1). A schematic illustration of the experimental procedure required when using proteomics study of microbial interactions.

We wanted to elucidate molecular responses in the fungus after encountering these V-ATPase-inhibiting antibiotics. For these studies, we decided to work with the filamentous fungus *A. nidulans*, which beside *N. crassa*, is the most studied model organism among the filamentous fungi. In the first study, we used RNA-DD to study responses in *A. nidulans* treated with bafilomycin B1 compared to non-treated fungus (Melin *et al.*, 1999). To compare an mRNA-based with a protein-based approach, we set up a similar system for proteomics using a second antibiotic, concanamycin A, known to work through the same mechanism of action. The experimental approach was as follows: Fungal conidia were inoculated in a rich medium for a total of 48 hours. After 24 hours, concanamycin A and ^{14}C -labelled amino acids were added to the media. Since concanamycin has a very dramatic effect on fungal growth, we could not add the compound directly to non-germinated conidia; the extracted protein was insufficient for 2D-PAGE analysis. Proteins were separated in the first dimension with a pI range from 3 to 10. For separation in the second dimension we used 10 and 15 % (w/v, acrylamide) PAGE.

We detected 20 proteins with changed abundances after treatment with the antibiotic (Melin *et al.*, 2002). Five of these proteins were identified using Mass Spectrometry (MS). Two down-regulated proteins had previously been described in *A. nidulans*: CpcB, a G-like protein involved in global amino acid control and initiation of sexual development (Hoffmann *et al.*, 2000); GpdA, glyceraldehyde-3-phosphate dehydrogenase (Punt *et al.*, 1988). There are no obvious explanations why the abundances of these two proteins should be decreased due to treatment with concanamycin. The reduced abundance of GpdA may be due to the overall reduced growth caused by concanamycin. The three up-regulated proteins were previously not described in *A. nidulans*. The proteins were named CipA to CipC (concanamycin induced protein); CipA is homologous to a *Candida* protein whose gene expression is strongly up-regulated by cadmium (Hong *et al.*, 1998); CipB, is homologous to an *Aspergillus terreus* protein involved in the synthesis of lovastatin (Kennedy *et al.*, 1999), which is a secondary metabolite working as a cholesterol-decreasing agent (Alberts *et al.*, 1980); CipC a previously totally unknown protein. The up-regulation of CipA might be explained if there is a connection between cadmium-induced stress and the concanamycin-induced stress. This also holds for CipB if the synthesis of lovastatin is stress-induced. A few conclusions can be drawn from this study: The abundance of several proteins is altered in *A. nidulans* upon treatment with concanamycin A. Although the growth rate is reduced and the morphology is severely changed, only a minor fraction of the most prominent proteins have their abundance changed. This may be explained by the fact that the growth and morphology of filamentous fungi can be altered drastically if a carbon source is changed, if treated with specific substances, or if point mutations are induced (Harris *et al.*, 1999; Trinci, 1984).

Summary of the comparison between proteomics and RNA-DD: In the mRNA-based approach we found and identified five genes with at least ten-fold change in expression when the fungus was treated with bafilomycin B1 (Melin *et al.*, 1999). Using 2D-PAGE we found 20 proteins with changed abundance of at least two-fold (Melin *et al.*, 2002). The differences in numbers and magnitudes of affected genes or proteins reflect the sensitivities but also the limitations of each of the approaches. In RNA-DD, a majority of the detected DNA fragments turned out not to be differentially expressed when follow-up control experiments were conducted, and were discarded as false positives. In 2D-PAGE analyses, it is relatively straightforward to repeat experiments with high reproducibility. Therefore, it is possible to detect relatively small but significant changes in protein abundance. However, false positives are also encountered when using 2D-PAGE, but, in our experience, they occur less frequently than in mRNA differential display.

The *Streptomyces*-produced bafilomycins and concanamycins are, due to their high specificity, suitable model-antibiotics for studies of fungal-bacterial interactions. However, these substances are also very toxic to all eukaryotes, so the antibiotic-producing *Streptomyces* strain can be used neither therapeutically nor for biocontrol purposes. In a screen for antifungal LAB at our laboratory, a *Lactobacillus plantarum* strain was identified that produces

antifungal dipeptides (Ström *et al.*, 2002). This bacterium also produces lactic acid that reduces the pH in the surrounding medium. The antifungal effect is due to contributions of both the dipeptides and reduced pH. We have set up an experimental system in which we co-culture *A. nidulans* with *L. plantarum* followed by extraction of fungal proteins. In the experiments, the two organisms are separated by a filter, which is permeable for the dipeptides (Ström, Melin and Schnürer, manuscript in preparation).

Yeasts

The most studied of all eukaryotic microorganisms is the common yeast *Saccharomyces cerevisiae*. With all protocols and tools in molecular biology and genetics, and the availability of the complete genomic sequence (Goffeau *et al.*, 1996), *S. cerevisiae* is a convenient model organism. When studying microbial interactions and aiming to reveal the target of a specific drug, which affects most eukaryotic organisms, *S. cerevisiae* would be a likely choice. However, this organism is quite unique and data obtained with *S. cerevisiae* cannot unambiguously be transferred to other fungi. Moreover, results obtained in lab strains of *S. cerevisiae* are not necessarily relevant for wild-type *S. cerevisiae* strains used in winemaking (Perez-Ortin *et al.*, 2002). Among the large numbers of papers dealing with the *S. cerevisiae* proteome only a few fall within the scope of this review, e.g. changes in protein spot intensities after *S. cerevisiae* was treated with the immunosuppressive drug mycophenolic acid (Escobar-Henriques *et al.*, 2001), and the modified protein pattern in pleiotropic drug-resistant mutants (Nawrocki *et al.*, 2001).

One of the major fungal opportunistic pathogens is the dimorphic fungus *Candida albicans*. Normally, *C. albicans* grows as yeast, but upon invading tissue the fungus forms filaments. One of the most widely used antibiotics against candidiasis is amphotericin B. This drug is nephrotoxic for humans limiting its use (Finkelievich *et al.*, 2000). Other frequently used drugs are imidazole and triazole drugs, but *C. albicans* usually develops resistance to these antibiotics (Georgopapadakou, 1998). There is much effort to find new and better antibiotics to stop *C. albicans* infections. Proteomics has been used in several studies to learn more about the physiology and responses to antibiotics in *C. albicans*. The prospects of using proteomics in order to study *C. albicans* was proposed and reviewed (Niimi *et al.*, 1999). Two examples of proteomic studies of ecological impact will be described below. However, the scope of these researches was to find means to prevent *Candida* infections.

In the first of these studies, (De Backer *et al.*, 2000) disrupted one allele of *cgt1*, the gene encoding 5'guanylyl-transferase (mRNA capping enzyme) in *C. albicans*. The mutant colonies exhibited an aberrant morphology (wrinkled colonies instead of normally smooth ones). The mutant strain was significantly more resistant to hygromycin and to heat-stress, though its virulence was similar to that of the wild-type *C. albicans*. A subsequent proteomic study resulted in some interesting observations, when overall protein patterns in the mutant and the wild type were compared. Three up-regulated proteins were excised from the gel and identified by MS: A translation elongation factor, Efl- p (Sundstrom

et al., 1990), a heat-shock protein, Ssa2p (LopezRibot *et al.*, 1996), and a ribosomal protein, Rps5p (Ignatovich *et al.*, 1995). The increased abundance of Efl- p might explain the increased resistance to hygromycin since this antibiotic inhibits translational elongation. The observation that an increase of a heat shock protein results in heat-stress tolerance is similarly expected. No obvious conclusion can be drawn from the increased abundance of the Rps5p protein.

In the second study of *C. albicans*, changes in the protein pattern were observed in the fungus after treatment with some antifungal agents (Bruneau *et al.*, 2003). *C. albicans* was treated with the following antibiotics: Mulundocandin, a derivative of mulundocandin, fluconazole and itraconazole. The mulundocandins are inhibitors of (1,3) glucan synthase but have also other activities (Hawser *et al.*, 1999; Roy *et al.*, 1987). The triazoles, fluconazole and itraconazole inhibit the synthesis of ergosterol, a sterol unique to fungi. These substances are widely used in antifungal treatment (Georgopapadakou, 1998). Hence, it is important to construct a proteome map that can be used to study responses to several known and unknown antifungal compounds, and in particular to mulundocandin. A substantial amount of specific results are available from this study. The authors found that the expression of 46 proteins that were induced while that of 22 proteins were repressed by mulundocandin. In addition, they showed that 69 % of the observed changes were similar for both mulundocandins. For the two azoles this number was 62 %. This indicates that the responses to related antibiotics are similar in *C. albicans*. Also the fact that the abundances of approximately one third of the proteins behaved in a different manner is interesting and indicates that a minor change in the antibiotic structure can change the proteome in the target organism.

Bacterial Studies

Among the proteomics publications on bacteria, several deal with pathogenicity and physiology. Like the fungus *C. albicans*, pathogenic bacteria can cause lethal infections in humans, and it is essential to learn more about these pathogens. Thus, the proteomics work in the gram-positive bacterium *S. aureus*, which frequently develops antibiotic resistance, has recently been reviewed and discussed in (Hecker *et al.*, 2003). Throughout this review the term microbial interaction have referred to an interaction of at least two kinds of microbes. There are, among the bacteria, also single species interactions, e.g. quorum sensing, (reviewed in Miller and Bassler, 2001) and one such proteomics study has been published (Riedel *et al.*, 2003). Two examples of proteomics studies, which address an understanding of changes in protein synthesis when the bacterium is treated with, or is producing antibiotics, will be described below.

It is commonly hypothesised that the synthesis of certain proteins is induced as a response to treatment with cell wall-active antibiotics, such as the β -lactams. To support this hypothesis Singh *et al.* (2001) performed proteomic studies on *S. aureus* and identified at least nine proteins that became prominent after 2.5 hours of oxacillin (a β -lactam antibiotic) stress. Of these nine proteins, five were further characterised using MS. One of these five proteins was identified as a

putative methionine sulfoxide reductase, MsrA, an enzyme that catalyses the reduction of methionine sulphoxides in proteins to methionine (Brot *et al.*, 1981). When using other cell wall-active antibiotics, the same proteins were induced. This also holds true, at the mRNA level, for MsrA. Other antibiotics, e.g. those targeting protein synthesis, did not affect the abundance of the nine proteins, or the expression of the MsrA-encoding gene. This indicates that there are general responses in the bacterium to different antibiotics (but with related targets). This is in contrast to the more variable responses obtained in the eukaryote *C. albicans* (Bruneau *et al.*, 2003).

Most of the publications reviewed here deal with the responses to certain antibiotics. In order to study the mechanisms behind the competition, it is also relevant to study the changes in the proteomes of antibiotic-producing organisms while synthesising these compounds. This has been achieved in *Streptomyces lividans* Lai *et al.*, (2002). Considering that most antibiotics known today are produced by various species within the genus *Streptomyces* (Demain, 1999), such an approach may be of great importance to learn more about general aspects of antibiotic synthesis. In the study by Lai *et al.* (2002) the protein pattern of wild-type *S. lividans* was compared to that of a strain that carried mutations introduced into the *rpoB* gene, encoding the β subunit of RNA polymerase. These mutants are resistant to rifampicin and antibiotic production is activated. The proteomics study of this work identified the two enzymes glutamine synthase and oxidoreductase as only being present in the mutant, and only during the late growth phase.

FINAL COMMENTS AND THE FUTURE

Proteomics is a comparatively new approach in functional biology. It has been proven useful when elucidating molecular responses in microorganisms. When studying microbial interactions, proteomics can also be used but some inherent limitations are noticed. One fundamental problem with this technique is the choice of a system that faithfully mimics the interaction of choice. Another aspect is that, during competition, the microbe may not change its protein production significantly (or detectably). The molecular response to an antibiotic may be extreme during laboratory conditions but, in the field, the concentrations of antibiotic secondary metabolites may not be high enough to cause the same changes in protein synthesis. Nevertheless, knowledge of the microbial responses to antibiotics is very important in medicine, and proteomics is a convenient method to investigate the general responses in the target microbe. In the near future, proteomics will develop into a standard method for analysing mutant phenotypes and identifying targets for new antibiotics. In biotechnology, mutant strains will be used in biocontrol, bioremediation etc. To predict the potential risks, it is important to analyze these mutants, e.g. by proteomics. The impact of a proteomics study should, in many cases, certainly be improved by the use of complementary approaches such as RNA-DD, microarray, and metabolomics (one of the latest "omics"), e.g. in *S. cerevisiae* it has been reported that the entire metabolome consists of approximately 600 metabolites (Oliver *et al.*, 1998). With such a combined approach it should be possible to link proteins to their metabolites, i.e. the enzymes

involved in the synthesis of specific metabolic compounds, e.g. an antibiotic can be revealed.

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ABBREVIATIONS

2D-PAGE	= Two-dimensional polyacrylamide gel electrophoresis
RNA-DD	= mRNA differential display
PCR	= Polymerase chain reaction
LAB	= Lactic acid bacteria
FISH	= Fluorescent <i>in situ</i> hybridisation
MS	= Mass Spectrometry

REFERENCES

- Alberts, A.W., Chen, J., Kuron, G., Hunt, V., Huff, J., Hoffman, C., Rothrock, J., Lopez, M. *et al.* (1980) Mevinolin: A highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent. *Proc. Natl. Acad. Sci. USA*. **77**: 3957-3961.
- Bowman, E.J., Kendle, R. and Bowman, B.J. (2000) Disruption of *vma-1*, the gene encoding the catalytic subunit of the vacuolar H⁺-ATPase, causes severe morphological changes in *Neurospora crassa*. *J. Biol. Chem.* **275**: 167-176.
- Bowman, E.J., Siebers, A. and Altendorf, K. (1988) Bafilomycins: A class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. *Proc. Natl. Acad. Sci. USA*. **85**: 7972-7976.
- Brot, N., Weissbach, L., Werth, J. and Weissbach, H. (1981) Enzymatic reduction of protein-bound methionine sulfoxide. *Proc. Natl. Acad. Sci. USA*. **78**: 2155-2158.
- Brown, P.O. and Botstein, D. (1999) Exploring the new world of the genome with DNA microarrays. *Nature Genet.* **21**: 33-37.
- Bruneau, J.M., Maillet, I., Tagat, E., Legrand, R., Supatto, F., Fudali, C., Le Caer, J.P., Labas, V., Lecaque, D. and Hodgson, J. (2003) Drug induced proteome changes in *Candida albicans*: Comparison of the effect of beta(1,3) glucan synthase inhibitors and two triazoles, fluconazole and itraconazole. *Proteomics* **3**: 325-336.
- Cash, P., Argo, E., Ford, L., Lawrie, L. and McKenzie, H. (1999) A proteomic analysis of erythromycin resistance in *Streptococcus pneumoniae*. *Electrophoresis* **20**: 2259-2268.
- Cordwell, S.J., Larsen, M.R., Cole, R.T. and Walsh, B.J. (2002) Comparative proteomics of *Staphylococcus aureus* and the response of methicillin-resistant and methicillin-sensitive strains to Triton X-100. *Microbiology* **148**: 2765-2781.
- Cowen, L.E. (2001) Predicting the emergence of resistance to antifungal drugs. *FEMS Microbiol. Lett.* **204**: 1-7.
- De Angelis, M., Bini, L., Pallini, V., Cocconcini, P.S. and Gobetti, M. (2001) The acid-stress response in *Lactobacillus sanfranciscensis* CB1. *Microbiology* **147**: 1863-1873.
- De Backer, M.D., de Hoogt, R.A., Froyen, G., Odds, F.C., Simons, F., Contreras, R. and Luyten, W. (2000) Single allele knock-out of *Candida albicans* CGT1 leads to unexpected resistance to hygromycin B and elevated temperature. *Microbiology* **146**: 353-365.
- Demain, A.L. (1999) Pharmaceutically active secondary metabolites of microorganisms. *Appl Microbiol. Biotech.* **52**: 455-463.
- Dröse, S. and Altendorf, K. (1997) Bafilomycins and concanamycins as inhibitors of V-ATPases and P-ATPases. *J. Exp. Biol.* **200**: 1-8.
- Druevfors, U., Jonsson, N., Boysen, M.E. and Schnürer, J. (2002) Efficacy of the biocontrol yeast *Pichia anomala* during long-term storage of

- moist feed grain under different oxygen and carbon dioxide regimens. *FEMS. Yeast Res.* **2**: 389-394.
- Duggan, D.J., Bittner, M., Chen, Y.D., Meltzer, P. and Trent, J.M. (1999) Expression profiling using cDNA microarrays. *Nature Genet.* **21**: 10-14.
- Escobar-Henriques, M., Balguerie, A., Monribot, C., Boucherie, H. and Daignan-Fornier, B. (2001) Proteome analysis and morphological studies reveal multiple effects of the immunosuppressive drug mycophenolic acid specifically resulting from guanylic nucleotide depletion. *J. Biol. Chem.* **276**: 46237-46242.
- Finquelievich, J.L., Odds, F.C., Queiroz-Telles, F. and Wheat, L.J. (2000) New advances in antifungal treatment. *Med. Mycol.* **38**: 317-322.
- Forgac, M. (1989) Structure and Function of Vacuolar Class of Atp-Driven Proton Pumps. *Physiol. Rev.* **69**: 765-796.
- Frändberg, E. and Schnürer, J. (1998) Antifungal activity of chitinolytic bacteria isolated from airtight stored cereal grain. *Can. J. Microbiol.* **44**: 121-127.
- Frändberg, E., Petersson, C., Lundgren, L.N. and Schnürer, J. (2000) *Streptomyces halstedii* K122 produces the antifungal compounds bafilomycin B1 and C1. *Can. J. Microbiol.* **46**: 753-758.
- Freeman, W.M., Walker, S.J. and Vrana, K.E. (1999) Quantitative RT-PCR: Pitfalls and potential. *Biotechniques*, **26**: 112-125.
- Garbaye, J. (1994) Helper Bacteria - a New Dimension to the Mycorrhizal Symbiosis. *New Phytologist*. **128**: 197-210.
- Georgopapadakou, N.H. (1998) Antifungals: mechanism of action and resistance, established and novel drugs. *Curr. Opin. Microbiol.* **1**: 547-557.
- Gerhardson, B. (2002) Biological substitutes for pesticides. *Trends Biotech.* **20**: 338-343.
- Goffeau, A., Barrell, B.G., Bussey, H., Davis, R.W., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, J.D. et al. (1996) Life with 6000 genes. *Science* **274**: 546-567.
- Goh, E.B., Yim, G., Tsui, W., McClure, J., Surette, M.G. and Davies, J. (2002) Transcriptional modulation of bacterial gene expression by subinhibitory concentrations of antibiotics. *Proc. Natl. Acad. Sci. USA* **99**: 17025-17030.
- Gottlieb, D. (1976) The production and role of antibiotics in soil. *J. Antibiot.* **29**: 987-1000.
- Harris, S.D., Hofmann, A.F., Tedford, H.W. and Lee, M.P. (1999) Identification and characterization of genes required for hyphal morphogenesis in the filamentous fungus *Aspergillus nidulans*. *Genetics* **151**: 1015-1025.
- Harry, J.L., Wilkins, M.R., Herbert, B.R., Packer, N.H., Gooley, A.A. and Williams, K.L. (2000) Proteomics: Capacity versus utility. *Electrophoresis* **21**: 1071-1081.
- Hawser, S., Borgonovi, M., Markus, A. and Isert, D. (1999) Mulundocandin, an echinocandin-like lipopeptide antifungal agent: Biological activities *in vitro*. *J. Antibiot.* **52**: 305-310.
- Hecker, M., Engelmann, S. and Cordwell, S.J. (2003) Proteomics of *Staphylococcus aureus* current state and future challenges. *J. Chromat. B. Anal. Tech. Biom. Life. Sci.* **787**: 179-195.
- Hoffmann, B., Wanke, C., LaPaglia, S.K. and Braus, G.H. (2000) *c-jun* and RACK1 homologues regulate a control point for sexual development in *Aspergillus nidulans*. *Mol. Microbiol.* **37**: 28-41.
- Honegger, R. (1991) Functional aspects of the lichen symbiosis. *Ann. Rev. Plant Phys. Plant Mol. Biol.* **42**: 553-578.
- Hong, Y.M., Park, S.W. and Choi, S.Y. (1998) Expression of the CIP1 gene induced under cadmium stress in *Candida sp.* *Mol. Cells* **8**: 84-89.
- Hughes, D. (2003) Exploiting genomics, genetics and chemistry to combat antibiotic resistance. *Nature Rev. Genet.* **4**: 432-441.
- Ignatovich, O., Cooper, M., Kulesza, H.M. and Beggs, J.D. (1995) Cloning and Characterization of the Gene Encoding the Ribosomal-Protein S5 (Also Known as Rp14, S2, Ys8) of *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **23**: 4616-4619.
- Jijakli, M.H. and Lepoivre, P. (1998) Characterization of an exo-beta-1,3-glucanase produced by *Pichia anomala* strain K, antagonist of *Botrytis cinerea* on apples. *Phytopathology* **88**: 335-343.
- Kennedy, J., Auclair, K., Kendrew, S.G., Park, C., Vederas, J.C. and Hutchinson, C.R. (1999) Modulation of polyketide synthase activity by accessory proteins during lovastatin biosynthesis. *Science* **284**: 1368-1372.
- Kent, A.D. and Triplett, E.W. (2002) Microbial communities and their interactions in soil and rhizosphere ecosystems. *Ann. Rev. Microbiol.* **56**: 211-236.
- Lai, C.X., Xu, J., Tozawa, Y., Okamoto-Hosoya, Y., Yao, X.S. and Ochi, K. (2002) Genetic and physiological characterization of *rpoB* mutations that activate antibiotic production in *Streptomyces lividans*. *Microbiology* **148**: 3365-3373.
- Liang, P. and Pardee, A.B. (1992) Differential Display of Eukaryotic Messenger RNA by Means of the Polymerase Chain Reaction. *Science* **257**: 967-971.
- Lindgren, S.E. and Dobrogosz, W.J. (1990) Antagonistic Activities of Lactic-Acid Bacteria in Food and Feed Fermentations. *FEMS Microbiol. Rev.* **87**: 149-163.
- Lipsitch, M. (2001) The rise and fall of antimicrobial resistance. *Trends Microbiol.* **9**: 438-444.
- LopezRibot, J.L., Alloush, H.M., Masten, B.J. and Chaffin, W.L. (1996) Evidence for presence in the cell wall of *Candida albicans* of a protein related to the hsp70 family. *Infect. Immun.* **64**: 3333-3340.
- Maplestone, R.A., Stone, M.J. and Williams, D.H. (1992) The Evolutionary Role of Secondary Metabolites - a Review. *Gene* **115**: 151-157.
- Melin, P., Schnürer, J. and Wagner, E.G.H. (1999) Changes in *Aspergillus nidulans* gene expression induced by bafilomycin, a *Streptomyces*-produced antibiotic. *Microbiology*, **145**: 1115-1122.
- Melin, P., Schnürer, J. and Wagner, E.G.H. (2002) Proteome analysis of *Aspergillus nidulans* reveals proteins associated with the response to the antibiotic concanamycin A, produced by *Streptomyces* species. *Mol. Genet. Genom.* **267**: 695-702.
- Miller, M.B. and Bassler, B.L. (2001) Quorum sensing in bacteria. *Ann. Rev. Microbiol.* **55**: 165-199.
- Moter, A. and Gobel, U.B. (2000) Fluorescence *in situ* hybridization (FISH) for direct visualization of microorganisms. *J. Microbiol. Meth.* **41**: 85-112.
- Navarre, C., Degand, H., Bennett, K.L., Crawford, J.S., Mortz, E. and Boutry, M. (2002) Subproteomics: Identification of plasma membrane proteins from the yeast *Saccharomyces cerevisiae*. *Proteomics* **2**: 1706-1714.
- Nawrocki, A., Fey, S.J., Goffeau, A., Roepstorff, P. and Larsen, P.M. (2001) The effects of transcription regulating genes PDR1, *pdr1-3* and PDR3 in pleiotropic drug resistance. *Proteomics* **1**: 1022-1032.
- Niimi, M., Cannon, R.D. and Monk, B.C. (1999) *Candida albicans* pathogenicity: A proteomic perspective. *Electrophoresis* **20**: 2299-2308.
- Oliver, S.G., Winson, M.K., Kell, D.B. and Baganz, F. (1998) Systematic functional analysis of the yeast genome. *Trends Biotech* **16**: 373-378.
- Pardo, M., Ward, M., Bains, S., Molina, M., Blackstock, W., Gil, C. and Nombela, C. (2000) A proteomic approach for the study of *Saccharomyces cerevisiae* cell wall biogenesis. *Electrophoresis* **21**: 3396-3410.
- Perez-Ortin, J.E., Garcia-Martinez, J. and Alberola, T.M. (2002) DNA chips for yeast biotechnology. The case of wine yeasts. *J. Biotech.* **98**: 227-241.
- Punt, P.J., Dingemans, M.A., Jacobsmeijns, B.J.M., Pouwels, P.H. and Van den Hondel, C. (1988) Isolation and Characterization of the Glyceraldehyde-3- Phosphate Dehydrogenase Gene of *Aspergillus nidulans*. *Gene* **69**: 49-57.
- Riedel, K., Arevalo-Ferro, C., Reil, G., Gorg, A., Lottspeich, F. and Eberl, L. (2003) Analysis of the quorum-sensing regulon of the opportunistic pathogen *Burkholderia cepacia* H111 by proteomics. *Electrophoresis* **24**: 740-750.
- Riley, M.A. and Wertz, J.E. (2002) Bacteriocins: Evolution, ecology, and application. *Ann. Rev. Microbiol.* **56**: 117-137.
- Roy, K., Mukhopadhyay, T., Reddy, G.C.S., Desikan, K.R. and Ganguli, B.N. (1987) Mulundocandin, a New Lipopeptide Antibiotic .1. Taxonomy, Fermentation, Isolation and Characterization. *J. Antibiot.* **40**: 275-280.
- Singh, V.K., Jayaswal, R.K. and Wilkinson, B.J. (2001) Cell wall-active antibiotic induced proteins of *Staphylococcus aureus* identified using a proteomic approach. *FEMS Microbiol. Let.* **199**: 79-84.
- Stone, M.J. and Williams, D.H. (1992) On the Evolution of Functional Secondary Metabolites (Natural- Products). *Mol. Microbiol.* **6**: 29-34.
- Ström, K., Sjögren, J., Broberg, A. and Schnürer, J. (2002) *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo(L-Phe-L-Pro) and cyclo(L-Phe-trans-4- OH-L-Pro) and 3-phenyllactic acid. *Appl. Environ. Microbiol.* **68**: 4322-4327.
- Sundstrom, P., Smith, D. and Sypher, P.S. (1990) Sequence-Analysis and Expression of the 2 Genes for Elongation Factor-1-Alpha from the Dimorphic Yeast *Candida albicans*. *J. Bact.* **172**: 2036-2045.
- Torsvik, V. and Ovreas, L. (2002) Microbial diversity and function in soil: from genes to ecosystems. *Curr. Opin. Microbiol.* **5**: 240-245.

Trinci, A.P.J. (1984) Antifungal agents which affect hyphal extension and hyphal branching. In Trinci, A.P.J. and Ryley, J.F. (eds.), *Mode of actions of antifungal agents*. British mycological society, Great Britain.

Vinas, I., Usall, J., Teixido, N. and Sanchis, V. (1998) Biological control of major postharvest pathogens on apple with *Candida sake*. *Int. J. Food Microbiol.* **40**: 9-16.