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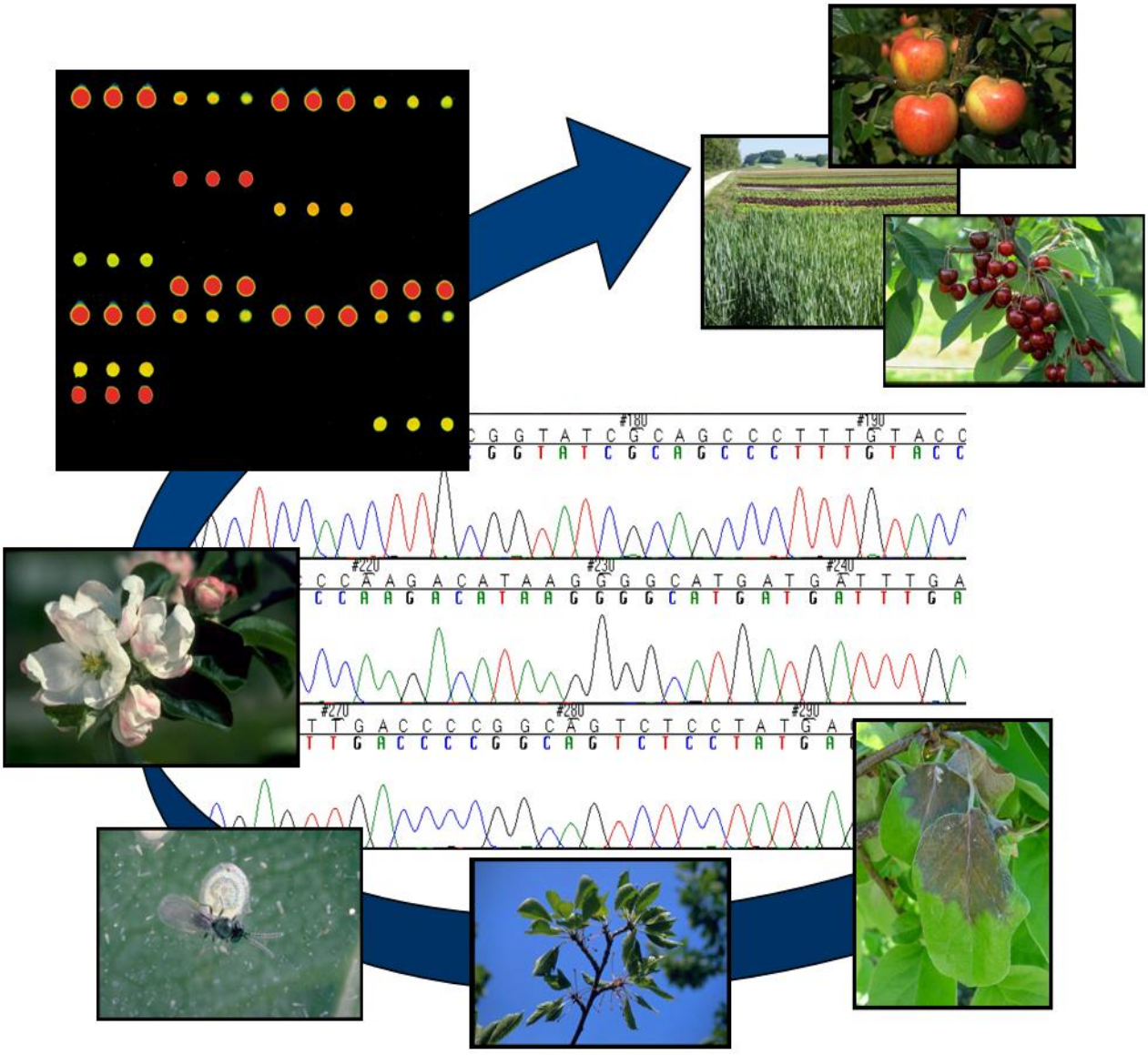
Agricultural Biomarkers for Array Technology



Edited by
Jürg E. Frey and
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COST Action 853

COST- the acronym for European **CO**operation in the field of **S**cientific and **T**echnical Research- is the oldest and widest European intergovernmental network for cooperation in research. Established by the Ministerial Conference in November 1971, COST is presently used by the scientific communities of 35 European countries to cooperate in common research projects supported by national funds.

The funds provided by COST - less than 1% of the total value of the projects - support the COST cooperation networks (COST Actions) through which, with EUR 30 million per year, more than 30.000 European scientists are involved in research having a total value which exceeds EUR 2 billion per year. This is the financial worth of the European added value which COST achieves.

A “bottom up approach” (the initiative of launching a COST Action comes from the European scientists themselves), “à la carte participation” (only countries interested in the Action participate), “equality of access” (participation is open also to the scientific communities of countries not belonging to the European Union) and “flexible structure” (easy implementation and light management of the research initiatives) are the main characteristics of COST.

As precursor of advanced multidisciplinary research COST has a very important role for the realisation of the European Research Area (ERA) anticipating and complementing the activities of the Framework Programmes, constituting a “bridge” towards the scientific communities of emerging countries, increasing the mobility of researchers across Europe and fostering the establishment of “Networks of Excellence” in many key scientific domains such as: Biomedicine and Molecular Biosciences; Food and Agriculture; Forests, their Products and Services; Materials, Physical and Nanosciences; Chemistry and Molecular Sciences and Technologies; Earth System Science and Environmental Management; Information and Communication Technologies; Transport and Urban Development; Individuals, Societies, Cultures and Health. It covers basic and more applied research and also addresses issues of pre-normative nature or of societal importance.

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Agricultural Biomarkers for Array Technology

2001-2007

Edited by

Jürg E. Frey

and

Frédérique Pasquer

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Foreword

I had the pleasure of managing this COST Action at two distinct phases. First, at its inaugural meeting and later, having re-joined the new COST office, when I became involved in organizing its final conference held in 2007. I left the COST office shortly after this, and am therefore in the fortunate position of writing this foreword with the benefit of both hindsight and a certain detachment.

Possibly the most important attribute of COST 853 was its openness. COST Actions are always intended to be open frameworks for cooperation, but Jürg Frey and his colleagues pursued this idea relentlessly. At the outset, the idea for this COST Action arose from a group of scientists whose backgrounds were largely but not exclusively in plant agriculture. At the zenith of the Action, the spectre had widened considerably, as the content of this book demonstrates. This is a very real example of multidisciplinarity, the coming together of scientists from varied backgrounds and interests working together around a common theme - microarray technology.

This openness was also evident by the constant involvement of partners from industry which enabled a fruitful exchange between those developing the newest techniques and those who could potentially apply them in a variety of different fields and circumstances. Without this mix of industry and public sector, the Action would not have progressed as much as it did since microarray technology is a very rapidly developing field. The rapidity and accuracy of the analyses we can perform in 2007 is very different from when the Action started in 2001, but COST 853 managed to successfully accompany this exciting development.

This foreword would not be complete without a word for the chair of the Action, Dr. Jürg Frey and the quite remarkable core team of scientists who worked closely with him at every stage. To say it was a pleasure to work with them is banal, it was in fact an immense pleasure! Jürg was also helped by an exceptional Management Committee and also by his trusted co-workers, first Monica Pfunder and then Frédérique Pasquer taking care of the detailed tasks that made every meeting so enjoyable, productive and scientifically successful.

Professor John Williams
I.N.R.A., Nouzilly, France

December 2007

Five years of COST Action 853 – a short overview

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Introduction

The main objective of the COST Action 853 was to establish and support microarray technology as a new tool for breeding, diagnosis, and high throughput screening in the field of agriculture.

A major motivation behind tackling this task was an unsatisfactory situation in diagnostics of agronomically relevant organisms. At the onset of this action, many different gene fragments of diverse genes were used by different laboratories for taxon identification. Thus, each diagnostic laboratory had to master a broad range of different analytical details, making the task of molecular diagnostics cumbersome and laborious. Therefore, simple and economic methods were sought to allow robust identification at the required taxonomic level for a wide range of organisms.

Microarray technology seemed to provide the potential to solve this problem by its virtually unlimited power for parallel analysis. One simple strategy was to use several genes with an optimal discrimination capability at the required taxonomic level, i.e., species, strain, serogroup, etc., and to design several diagnostic probes per taxon to maximise the accuracy and robustness of the assay. Basically, this technology enables the production of microarray chips that can be used for large taxonomic groups such as all agronomically relevant bacteria, nematodes, insects, etc. As the result of such an assay is a simple pattern of spots, data analysis and interpretation should not present any difficulties. Because such an assay would be simple to perform it could easily be adapted in diagnostic laboratories.

COST action 853 was organised in five strongly networking working groups (Figure 1): WG1 – Nucleic-Acid Based Microarrays, led by Dr. P. Bonants (HRI, NL), with the main objective to collect all available information on suitable DNA and RNA markers for phytodiagnostic purposes. Furthermore, members of this group should explore different microarray methods. WG2 – Protein Based Microarrays, led by Drs. I. Barker and N. Boonham (CSL, UK), had the same objective as WG1 but with a focus on proteins. WG3 – Bioinformatics and Information Dissemination, led by Drs. P. von Rohr and U. Wagner (FGCZ, UNI/ETH Zurich, CH), provided the necessary expertise in bioinformatics and a platform for coordination and information dissemination. The latter two topics were covered mainly by a subgroup "Internet Office" (J.E. Frey, Agroscope ACW, CH). WG4 – Chip Production and Analysis, led by Prof. D. Blohm (University of Bremen, DE), provided sophisticated equipment for spotting, microarray hybridization and reading, together with the required expertise that was very beneficial to all members of this action. WG5 – Microarray Technology for Environmental Monitoring, led by Dr. X. Nesme (University of Lyon, F), had the objective to collect information to develop microarray technology for environmental monitoring in agro-ecosystems. The action was chaired by Dr. J.E. Frey and Prof. G. Adam. Within this consortium we could offer the full set of necessary equipment, such as spotters, hybridisation tools and readers, as well as the required skills to all action members.

The concept of this action was attractive to many researchers working in the field of agronomy and so the action grew rapidly to 21 signatory countries.

The individual research teams involved in this COST Action work in many different fields in the area of agricultural research. Identification of plant pathogens and characterization of genes relevant for crop breeding are among the main topics common to all members of this Action. DNA-based identification of pests and pathogens was already well established before this Action was initiated. However, as mentioned

above, major problems existed because of the great diversity of pests and pathogens that had to be dealt with and the lack of harmonization with respect to the gene(s) and gene fragments best suited for diagnosis of this broad range of target organisms.
In the course of our action we spent much effort for the development of specific techniques, probe design and strategies for target labelling. Many interesting achievements were first communicated at some of our Working Group Meetings. Furthermore, many new contacts were established, eventually resulting in a dense network that included companies and other research organisations, for example via several FP6 projects of the European Union.

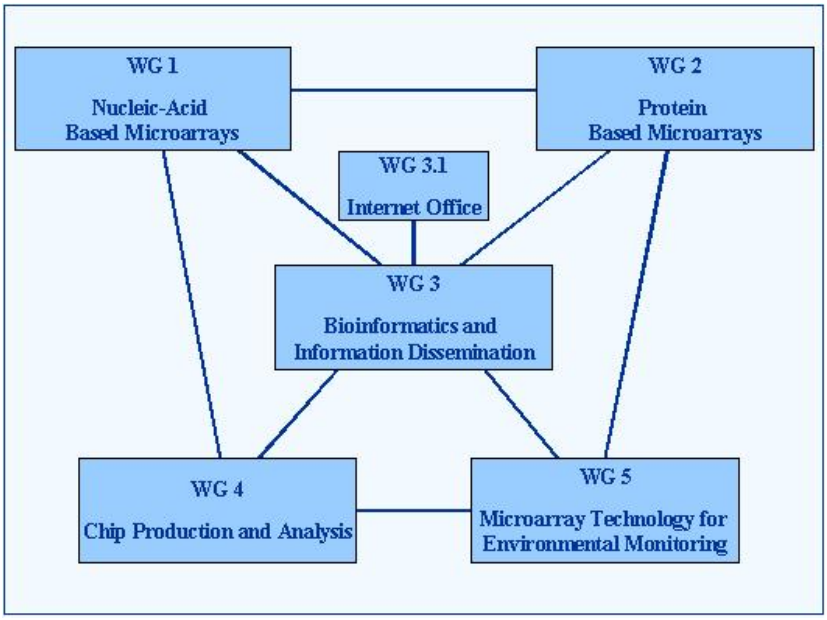

Figure 1: Organisation of the COST Action 853 in five interconnected working groups.

In the following, we will give a short overview on some of the highlights, successes and problems encountered in the course of COST Action 853.

Promises and achievements of the Action

In the Memorandum of Unterstanding of our Action, the following main benefits were expected. First and most importantly, the proposed Action should provide a platform to introduce and support this important new technology to many European countries. Secondly, the broad availability of this widely applicable technology thus provided should promote and protect development of agricultural products in Europe. The expected benefits were summarized as follows:

1. Assessment of the suitability of different microarray techniques to the field of phytodiagnostics and animal/plant breeding
2. Establishment of a database containing all relevant information on nucleic acid sequences and proteins suitable for microarray - based phytodiagnosis
3. Coordination/standardisation of microarray chip composition and production, profiting from the combined knowledge of all participating countries
4. Implementation of specific microarray technologies in participating countries, thereby establishing this important new technology in European phytodiagnosis
5. Significant improvement in harmonization of European phytodiagnosis
6. Support of this new phytodiagnostic technique on a broad European level

The contributions in this book provide convincing evidence that these major goals were clearly achieved.
Within the course of our Action, all collaborators and their laboratories were fully or partially financially supported by their COST member countries providing salaries and equipment. The collaborators used these resources for top-level research and development of methods which resulted in a wealth of new discoveries. Most of the efforts undertaken for our Action had an agricultural basis/aim and thus, the results are directly in line with the expected benefits for our Action. Thus, we assessed a wide range of different microarray

techniques, and successfully coordinated microarray chip production and implementation of specific technologies. Thereby we provided a platform for this technology and significantly contributed to the harmonization of European phytodiagnosis. One aim was, however, only partially achieved. Initially, we intended to assemble a database containing all relevant information for diagnosis (point 2 above). However, one of the important lessons learned in this Action was that probe design is intimately dependent on the specific technical parameters used and that no algorithms exist that satisfyingly predict probe behaviour (see below). Thus, probes with acceptable hybridization characteristics when used in one specific assay proved to be virtually non-functioning in another. Furthermore, as some of the research was performed together with private companies, it was impossible to obtain all relevant information for creating this cumulative database. Despite these problems, we were able to collect all important progresses and achievements of this Action and we made the relevant information available on our homepage (www.cost853.ch).

Highlights and successes of the Action

Highly successful meetings

In the course of this action we convened seven management committee meetings and five meetings of chairpersons and WG leaders. These administrative meetings are important to the overall success of any Action as it is the place where strategies are decided and tasks and duties are distributed. The management committee members of this Action always participated with great enthusiasm and thus built a robust collaborative and scientific basis for our Action. In addition all nine working group meetings were very interesting and stimulating. These working group meetings were always very much up-to-date as internationally renowned experts in the respective fields were invited in all of them. The meetings were organized in a way allowing profound discussions and thereby a thorough comprehension of the presented materials and techniques.

New STSM workshop format

At the onset of this Action, only few of the collaborating scientists had hands-on knowledge on microarray technology or direct access to it. There was thus an urgent need for this kind of training. However, because of the high costs of the equipment and the fact that it was a very young technology, only few members of our consortium could offer the required equipment and space. This represented a major problem, as the organisational tool provided by COST for such training was the short term scientific mission (STSM). These missions were originally designed to allow individual scientists visiting a specialist laboratory to obtain training in a specific technique. Thus far, none of the potential hosts had the capacity to provide training for more than 40 individual scientists which was the initial number of applications we obtained in response to our first query. To enable accommodation of the observed need in hands-on microarray and bioinformatics training, we initiated the new COST format of “STSM workshops”. Such workshops may accommodate up to 20 trainees and were thus an ideal format for our needs. Between 2003 and 2006, we conducted three highly successful STSM workshops totalising 43 participants. This training together with the many fruitful contacts within our Action may have contributed to the significant increase in progresses that could be observed in this field as our Action went on.

Evaluation and validation of various microarray systems

Many different microarray systems have been proposed within our Action frame and much effort went into testing their respective pros and cons. To list all individual technologies covered within this Action is not possible in this short overview and therefore, we just highlight few and refer to the remainder of this book and our homepage for the others.

Among the simplest systems is one that uses fluorescently labelled PCR products for hybridization on oligonucleotide microarrays. In this method, gene fragments containing the diagnostic information (e.g., the mitochondrial cytochrome oxidase I gene in many insects) for taxon identification are PCR amplified using fluorescently labelled primers. The amplicons are then hybridized to a microarray slide containing discriminatory oligonucleotide probes. This simple concept was shown in our laboratory as well as in others to be very robust and simple to use, and it was shown that it is applicable to diagnostic problems in all organisms including viruses, bacteria, insects, mammals and plants (e.g. chapters Boonham and Tomlinson, and Aittamaa et al.; Deyong et al., 2004; Tobler et al., 2006; Pfunder et al., 2004; Frey and Pfunder, 2006;

2006, and Pasquer et al., 2008).
The same approach was initially used by another laboratory in our consortium from Vienna (Austria) for detection and identification of microorganisms in environmental samples using 16S probes (Bodrossy and Sessitsch, 2004). However, it was shown that in mixed samples, the detection limit of individual species was only around 5% which was considered too high. This group therefore developed an alternative labelling strategy, sequence-specific end-labelling of oligonucleotides (SSELO; Kostic et al., 2007). Here, labelling occurs on the chip after hybridization. The probe has a free 3' end and upon binding a matching target sequence, a single base extension reaction is performed using fluorescently labelled nucleotides. With this strategy it was possible to lower/increase the detection limit of individual microorganisms in mixtures to around 0.1% (chapter Bodrossy and Sessitch).
All above mentioned approaches use an initial PCR amplification step to produce enough labelled DNA for hybridization. For the analysis of mixed samples or heterogeneous templates, this step comes with the disadvantage that unequal amplification of different target sequences may distort the quantitative representation of the original occurrence in the PCR products. The use of Padlock probes provides an elegant solution to this problem (chapter Bonants et al.). These probes contain two probe sites oriented head to head together with primer sites and a zip-code sequence. The two probe sites hybridize to a matching target site and, with a full match, the gap between the two probe sites is closed with a ligation reaction. Then, the circularized Padlock probe is amplified using the in-built primer sites. Finally, the amplified probes are de-multiplexed on a microarray carrying the inverse recognition sequences for the zip-codes. This system is highly sensitive and specific, and because all amplicons have identical primer sites the quantitative representation is maintained.
To characterize microbial soil communities our colleagues from Lyon (France) followed another strategy. They use high-density microarrays covering the 16S-rDNA. This strategy allows to perform high-resolution differentiation among different microbial soil communities (chapter Nesme and Oger-Desfeux). One drawback of this solution is that data analysis is relatively complex and requires good statistical knowledge.
Quantification is inherently difficult with microarrays and if needed for a specific application represents an additional level of complexity for the development of diagnostic microarrays. Our colleagues from Wageningen (The Netherlands) followed a unique way to circumvent these problems by using the microarray format for highly parallel quantitative real-time PCR (chapter Van Doorn et al.). In collaboration with BioTrove Inc., they developed a microarray allowing >3000 real-time PCR reactions in parallel starting with the ligated Padlock/PRI-lock probes as template. This system combines the advantage of highly precise quantification of real-time assays with the high degree of multiplex analysis provided by the microarray format. The drawback of this approach is the need for high precision optics on a special analytical system and thus it is currently not widely applicable.
Alternatives to optical detection were also developed in our consortium. Our colleagues from Lithuania are studying systems to allow highly sensitive capture of target molecules by biosensors and amperometric detection of electrochemical signals (chapter Ramanavicius and Ramanaviciene.). This work is of high relevance as it lends itself to miniaturization and avoids expensive optical detection. However, in contrast to the above mentioned technologies this approach is still in its infancy.

Protein detection

Most of the efforts of our COST Action 853 went into the field of DNA arrays and only limited resources were spent for Working Group 2, protein arrays. This had several reasons. First, it still is very much simpler to work with DNA than with proteins because DNA is a very robust molecule that can easily be stored and handled and will not rapidly deteriorate at room temperature. Second, while DNA or RNA are relatively easily accessible for analysis, protein diagnostics requires antibodies or alternative capture probes that first have to be developed for most organisms. Finally, nucleic acid and protein assays cannot easily be mixed. Furthermore, if antibodies for only one individual in a set of organisms to be diagnosed are lacking, there is a need for both tests (protein and nucleic acid assay). Thus, only few of our collaborators were studying protein arrays. Poltronieri and collaborators managed to produce an interesting chip allowing detection of important allergens due to mites in food product and dust. This original approach allows the detection of health-threatening proteases at an extremely low level compared to usual detection techniques (chapter Cimaglia et al.).
Nevertheless, protein detection today is a very frequently used tool for virus detection. For example, the

group of Jan Bergervoet is greatly improving the detection sensitivity of potato viruses by immunoassays using the Luminex xMAP technology (chapter Bergervoet et al.).

Gene expression studies

Finally, some members of our consortium were involved in the "original" approach of microarray technology, i.e., gene expression analysis. The group of Marusa Pompe-Novak (chapter Kogovšek and Pompe-Novak) was studying plant-pathogen interactions and the lessons learned in this project were of great value to many colleagues in the action who, as an extension of their skills in diagnostic microarrays, continue with projects on gene expression or start to use quantitative information in the analysis of their diagnostic microarrays.

Progress in general knowledge of microarray probe design

The major challenge in all microarray based diagnostic systems is probe design. The goal is to find genetic regions that show no or low variation between individuals of a taxon but with very clear differentiation between different taxa (e.g., chapter Adam). The search for such regions is usually based on DNA sequence alignments of candidate genes. For microorganisms, the "gold-standard" for such a gene is the 16S ribosomal RNA. Over a decade ago, (Ludwig et al., 2004)started to build a database, ARB, containing all known 16S rDNA sequences of microorganisms, and which includes sophisticated algorithms for alignment and primer and probe design (chapter Glöckner and Peplies, chapter Nesme and Oger-Desfeux). The program may also be used with other genes (chapter Bodrossy and Sessitch). Within our consortium, we had several training courses for this important tool.

One of the problems with probe design is the huge variation in hybridization performance of different probes. We soon found that there is a serious gap between the performance of probes on the chip surface as observed in our assays compared to the expected performance based on our calculations of expected melting behaviour (Pfunder et al., 2004), a serious problem that is still not solved (e.g. chapter Sip and Pozhitkov et al., 2006). The most frustrating consequence of this problem is that obtaining optimized probe sets requires on-chip selection. This implies the design of several probes per target sequence hoping that at least one will perform well for the test under development. In cases with limited sequence divergence between taxa, there are narrow limits to the range of possibilities for probe design. It is not uncommon to find that, although the target sequence initially looked promising, none of the possible probes performs well enough to be of practical use and that, therefore, it is necessary to search for alternative target sequences. It is an unfortunate fact that the availability of such alternatives is to date still very restricted in many important pest organisms. The process of microarray optimization is therefore sometimes both very time-consuming and expensive.

Unfortunately, as mentioned above, our work also revealed that probes cannot easily be transferred between systems. As a matter of fact, the hybridization behaviour of probes that were selected in an optimization scheme for one probe set is often less than acceptable if used in other hybridisation conditions. This is the main reason why we have abstained from establishing a probe database. All relevant information may be obtained from the respective publications that are listed on our website.

Perception of COST Action 853

Our Action has raised a lot of interest in many other COST Actions and generally in the diagnostic field across Europe, including the private sector. Many of our meetings were visited by interested scientists not directly involved in COST themselves, and by representatives of companies working in this field. The positive impression made by the presentations of COST members was a major incentive for many companies to start collaborations with individual laboratories of our Action. Importantly, it initiated science networks and set the grounds for many parallel research projects, such as FP6 projects (e.g., Fish&Chips Project of Prof. Blohm; http://www.fish-and-chips.uni-bremen.de/PostNuke/html/) and nationally funded projects.

To accommodate the general interest in our Action, we were invited by the COST office to organise a Trans-COST meeting covering all important aspects of our Action and including all relevant development of the other interested COST Actions. Members of COST Actions 853, 858, 861, 863 and 926, and of several private companies were participating at this meeting held on May, 22-24, 2007, in St. Feliu de Guixols (Spain) together with the last meeting of our Action. This very interesting and stimulating joint meeting was attended by many top experts in the field of diagnostics and was a formidable demonstration of the great

overall success of our COST Action 853, both with respect to the scientific and technological achievements and to the positive impression we made in the scientific community and in the public appearance.

Conclusion

COST Action 853 was a very timely action that enabled the collaborating scientists to participate in the development and establishment of a new and highly relevant technology. Many different microarray technologies were evaluated and the experience gained represents a highly valuable reference for further work. All members of the Action were very active and acted as ambassadors for the Action within their countries. This is the main reason for its wide public appearance. This Action has significantly contributed to improve European collaboration and an increased level of harmonisation in agricultural diagnosis. Overall, COST Action 853 was highly successful and the established knowledge will be an important cornerstone for continuing efforts in the field of microarray diagnostics for many years to come.

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Microarray hybridization

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Abstract

Design of microarrays for diagnostic purposes is based on rules derived mainly from general concepts of hybridization and probe design in bulk solutions. The real conditions on a microarray surface significantly differ from bulk solution conditions as a result of the immobilization of probes and interactions of probes and targets with the surface. Multiple interactions of probe fragments with the surface, dynamics of the system and statistics of fragment binding modes should be taken into account. In this article we present a brief summary of requirements concerning the melting temperature, the probe similarity, the longest identical sequence stretch and the steric hindrance of the probes. The influence of the microarray surface and the various types of microarray surface chemistry that can compromise theoretical predictions of quality of probes based on state-of -the-art algorithms is discussed.

Introduction

The main attraction of using nucleic acids in detection and diagnostics is i) the specificity of interaction that makes it possible to distinguish two molecules differing in only one nucleotide and ii) the apparent simplicity to predict and to design such a specific interaction. To find a particular nucleic acid sequence among many others, just take its complementary sequence, label it, mix and follow the hybridization pattern. Every living organism, from viroids that are nothing but tiny pieces of nucleic acids up to human beings, is characterized by its genetic information written in form of a nucleotide sequence. The microarray detection method is based on fragments of nucleic acids (probes) that selectively hybridize to the nucleic acid sequences present in the sample (targets).

The idea of a DNA-microarray is to have a large number of such probes (virtually unlimited, practically up to hundreds of thousands) which can simultaneously interact with the labeled targets of the sample. To distinguish among individual probes, they are spotted or synthesized on a surface so that they together form a two-dimensional array, where each probe is characterized by its coordinates.

The microarray thus enables simultaneous detection of a great number of sequences in one reaction. The probe design must take into account that all spots/sequences on the microarray have to work under the same conditions (temperatures, solution composition, etc.) except for sequences of individual probes. Probe design for a microarray requires optimization of the whole set of probes and of the hybridization conditions to get the best performance. For a real microarray it is often difficult to attain optimum stringency for all spots, because it is impossible to individually tune all hybridization parameters for every spot.

General concepts of nucleic acids hybridization and probe design

Molecular hybridization processes on a microarray can be described and quantified in the first approximation by classical concepts of nucleic acids hybridization in solutions. The main factors influencing melting temperature and kinetics are the base sequence (Breslauer et al., 1986) and the composition of the solution which determines its ionic strength. Several points have to be considered in microarray probe design: the melting temperature, the probe similarity, the longest identical sequence stretch and the steric hindrance of the probes.

Melting Temperature

The melting temperature (Tm) should be approximately the same for all probes on the microarray. Tm is defined as the temperature at which half of the DNA strands are in the double-helical state and the other half are single-stranded. Software tools for probe design perform the melting temperature calculation using one of the empiric formulas. These calculations give a more or less precise starting value of Tm. Probably the best agreement with experimental values yields the so-called nearest neighbour thermodynamic theory. This concept recognizes the influence of sequence on Tm. It assumes that the binding between strands in a duplex depends not only on its base composition but also on its sequence (e.g. AT/TA binding parameters are different from those for TA/AT). The Tm in Kelvin is then calculated using the formula

$$T_m = \frac{dH}{dS + R\ln\frac{c}{4}} + 16.6\log_{10}[K^+]$$

where dH is the enthalpy for helix formation, dS is the entropy for helix formation, R is the molar gas constant and c is the total oligonucleotide strand concentration. The formula takes into account salt molar concentration [K^+]. For details and examples see the original article by Breslauer et al. (1986). The melting temperature reflects the free energy of duplex formation. The dS and dH parameters are determined experimentally and may considerably differ from the Breslauer's values. The thermodynamic parameters and nearest neighbours calculation method presented by Santa Lucia (1998) are widely used for T_m estimation in oligonucleotide probe design.

The strategy for optimum array performance especially when targeting a higher number of closely related genes is to design and maximize the number of oligonucleotide probes originating from a set of highly similar sequences. The use of multiple probes to cover a given target increases the assay reliability.

Overall probe similarity

The hybridization specificity may be affected by a variety of probe design factors, including the overall sequence similarity, the distribution and positions of mismatching bases and the size of the longest identical stretch as shown by Liebich (2006).

Hybridization specificities increase with overall similarities between probe and target sequences, as shown by Liebich et al., 2006. Under the described experimental conditions, they could establish a logarithmic relationship between probe-target similarity and the percentage of hybridized probes.

Longest identical sequence stretch

When the same data were analyzed in relation to the length of a common identical sequence stretch, a similar relationship to that for percent overall similarity was found. The percentage of probes that gave positive hybridization signals was considerably lower for probes with up to 20-bp identical sequence stretches to the target sequences (about 10%) than those with at least 22-bp identical sequence stretches (about 25%), suggesting that a 20-bp identical sequence stretch could be an appropriate cutoff value for probe design (Liebich et al., 2006). Another confirmation of these empirical rules was given by Bystricka et al.(2005) and He (2005).

The steric hindrance

At last but not at least, the design of probes and determination of experimental conditions should minimize any form of steric hindrance affecting the hybridization process. The most obvious form of steric hindrance concerns formation of higher order structures like hairpins in both the probe and target molecules that prevent the formation of the duplex. By choosing proper probe sequences and hybridizing non-modified nucleic acids in 'bulk' solution this problem can be minimized.

Particular problems of surface-array hybridization

The surface plays an important role. There are three major differences with respect to the bulk solution conditions:

1) The capture probes are immobilized to a certain extent by surface binding and therefore their molecular dynamics is different from that describing a system of two free strands in a solution. Kinetics of hybridization is much slower and the true equilibrium point is rarely reached in real microarray applications.

Kinetics were measured on photolithographic microarrays (Glazer et al., 2006), spotted surface microarrays and gel-based microchips (Sorokin et al., 2006). The hybridization kinetics is slower in gel-based microchips than in spotted surface microarrays but the fluorescence signals and mutation discrimination efficiency is better for the gel-based microarrays. The authors attribute this fact to better immobilization efficiency and to the higher thermodynamic association constants for duplex formation within gel pads. The hybridization kinetics can be accelerated by recirculation in microfluidic devices. Lee et al. (2006) report that recirculation shortened the time of perfect match target-probe hybridization from 6 hours to 2 hours compared to static hybridization. Fast kinetics are attained also in lateral flow microarrays (Carter and Cary, 2007) which allow analyte detection times of <120 s and sub-femtomole sensitivity with ten-microliter sample volume (excluding sample preparation and amplification).

2) The presence of the surface affecting all processes in its vicinity and especially the interactions of the capture probe with the surface represent an important steric hindrance making single strand – double strand transitions more difficult. A microarray surface effect on binding affinities was confirmed recently by Zhang et al. (2007) but so far no practical tool has been developed considering this effect for the probe design.

3) The surface, charged or uncharged, represents an electrostatics generating artefact influencing the concentration profiles of ions in the vicinity of bound molecules. Therefore, the real values of concentrations influencing the duplex formation may be different from those expected.

Most problems with a considerable percentage of flawed capture probes that are theoretically fulfilling all the requirements but practically do not meet the expectations arise from a total neglect of the above-mentioned surface phenomena on an array during the standard probe design.

The surface of a microarray is even in the simplest cases very complex. While the target binds to the probe always by weak hydrogen bonds to form a duplex, the probes bind to the surface by weak non-covalent and/ or strong covalent bonds, depending on the used surface chemistry.

Although DNA can be printed directly onto a clean glass surface (Call et al., 2001), better results are generally obtained with modified surfaces such as poly-L-lysine, 3-aminopropyltrimethoxysilane, 3-glycidoxypropyltrimethoxysilane and aldehyde or carboxylic acid. Epoxide, isothiocyanate and aldehyde activated glass surfaces form covalent links with amine-terminated cDNA and amine-terminated oligonucleotides. Non-modified DNA binds by weak interactions onto amine-functionalized surfaces such as poly-L-lysine and 3-aminopropyltrimethoxysilane. Surfaces modified with polyamidoamine dendrimer bind non-modified DNA electrostatically. Impact of surface chemistry and blocking strategies on DNA microarrays was studied by Taylor et al. (2003).

The picture of a microarray containing probes as fragments that are attached to the surface just by one end and otherwise extend free into the solution is obviously not true. A more realistic image must consider additional aspects like multiple interactions of probe fragments with the surface, dynamics of the system and the fact that the intensity of a particular spot is given by a statistical set of many fragments hybridizing to that spot.

Multiple interactions of the fragments are caused mainly by negative charges of nucleic acids located on phosphate groups. With positively charged surfaces like poly-L-lysine, the attractive interaction is direct. However, DNA binds by multiple interactions onto negatively charged surfaces if positively charged ions are present in a sufficient concentration in the solution. Indeed, divalent cations can be used to immobilize nucleic acids for AFM imaging (Atomic Force Microscope imaging; Pastushenko, 2002). AFM imaging clearly shows the DNA molecules collapsed onto the planar surface.

The system of non-covalently bound probes is highly dynamic. At room temperature the probes are subject to thermal fluctuations resulting in partial detachment from the surface and a transient exposure of binding sites that enable formation of the duplex with the target fragment.

The signal of a spot is the sum of signals emitted by labelled targets situated within the spot. The conformational space of duplex formation is explored by a large set of molecules, exceeding in most cases 10^6 molecules (probes) per spot. For example, this number ranges from several millions of capture probes per spot for Affymetrix and NimbleGen arrays to around 10^8 probes per spot for cDNA arrays and extends to over 10^{10} for oligonucleotide-based arrays. The targets that found favourable conditions for binding in the context of both capture probes and local surface remain attached to the probe and can emit a signal. The large number of involved molecules statistically averages out the influence of local surface inhomogeneities and enables hybridization with a subset of randomly bound probes.

Conclusion

All the above-mentioned factors should be taken into account as parameters during the process of microarray probe design. Due to the complexity of such a task, no state-of -the-art algorithms are capable of predicting exactly the quality of probes for microarray hybridization. A detailed study aimed at short oligonucleotide probes clearly shows the limits of standard algorithms (Pfunder and Frey, 2005) .

Even commercial microarrays suffer from a high percentage of poorly performing probes. One way how to minimize this drawback is to adopt special methods of data analysis taking into account the probe performance (Expression Analysis).

As long as theoretical predictions of probe performance remain only the first approximation, the importance of experimental validation of probes cannot be overestimated. During the design of microarrays for practical routine applications, excessive testing should be performed to show the performance of the chip under various conditions. This should be stressed especially for applications involving complex samples, containing many target sequences, where mutual interactions and cross-hybridizations are possible. The following chapters present convincing evidence of successful microarray design for agricultural practice.

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ARB and SILVA: A software environment and databases for ribosomal RNA sequence data

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Abstract

Sequencing the ribosomal RNA (rRNA) genes is currently the method of choice for nucleic acid-based detection of microbes, assessment of microbial diversity, and phylogenetic reconstruction. The ARB (latin, 'arbor' = tree) software suite has been accepted by researchers worldwide as a standard tool for large scale ribosomal RNA analysis. More than 12 years of development have already been invested in the software package and the corresponding high quality rRNA alignments. The interactive software tool ARB was recently supplemented by SILVA (Latin, 'silva' = forest), a set of up to date, quality controlled, and aligned rRNA datasets comprising *Bacteria*, *Archaea,* and *Eukarya.* SILVA is designed as an automatic software pipeline for sequence retrieval, quality assignment, and the alignment of nucleic acid sequences based on the latest ARB alignments. Two precompiled sequence datasets for ARB are offered for download on the SILVA website: (1) the reference (Ref) datasets, comprising only high quality, nearly full length sequences suitable for in-depth phylogenetic analysis and probe design and (2) the comprehensive Parc datasets with all publicly available rRNA sequences longer than 300 nucleotides suitable for biodiversity analyses. ARB and SILVA are freely available at www.arb-home.de and www.arb-silva.de, respectively.
Keywords: ribosomal RNA, ARB, database, SILVA, alignments, phylogeny, FISH, diagnostics

Introduction

Large scale sequencing of the three domains of life has become the standard approach to access the genetic potential of organisms. The increasing amount of sequence data is of central importance for molecular taxonomy, diversity analysis and the identification of microorganisms. Initiated by the pioneering studies of Pace, Olsen, Giovannoni and Ward (Pace *et al.* 1985; Olsen *et al.* 1986; Giovannoni *et al.* 1988; Ward *et al.* 1990) over 15 years ago, the ribosomal RNA (rRNA) molecule has been established as the "gold-standard" for the investigation of the phylogeny and ecology of microorganisms (Amann *et al.* 1995; Pace 1997). Today more than 500,000 publicly available small and large subunit (SSU and LSU) rRNA sequences ask for appropriate software tools and specialized quality controlled databases. In anticipation of this impending deluge of rRNA data, the development of the ARB software suite and the curation of its associated databases began more than 12 years ago (Ludwig *et al.* 2004). ARB offers a graphical user interface and a wide variety of interacting software tools built around a common database. The ARB project has been recently supplemented by SILVA which provides structured, integrative knowledge databases for small and large subunit rRNAs (Prüsse *et al.* 2007). Although several other databases exist, the ARB/SILVA project is currently the only one which incorporates homologous small subunit sequences (SSU) from Eukaryotes (18S) and additionally offers large subunit (LSU) databases (28S/28S) comprising sequences from all three domains. Based on regularly offered international workshops and the ARB mailing list, it is currently estimated that the ARB software suite and its databases are employed worldwide by several thousand users from academia and industry.

Materials and Methods

The ARB software was developed for UNIX systems and their derivatives. The major part of the source code was written in C++ and C; some parts were written in Perl and Java and other script languages. The graphical environment is based upon the Open Motif library. Besides proprietary developments like the ARB parsimony tool and the interactive sequence and secondary structure editor as well as probe design and evaluation functionalities, some programs of the PHYLIP package for phylogeny inference (http://evolution.genetics.washington.edu/phylip.html) were incorporated as components directly interacting with the central database. Recently, PhyML (Guindon and Gascuel 2003) and RAxML (Stamatakis *et al.* 2005) were included for fast maximum-likelihood-based phylogenetic analyses of nucleic and amino acid sequence data.

SILVA provides two types of precompiled databases for both small and large subunit ribosomal RNA sequences in ARB format: the high-quality Ref databases and the comprehensive Parc databases. The Ref databases are subsets of Parc, which are exclusively comprised of nearly full length 16S/18S and 23S/28S rRNA sequences. A sequence is accepted if it is at least 1200 bases long. Additionally, sequences as short as 900 bases are included if they belong to the domain *Archaea*. Sequences in the LSU Ref database have to be at least 1900 bases long. For quality control, all sequences that could not be unambiguously aligned or are of low quality were removed from the Ref databases. Both Ref databases are supplemented with a guide tree. The rRNA Parc databases are a collection of all quality checked and automatically aligned rRNA sequences longer than 300 bases. All sequences in the ARB/SILVA databases are associated with a rich set of sequence and process parameters. The databases are regularly updated and follow the release cycle and numbering of the EMBL database.

Results and Discussion

ARB is a comprehensive software package originally developed for ribosomal RNA (rRNA) data. Nowadays, it can be used for any nucleic- or amino-acid sequence data. The central idea is to arrange a database of sequences and associated descriptive information according to the phylogenetic relationships of the corresponding organisms. This phylogenetic tree is visualised and can be used for walking through the database via simple mouse clicks (Figure 1). Furthermore, to facilitate in-depth analysis of molecular data, a comprehensive selection of software tools is integrated into ARB. These are controlled via a common graphical user interface and they interact directly with one another as well as with the database.

For rRNA data, the SILVA-project provides databases with quality checked and aligned sequences. Any additional data related to the individual sequences can be stored in structured database fields or linked via local or worldwide networks. With the “search and query” system for database management, users are able to perform complex queries to e.g. create sequence subsets according to the rich amount of contextual and processing information provided for each rRNA sequence by SILVA. Detailed numerical information about the sequence and alignment quality, as a well as the potential for sequence anomaly (chimeras) facilitate the selection of sequence subsets for high quality tree reconstructions.

All information stored in the database, along with the sequence data such as bibliography, user-made entries or information calculated on-line from the database entries, can be shown at the terminal nodes of the tree.

Sequence editor and alignment

A powerful sequence editor which can be used for nucleic acid and amino acid sequences allows sequence editing, string search, visualisation of base pairing and positional variability. All colours and symbols are user-defined, and separate “align” and “edit” modes prevent erroneous changes. Furthermore, the editor hosts an automatic aligner that is able to align new sequences according to a reference alignment. For DNA and RNA sequences it is supported by a powerful suffix tree server which automatically picks closely related sequences from the reference alignment as templates. The editor is also capable to handle protein sequence data and synchronisation of nucleic acid and amino acid sequence alignments is possible. Pattern search functionalities and the simultaneous secondary structure editor help the scientist to evaluate probe or primer targets.

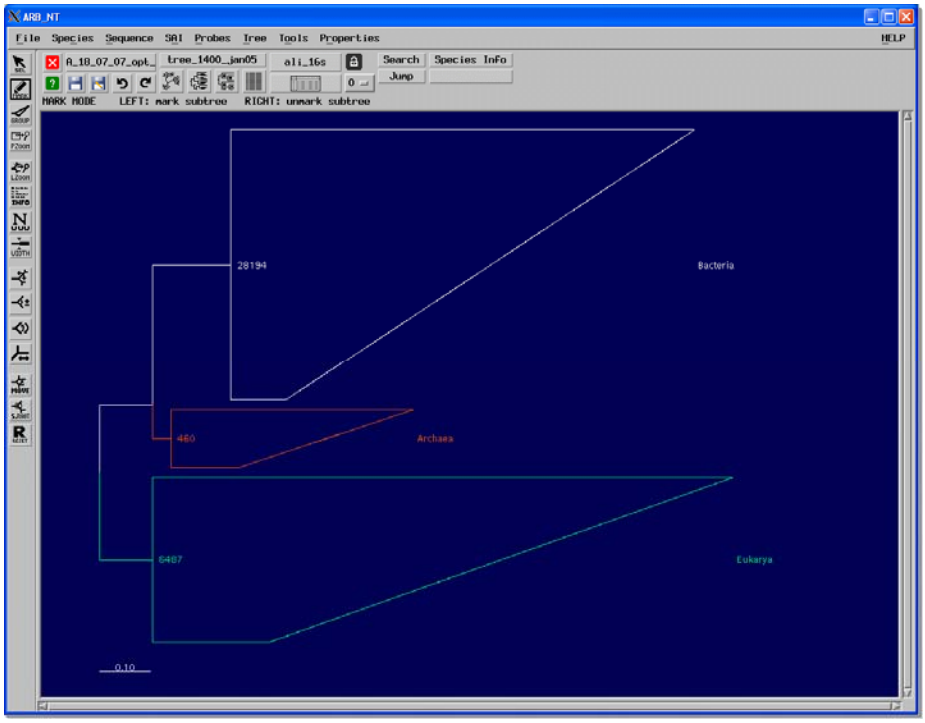

Figure 1: The ARB workbench. All functionalities can be accessed from a central graphical user interface grouped around a tree.

Phylogenetic tree reconstruction

For phylogenetic tree reconstruction, several methods are available as part of the package. This involves distance matrix, parsimony and maximum likelihood approaches. Furthermore, a special maximum parsimony approach has been implemented in ARB that allows the reconstruction and evaluation of large trees. One prominent feature is the possibility to add sequences of different length to an existing tree without changing the overall topology. Optimisation of trees can be applied to complete and user-selected sub-trees and intermediate stages can be stored.

Probe design and evaluation

In just three steps high-quality molecular probes can be designed and visualized including selection of target organisms, probe calculation and probe match. For fluorescence *in situ* hybridisation (FISH), the additional ARB multi-probe software and the visualization of *in situ* accessibility is especially useful. The probe design function of ARB offers various settings to define e.g. target group coverage and the number of allowed out-group hits in a flexible way, a clear must for probe design based on the extremely diverse and large rRNA databases. All potential target binding sites for the selected group of sequences are then calculated by ARB and displayed in a concise table which allows easy assessment of the candidates by the user. Afterwards the preferred candidates can be manually evaluated by various search and visualization tools. The probe match tool allows a fast search of all probe binding sites within the complete dataset, including a user-defined number of mismatches. Quality and position of the mismatches are indicated and target and/or non-target sequences are automatically shown in the tree of the ARB main window for an intuitive overview of probe coverage and specificity. Moreover, probe binding sites including mismatches can be visualized on the alignment level as well as in the secondary structure viewer of ARB (Figure 2). In the ARB editor and the probe match tool, the pre-calculated probe sequences can also be altered by hand to easily access the impact of these modifications.

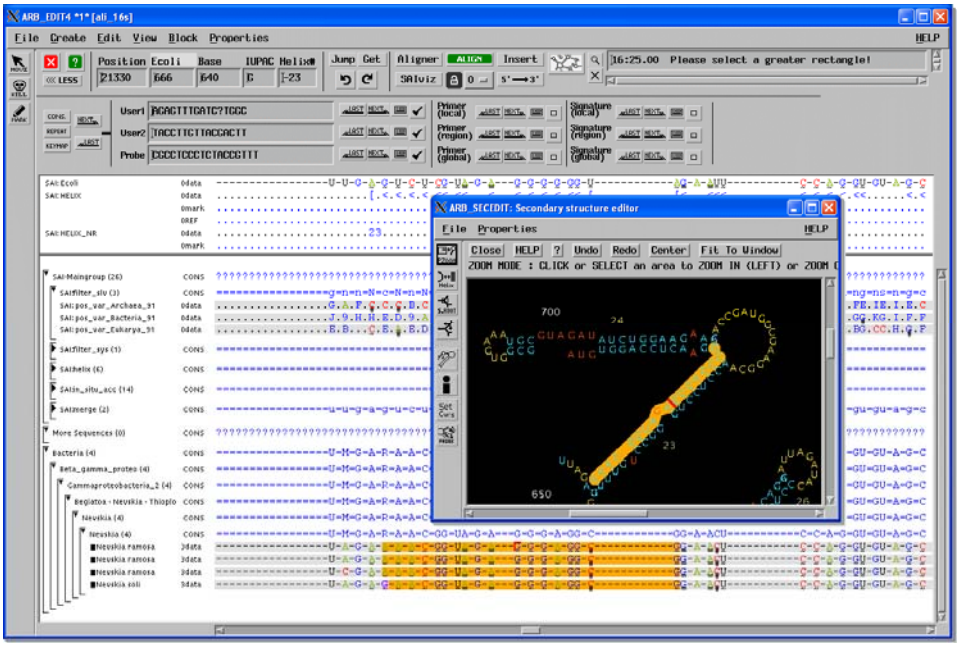

Figure 2: The ARB primary and secondary structure editor. Probes or primers can be easily visualized (orange).

Conclusions

The combination of ARB and SILVA provides a flexible software workbench for sequence analysis as well as comprehensive, quality controlled, richly annotated and aligned reference rRNA databases to support the molecular assessment of biodiversity, as well as investigations of the evolution of organisms. Applications of the databases range from basic research in microbiology and molecular ecology to the detection of contaminants and pathogens in biotechnology and medicine. Molecular taxonomy and diagnostics have already revolutionized our view on microbial diversity on Earth (Hong *et al.* 2006; Pedros-Alio 2006; Sogin *et al.* 2006), and the added value of molecular techniques for the determination of eukaryotic diversity has recently been documented by Tautz et al. (2002). The ARB/SILVA duet is designed to assist in the daily effort to keep pace with the increasing amount of data flooding our general-purpose primary databases.

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Development of genus specific primers for identification of Tobamoviruses

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Abstract

The detection and differential diagnosis of plant pathogens via polymerase chain reaction (PCR) is already routine and important especially in cases for which no suitable antibodies are available or the highest sensitivity is of importance. As in most of cases the detection of a virus in the sample is enough to exclude it from further processing, a genus or even family specific test would suffice. This reduces costs and time for testing. However, a second test using the amplicons allows differential diagnosis down to the species or even beyond. In this paper we describe the development of genus specific primers suitable to detect species of the genus *Tobamovirus*.

Introduction

With the increase of sequence information we have now the ability to develop not only species-specific primers, but also primer pairs that are suitable to amplify whole genera or even families. The facts that especially support this for plant viruses are briefly:

1. the tendency of plant viral coat proteins (CP) to be highly conserved at the species level due to the lack of CP-directed antibodies in their hosts
2. the additional function of the CP for vector transmitted viruses to support transmission and its function for long distance transport in the plant hosts
3. the over centuries proven suitability of the CP as taxonomically most important protein for species demarcation by serology.

The latter point indicates in addition that enough diversity exists inside the CP to allow for differentiation using the amplified CP ORF as shown by Letschert *et al.* (2002).

The first paper on this topic was published in 1999 by van der Vlugt *et al.* who demonstrated that many members of the family *Potyviridae* could be detected by one primer pair flanking part of the CP. Since then many more, at least genus-specific, primers have been described: for Tobamoviruses (Letschert *et al*., 2002), Potex-viruses (van der Vlugt & Berendsen, 2002), Viti- and Foevaviruses (Dovas & Katis, 2003) and Nepoviruses (Digiaro *et al.*, 2007).

The identification of genome areas suitable for general primers with the ability to react with more than one species of a taxon can be done with many different approaches. Alignment of known sequences would appear to be the simplest and led to success in case of the Potyvirus primers. Choosing other targets with even more conservation to be expected could be a solution as shown by the work published by van der Vlugt and Berendsen (2002) or Dovas and Katis (2003) for Closteroviruses where the RNA dependent RNA polymerase was selected. In these cases, highly degenerated primers were necessary for success.

For the genus *Tobamovirus*, we have chosen another approach to ascertain that the primers to be developed were safe against genetic diversity. For Tobamoviruses, two regions flanking the coat protein are highly conserved in their sequence resulting in specific structures of the unpacked RNA. These structures are necessary for viral replication and particle formation. At the very 3'-end, the RNA can fold into a tRNA-like structure that becomes aminoacylated with histidin (Figure 1a). Upstream of this, mostly in the movement

protein ORF, is a second highly conserved loop structure which is necessary to initiate particle assembly, called "origin of assembly" (Figure 1a). For these two sequence stretches forward and reverse primers were developed (Figure 1c) which at least for the subgroup (sg) 1 cluster allowed the amplification for all member species (Figure 1b).

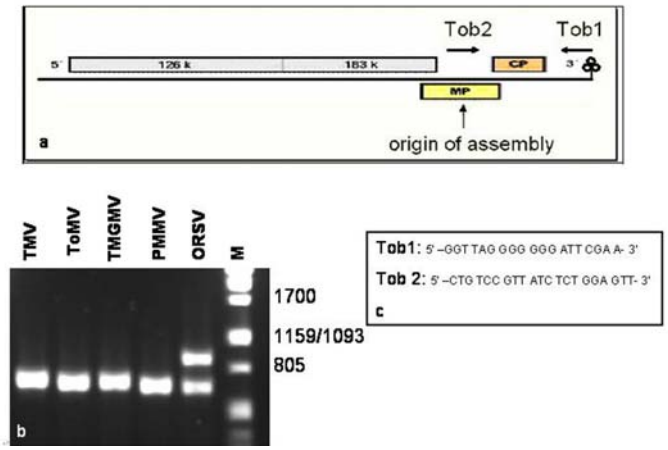

Figure 1: Development of general primers for subgroup 1 of Tobamoviruses. a: Genome structure of TMV and position of the primers; b: Amplification results with different virus species of sg 1 (the double band with ORSV is due to a duplication of the reverse primer sequence, see also Figure 3); c: Sequence of the primers

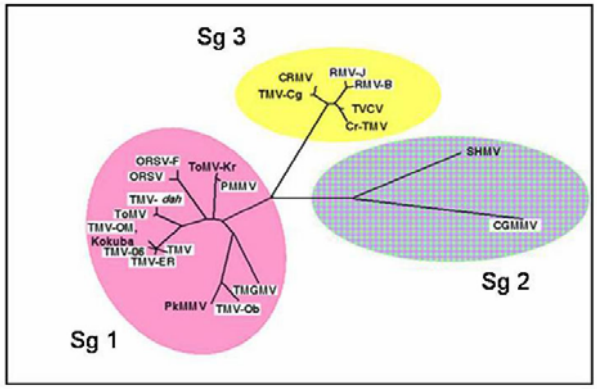

Figure 2: Clustering of the genus Tobamovirus into subgroups (modified from Lartey et al., 1996).

According to the nucleic acid sequence data of their CP, members of the genus *Tobamovirus* form three clusters that correlate with host range differences and serological crossreactions (Lartey *et al.*, 1996; Heinze *et al.*, 2006) (Figure 2). Members of sg 2 and sg 3 could not be amplified by the primer pair for sg 1. However, available sequence data of the sg 2 and sg 3 species indicated that the reverse primer Tob 1 should fit and only the forward primers had to be adapted for each subgroup (Heinze *et al.*, 2006).

Material and Methods

The virus isolates that we have used are summarized in detail in two publications (Letschert *et al.*, 2002; Heinze *et al.*, 2006). We have used at least seven different isolates per virus species. Each isolate was cloned by three successive local lesion transfers before propagation on systemic hosts.

The amplified CP of each isolate was inserted into a plasmid and transformed into *Escherichia coli* (Letschert *et al.*, 2002). Five independent transformed colonies were picked, their plasmid re-isolated and the insert sequenced. Only if all five sequences revealed no significant differences and their serological reactions in ELISA as well as immuno-electron-microscopy were unequivocal, the viral isolate was declared clean.

From the determined sequences, we have developed species-specific forward primers inside the CP ORF (Table 1) allowing either species identification by semi-nested PCR starting from the PCR-products obtained with genus-specific primers, or a species-specific RT-PCR using Tob1 as reverse primer.

The species-specific primer oligonucleotides were also tested *in silico* with the program "Amplify" for Macinthosh computers. To accomplish this, a test-DNA sequence was generated from known amplicon sequences to be tested (see Figure 3 for accession numbers). If the specific forward primer binds on any other position in this generated test sequence, the used software would generate a virtual amplicon.

Table 1: Species-specific Primers used

Virus species	specific forward primer
TMV	5’ CGG TCA GTG CCG AAC AAG AA 3’
ToMV	5’ CGG AAG GCC TAA GGA AGG GAA GC 3’
TMGMV	5’ AAR TAA ATA AYA GTG GTA AGA AGG G 3’
PMMV	5’ GGG TTT GAA TAA GGA AGG GAA GC 3’
ORSV	5’ AGG TGA TAG TGA TGT TGG TAT T 3’

Results and Discussion

We could show that one reverse and three forward primers with only few degenerated nucleotides could successfully be used to amplify the complete or almost complete CP of the accepted and also tentative species of the genus *Tobamovirus*. We could also show that, for each species, a specific forward primer could be designed that allowed a differential diagnosis via a semi-nested PCR using the amplicons of the general RT-PCR (Figure 4).

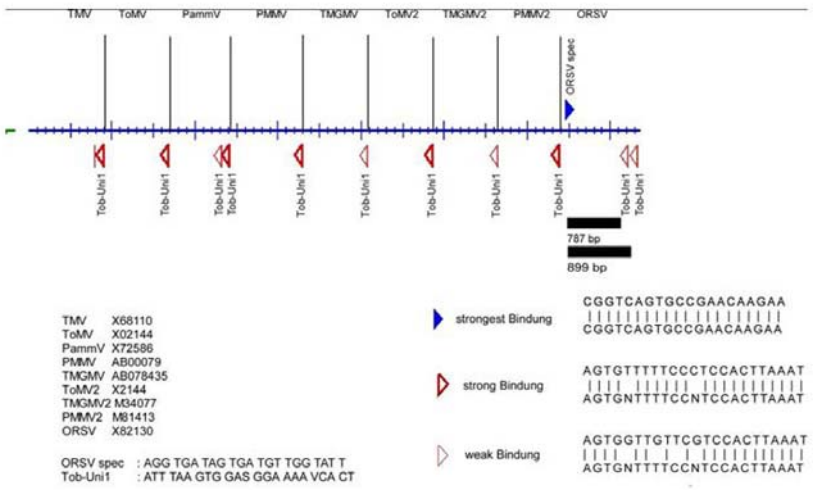

Figure 3: Virtual PCR with ORSV-specific oligos. Tobacco mosaic virus (TMV), Tomato mosaic virus (ToMV), Paprika mild mosaic virus (PaMMV), Pepper mild mosaic virus (PMMV), Tobacco mild green mosaic virus (TMGMV), Odontoglossum ring spot virus (ORSV).

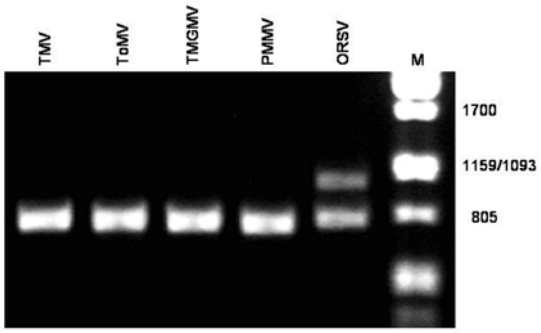

Figure 4: Semi-nested PCR with species-specific forward primers. After a general RT-PCR, part of the amplified products was used for a semi-nested PCR and the obtained products were separated on a 1% agarose gel. Acronyms are as in Figure 3. M = λ DNA digested with PstI (number of bp are indicated at right).

The results from the *in silico* testing (Figure 3) suggested that the unique oligonucleotides should only prime with their specific sequences. This was confirmed by the results shown in Figure 4 where semi-nested PCRs amplified only the respective specific target.

These results show that specific oligonucleotides are suitable to serve as forward primer together with the general tobamovirus reverse primer for species specific RT-PCR even when mixed infections are present. It may be also possible to use these 22 to 25-mer oligonucleotides as capture probes in an array approach similar to the work described by Zhang *et al*. (2005).

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Applications in virus detection

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Abstract

Currently, a wide variety of methods is available to diagnostic testing services and is in use on a day-to-day basis. The methods used are diverse including morphological identification using microscopy or electron microscopy, methods detecting proteins from the organisms such as ELISA or electrophoresis, molecular methods identifying the nucleic acid of the organisms such as PCR, RT-PCR, real-time PCR, reverse PAGE, nucleic acid hybridisation and finally traditional bioassays such as inoculation of test plants or growth on selective media. For true diagnostic purposes i.e. identification of the causal agent of disease, often the more traditional investigational techniques are most useful. For viruses, this often means test plant inoculation and electron microscopy. In the context of diagnostics, microarray methods could enable the detection of large numbers of target pathogens using a generic technology.

Introduction

Accurate diagnosis of disease causing agents is an essential prerequisite for effective control. To this end a wide spectrum of methods have been developed and are currently in use in diagnostic laboratories on a day-to-day basis. Most of the methods used for viral disease diagnosis are geared towards specific detection of a single target. However, there are two scenarios when this is less than ideal, firstly when presented with a symptomatic plant with unknown aetiology, and secondly, when screening for the health of a given plant, e.g. during the vegetative propagation chain for ornamentals. In each of these cases, it is common for a number of different methods to be executed in parallel to reach a final result.

Some 'multi target' generic assays have been used for plant virus diagnosis, although most of these methods continue to be useful as investigational tools they can be limited to a small range of targets with a specificity often limited to the genus level. Electron microscopy has been used for many years as a 'multi target' assay and although very useful for detecting and discriminating rod shaped particles, the presence of spherical viruses is very difficult to detect. In each case it is common to only identify an unknown to the genus level. Assays based on infectivity, can also be described as multi target, however, completely 'universal' indicators do not exist for all viruses and many viruses are not mechanically transmissible. Detection of viral coat protein using mass spectrometry such as MALDI TOF (Matrix Assisted Laser DIssorption Time Of Flight mass spectrometry) has been reported for viruses (Thomas et al., 1998) and can also be described as a generic. However, these methods are only of very niche application to viruses which reach unusually high titres in some hosts and are of no use for viruses that do not have a protein target. All of these methods have a basic drawback in common, since each is based on a property that is common at the genus level (e.g. particle morphology, coat protein size or local lesion host). Diagnosis to species level will often require further testing with another method. Microarray technology could allow the detection of a large number of different viruses in a single generic assay (Boonham et al., 2007). This paper describes a method developed for the detection of plant viruses using a microarray based on oligonucleotides spotted onto glass slides and a dual colour reporting system.

Materials and methods

Design and printing of arrays

The arrays comprised of oligonucleotides, 50nt in length, amino-linked at the 5'end, spotted onto epoxy coated glass slides. The approach used for designing the oligonucleotides was to download all the sequences available for the viruses of interest from the EMBL database and compile multiple sequence alignments using the Clustal V method from the MegAlign multiple alignment package (DNA Star). Conserved regions were identified within the sequence of a given species, which would give discrimination between the species of interest. In addition the Basic Local Alignment Search Tool (BLAST at http://www.ncbi.nlm.nih.gov/BLAST/) was used to identify any homology with the chosen probe designs and other genes (in particular plant genes) present on the database.

The microarrays were fabricated by MWG Biotech (Ebersberg, Germany), and were based on 5′amino-modifed oligonucleotides spotted in duplicate to epoxy coated glass microscope slides (Nexterion E, Schott NA Inc.). The slides were stored in a cool dark place ready for hybridisation.

Nucleic acid extraction

RNA extractions were carried out using a CTAB method adapted from Chang et al., (1993) followed by further clean up using an RNeasy column (Qiagen Ltd., Crawley, UK). RNA was eluted from the column using 200µl of nuclease free water. An aliquot of the resulting RNA was diluted in nuclease free water and the concentration estimated following spectrophotometric estimation at 260 and 280 nm. RNA was extracted from plants infected singly with *Turnip mosaic virus* (TuMV), *Tomato bushy stunt virus* (TBSV), *Pepino mosaic virus* (PepMV) and from uninfected plants.

Labelling of cDNA

Synthesis, labelling and purification of cDNA were carried out from 50µg of RNA using the CyScribe™ Post-Labeling Kit (Amersham Pharmacia) following manufacturer's protocols. The procedure involves synthesis of cDNA incorporating amino allyl (AA) dNTP's followed by post-labelling of cDNA with reactive Cy Dye. The strategy of labelling was to extract total RNA from both infected and healthy plants. The RNA from the infected plants was converted to cDNA and labelled with Cy5 whilst the RNA from the infected plants was converted to cDNA and labelled with Cy3. The two labelled cDNA targets were then mixed together and hybridised to an array.

Hybridisation and washing

Array hybridisation was carried out using array buffers supplied by MWG Biotech and Gene Frames (ABgene) following the manufacturer's protocols. Briefly, labelled cDNA was mixed together and with 30µl of array hybridisation buffer and heat denatured for 3 min at 95°C, before being incubated on ice for 3 min. The labelled cDNA was then pipetted onto the array surface and gene-frame cover slip applied. Hybridisation was carried out in an array cassette to maintain humidity (HybChamber - Gene Machines, California, USA) for 16 h at 42°C. Following incubation the slides were washed for 5 min in 2x SSC containing 0.1% SDS, 5 min in 1x SSC and 5 min in 0.5x SSC. All wash steps were carried out in pre-warmed buffer at 30°C. The slides were dried and stored in opaque slide boxes at room temperature.

Microarray scanning

The hybridised arrays were scanned using an Axon Instruments GenePix 4000B array scanner (A1-Biotech, Bedford, UK), using GenePix Pro 3.0 software. Scanning was carried out at 532nm and 635nm with photomultiplier tube (PMT) settings in the range (600-950) in order to minimise the number of saturated pixels.

Results

Since a dual label approach was taken, cDNA for the control genes should be present in both the Cy3 and the Cy5 labelled reactions. Thus when this hybridisation is performed on the array, the control spots should give signals at both 532nm and 635nm and should appear yellow in a ratio/overlapping image. Any virus RNA from the test plant should be labelled only in the Cy5 reaction and the cDNA will not be present in the

healthy plant (labelled with Cy3) thus the virus spots should appear red in the ratio image. The results (figure 1) show that detection mostly occurred only with the homologous capture probes.

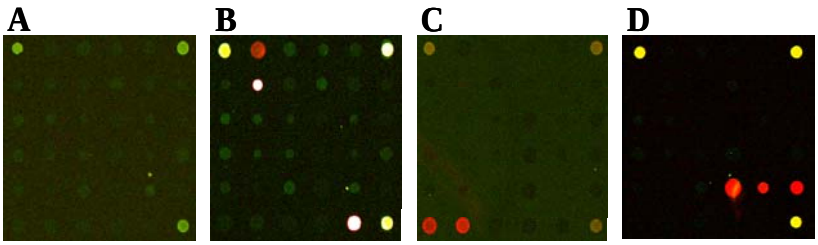

Figure 1: Examples of ratio array images from a healthy plant (A) and plants infected with (B) *Pepino mosaic virus, (C) Tomato bushy stunt virus* and (D) *Turnip mosaic virus*.

Discussion

Many methods, either molecular or serological, have been developed and published for virus diagnostics. Most of these methods are directed at very specific detection of an individual virus target. As such these methods are very suited to survey work, and also often used for diagnosis or screening. In this function, they are usually used as one assay in a panel of assays for the detection of possible disease causing agents (parallel testing) or to confirm the identity of a disease causing agent (identified or suspected) following the testing by another method (confirmatory testing). In an ideal world, diagnostic (as opposed to detection) methods tend to be much more 'investigational' in as much as they can be used for "finding the causal virus and recognising it" (Bos, 1999) with no prior knowledge of the agent being investigated. In the diagnostic arena, microarray methodology is currently being used to streamline parallel screening as it is currently carried out. These panel type screens are often based around hosts or groups of hosts, and array methods have been published for a number of plant infecting viruses (Boonham et al., 2003; Lee et al., 2003; Bystricka et al., 2003; Bystricka et al., 2005; Deyong et al., 2005; Agindotan and Perry, 2007).

For array techniques to really succeed in making an impact in the virus detection arena, a number of technical drawbacks, highlighted by initial work, need to be overcome. Firstly, the sensitivity of the approach is low when compared to PCR methods, and although useful level of sensitivity is achieved ideally a generic screen would be more sensitive than ELISA. Secondly, design of oligonucleotides is an issue in which a significant number of oligonucleotide probes designed do not perform as expected.

In addition to these technical problems, current approaches are based on the detection of a list of known targets, as such they do not answer one of the more fundamental problems, that of detection of unknowns. In areas of plant pathology where pests or disease causing organisms are isolated (either as individuals in the case of insects or in culture in the case of bacteria and fungal pathogens), the approach of sequencing conserved genes is becoming a tool of trade. The method has been used for many years, but is now generically referred to as 'DNA barcoding' and enables not only the identification of known (and sequenced) organisms but also the identification of unknowns. The method is based upon the generic amplification of conserved genes and as such enables the generation of sequence data from previously un-described organisms. Viruses do not have conserved genes that enable generic amplification, so a method based on array hybridisation to more broadly specific regions of sequence (perhaps at the genus level) may enable the detection of unknowns within known genera. This approach may however ultimately be superseded by modern *de novo* direct sequencing techniques. Methods based on pyrosequencing enable the generation of very large amounts of sequence from a library of the 'unknown'. If the host genome sequence is known, the pathogen sequence (including any new or unknown targets) can be extracted from it in an approach that could be completely generic regardless of the nature of the target pathogen or host (Cox-Foster et al., 2007).

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Electrochemical methods for DNA and protein detection

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Abstract

Recently, electrochemical methods have become an important tool for detection of high molecular weight analytes such as DNA and some proteins. The DNA and the majority of proteins are redox inactive at potentials in the range of -600 mV to +600 mV *vs.* Ag/AgCl that are used for amperometric detection. For this reason there are several general strategies for electrochemical detection of mentioned analytes: (i) based on application of redox labels and followed by amperometric detection; (ii) direct detection of formation affinity based complexes by registration of impedance changes. Direct detection methods are the most attractive as they facilitate and simplify the detection procedure. Some direct electrochemical methods for detection of DNA and proteins are introduced in this chapter. To perform impedimetric detection, special modification methods that form layers sensitive to target DNA or protein on the surface of electrodes are desired. In addition significant changes in capacitance and/or electrochemical resistance after formation of DNA or protein complex are requested. Some conducting polymers are applied for such modification purpose. Polypyrrole (Ppy) is one of the most extensively used conducting polymers in design of DNA and protein sensors. Here, we review recent advances in application of polypyrrole in immunosensors and DNA sensors. Significant attention is paid to immobilization of biologically active Ppy molecules during electrochemical deposition of this polymer. Some unique properties of this polymer, including formation of molecular imprints and prevention of some undesirable electrochemical interactions, are discussed.

Keywords: Bioelectrochemistry; Conducting polymers; Polypyrrole; Biosensor; DNA sensor; Immunosensor; Molecularly imprinted polymers; Nanotechnology; Electrochemistry; Fluorescence; QCM; SPR.

Introduction

Bioanalytical methods are rapidly evolving towards detection of target protein and DNA because both types of analytes are very important for biomedicine, forensic investigations, biochemistry and biotechnology. Various immunoassays and nucleic acid-based tests have been well established for many years as the cornerstone of detection technologies. These assays are sensitive, selective and, in general, highly resistant to interference from complex sample matrices. However, both antibody- and nucleic acid-based detection systems require *a priori* knowledge of the target and development of specific reagents; multiplexed assays can become increasingly problematic when attempting to detect a plethora of different targets, the identities of which are unknown (Ngundi et al., 2006). In an effort to circumvent many of the limitations inherent in these conventional assays, other biological recognition systems – biosensors – were developed as more competitive alternatives. Up to now the majority of DNA and protein detection strategies in biosensors, DNA-chips and protein chips were based on optical detection methods with clear domination of fluorescence detection. However, optical detection is not always optimal, especially if the samples are turbid and/or coloured and/or contain fluorescent materials or fluorescence quenchers. All these mentioned problems may be avoided if electrochemical methods are applied. Electrochemical methods are evolving rapidly towards solving of challenging bioanalytical problems, including specificity, stability and sensitivity.

Recently, electrochemical methods became an important tool for detection of high molecular weight analytes like DNA and some proteins. The DNA and the majority of proteins are redox inactive at potentials in the range of -600 mV to +600 mV *vs.* Ag/AgCl which potential is the most used for amperometric detection. For this reason, there are several general strategies for electrochemical detection of mentioned analytes: (i) based on application of redox labels and followed by amperometric detection; (ii) direct detection of formation of affinity based complexes by registration of impedance changes. Direct detection methods are the most attractive since they facilitate and simplify the detection procedure. Some direct electrochemical methods for detection of DNA and proteins are introduced in this chapter. To perform impedimetric detection, special modification methods that form layers sensitive to target DNA or protein on the surface of electrode are desired. In addition, significant changes in capacitance and/or electrochemical resistance after formation of DNA or protein complex are requested. Some conducting polymers can be exploited as an excellent tool for the preparation of biologically sensitive layers with sensitivity to target DNA and target proteins. Some conducting polymers doped and/or covalently modified by biomaterials exhibit unique affinity properties (Ramanaviciene and Ramanavicius, 2004a) that can be easily applied in the design of electrochemical sensors.

Polypyrrole is one of the most extensively used conducting polymers in design of bioanalytical sensors (Adeloju and Wallance, 1996). Versatility of this polymer is determined by a number of properties: redox activity (Han et al., 2005), ability to form nanowires at room temperature with conductivity ranging from 10^{-4} S cm^{-1} to 10^{-2} S cm^{-1} (Khomenko et al., 2005), ion-exchange and ion discrimination capacities (Johanson et al., 2005, Weidlich et al., 2005), electro-chromic effect depending on electrochemical polymerization conditions and charge/discharge processes (Krivan et al., 2005), strong absorptive properties towards gases (Chehimi et al., 2004), proteins (Azioune et al., 2005), DNA (Saoudi et al., 2000), catalytic activity (Raoof et al., 2004, Khomenko et al., 2005, Ramanavicius et al., 2004), corrosion protection properties (Hi et al., 2005), etc. Most of these properties are depending on the synthesis procedure as well as on the dopant's nature. Polypyrrole (Ppy) is one of the most extensively used conducting polymers in the design of DNA and protein sensors. Polypyrrole might be electrochemically generated and deposited as semiconducting layer on the electrodes made of different conducting materials including platinum, gold and carbon. Versatility of Ppy is determined by the following: its biocompatibility, its capability to transduce energy arising from interaction of analyte and analyte-recognizing-site into electrical signals that are easily monitored, its capability to protect electrodes from interfering materials, its easy electrochemical deposition on the surface of any type of electrodes. Nowadays, Ppy becomes an important tool for nanotechnological applications (Malinauskas et al., 2005). Electrochemical Ppy deposition technique is under development with various types of electrochemical sensors and biosensors. Dependent on biomolecules entrapped within Ppy during electrochemical deposition of this polymer, the electrochemical affinity sensors are divided into: (i) immunosensors based on immobilized proteins exhibiting affinity (Ramanaviciene and Ramanavicius, 2002), (ii) DNA sensors based on covalently immobilized and/or entrapped ssDNA (Wang, 1999, Wang and Jiang, 2000, Ramanaviciene and Ramanavicius, 2004c), (iii) affinity sensors based on molecularly imprinted polymers (Ramanaviciene and Ramanavicius, 2004b).

The aim of this study is to review major advances and applications of electrochemical methods in the design of affinity sensors including DNA sensors, immunosensors, and molecularly imprinted polymer based sensors. As conducting polymer, polypyrrole, among other polymers, is used most in design of electrochemical biosensors. Therefore, the main attention will be focused on applications of this polymer.

Discussion

Chemical and Electrochemical polymerization of polypyrrole

According to our knowledge, the conducting polymer polypyrrole was for the first time chemically synthesized by Angeli in 1912 (Angeli, 1916). But it did not receive any considerable attention until the conducting state of conjugated polymers was detected by AJ. Heeger, AG. MacDiarmid and H. Shirakawa (Chiang et al., 1977). Nowadays, polypyrrole is synthesized by conventional chemical methods and more innovative and versatile electrochemical methods. Several major ways are applied for polypyrrole synthesis which are based on induction of polymerization by different factors: (i) chemical initiation by oxidative agents (Angeli, 1916, Henry et al., 2001), (ii) photo induced synthesis (Fang et al., 2002), (iii) electrochemical polymerization by anodic current (Schuhmann et al., 1990). With all mentioned

technologies, synthesized Ppy is insoluble in all common solvents because of strong inter-chain interactions (Oh et al., 2001). All polymerization initiation methods mentioned have particular applications, e.g. chemical initiation by oxidative agents might be successfully applied if a large amount of polypyrrole is needed for application in the design of chromatography columns (Deore et al., 1999) or for some other purposes. By using chemical (Henry et al., 2001) or even biochemical methods (Ramanavicius et al., 2005), it is easy to prepare Ppy particles of different and/or controlled size, ranging from several nanometers up to several micrometers and/or containing various inclusions. Moreover, by chemical methods it is possible to uniformly perform over-oxidation of this polymer, which is of special interest for affinity chromatography since molecularly imprinted Ppy might be produced, which may exhibit selectivity to molecules ranging from the small organics (Liang et al., 2005, Ebarvia, 2005, Trojanowicz and Wcislo, 2005) to high molecular weight biomolecules (Ramanaviciene and Ramanavicius, 2004b). Photo-induced Ppy synthesis is attractive in photolithographic application of this polymer, since it allows alterations in synthesized Ppy morphology by change of excitation light wavelength and it might theoretically be applied for the design of electronic chips. However, because of a slow light induced polymerization rate, this polymerization type is still not very frequently applied compared to chemical or electrochemical polymerization.

By using chemically induced polymerization, the Ppy is mainly produced in bulk solution and just some amount of synthesized polypyrrole is covering the surface of introduced materials. It means that chemically induced polymerization is not very efficient with respect to deposition of Ppy on some surfaces. Moreover, Ppy is almost insoluble in common solvents, except when it is doped with proper agents that increase the solubility of this polymer (Pokrop et al., 2004), but it also means that deposition (e.g. by solvent evaporation) of this polymer from the solution is possible at the stage where the polymer is still in the form of colloid particles, i.e. before its precipitation (Ramanavicius et al., 2005). However, the major obstacle for use of this deposition method for designing of Ppy based sensors is a poor adherence of Ppy synthesized by this technique to the electrode surfaces. Such disadvantages might be reduced if electrochemical polymerization on specially pre-treated conducting surfaces is applied (Schuhmann et al., 1997). It allows deposition of Ppy over the electrodes placed in any type of electrochemical cell. The electrochemical polymerization has found an application as a general method for deposition of thin Ppy layers. By varying of the current passing through the electrochemical cell, potential thickness and morphology of deposited Ppy layer might be controlled (Schmidt et al., 1993). Different solvents including acetonitrile and water might be applied for electrochemical deposition of Ppy. As the Ppy synthesis might be performed from water solution at neutral pH, it is useful for entrapment of various biomaterials within polypyrrole. Such biomaterials might be small organic molecules, proteins, DNA, viruses and even living cells. Buffers with high buffering capacitance are requested for such electrochemical polymerization/entrapment. Otherwise, local production of a large amount of protons in the course of the polymerization may negatively affect the properties of the biomolecules to be entrapped within Ppy. Electrochemically synthesized Ppy has some attractive features, such as good conductivity and very high adherence of these films to the mostly used substrates for biosensor design leading towards sufficient stability of biosensors, even in a neutral pH region. On the other hand, the electrochemical properties of Ppy strongly depend on the redox state of this polymer. Moreover, electrochemical polymerization is applied for deposition of polypyrrole layers inside geometrically complicated electrochemical cells (Habermuller and Schuhmann, 1998) and there is almost no doubt that this polymerization method might be extremely useful for deposition of Ppy layers inside microfluidic devices including lab-on-a-chip devices and other bio-chips.

In particular cases, over-oxidized Ppy might be synthesized electrochemically. Then, entrapped molecules and/or dopants might be extracted from the Ppy structure by application of special solvents. In such cases, the so called molecularly imprinted polymers (MIP's) might be designed.

At positive potentials, an over-oxidation of Ppy is occurring, leading to lowering of Ppy conductivity and originating in an easier leakage of anionic molecules if they were included into the polymeric backbone. Over-oxidation of Ppy appears at lower positive potentials in a water and/or oxygen containing environment and in this case it is leading to partial destruction of the polymeric backbone and generation of oxygen-containing (carboxyl, carbonyl and hydroxyl) groups. Over-oxidized Ppy has been used in many electroanalytical applications that utilize its permselectivity and is often used as discrimination membrane, which significantly increases selectivity of electrochemical biosensors (Ramanavicius, 2000, Geise et al., 1991). This technology enables to prepare large variation of nanostructured polymeric layers with different analytical characteristics even if the same bulk solution is used for polymerisation. In terms of possible

variations and control of polymerization conditions, electrochemical polymerization is generally more versatile than the chemical one. It was demonstrated that the surface of electrochemically deposited Ppy after some additional procedures of electrochemical/chemical functionalization might be covalently modified by enzymes (Ramanavicius et al., 2000). Those structures were applied in a biocatalytic biosensor design, and it was demonstrated that Ppy layers modified by the same enzyme exhibit significantly different selectivity towards various substrates if different Ppy modification approaches are applied. Moreover, combination of electrochemical techniques with some chemical surface modification techniques is useful for development of new nanostructured polymers. In some particular cases, chemical methods are more convenient for technological applications. Chemically synthesized polypyrrole might be applied for affinity chromatography purposes (Trojanowicz and Wcislo, 2005). Chemical and electrochemical Ppy synthesis methods are finding application in different technological areas including affinity sensors. The capability of electrochemical polypyrrole synthesis is significantly extended, since some different electrochemical techniques might be applied for deposition of Ppy over the electrodes: constant potential electrodeposition, galvanostatic deposition, cyclic voltammetry, and potential pulse techniques (Schuhmann et al., 1997).
According to our experience based on application of conducting polymers in biosensor design, the pulsed potential technique is the most suitable for nanostructuring of Ppy by entrapment of biologically active materials within backbone of this polymer (Schuhmann et al., 1997).

Formation of biological recognition part of DNA and protein affinity sensors

In the majority of affinity sensors, immobilization of biomolecules including single-stranded DNA is a key factor during the creation of any type of biosensors and/or bio-chips. To reach a high analytical information density, miniaturization of separated bi- recognition parts is required (Livache et al., 1998). Several different strategies have been used in common DNA- and Protein-arrays: a mechanical spotting or synthesis of oligonucleotides (ODN) on an activated support (Yershov et al., 1996, Blanchard et al., 1996), a photochemical process (Pease et al., 1994) and an electrochemical process (Livache et al., 1994). Among mentioned methods only electrochemical polymerization involves the application of conducting conjugated polymers (CP) such as polypyrrole. Some steps of this technology were described in previous articles (Livache et al., 1994, Ramanavicius, 2006). The electrochemical oxidization of pyrrole forms, in one step, a solid polypyrrole film, which covers the surface of the electrode. The geometrical shape and size of the formed Ppy layer is limited to the electrochemically active surface of the electrode. Electropolymerization allows significant miniaturization of spots used for electrochemical detection. Efficiency of electrochemical Ppy deposition has been recently demonstrated in DNA-sensors devoted for genotyping (Livache et al., 1998). In addition, the versatility of this immobilization procedure allows the immobilization of polypeptides (Heiduschka et al., 1996) and proteins including enzymes (Ramanavicius et al., 1999, Schuhmann et al., 1997, Shin and Kim, 1996, Schuhmann and Kittsteiner-Eberle, 1991). In addition, molecular imprints for detection of proteins with rigid 3D structure might be formed if such proteins are added into polymerization bulk solution and are removed from the polymeric structure after formation of polymeric layer (Ramanaviciene and Ramanavicius, 2004b).

Electrochemical detection methods used for DNA and Protein detection

Concerning the detection of analytical signal, there are two major types of DNA and protein detections: i) one requires some additional electrochemically active labels, ii) the second is based on detection of electrochemically detectable changes without application of additional electrochemically active labels. Application of electrochemically active labels allows significant increase in sensitivity of bioanalytical systems for DNA and protein detection, as these analytes in most cases are almost electrochemically inactive. Application of additional redox labels allows to use more basic electrochemical detection techniques including amperometric detection at constant potential and/or application of cyclic amperometric detection. Additional electrochemically labelled compounds including ssDNA fragments or secondary antibodies that are labelled with redox active organic compounds and redox enzymes are applied in mentioned amperometric affinity sensors. Sometimes in DNA sensors, redox-active DNA intercalating compounds are applied that can generate specific amperometric signals.
However, working in “label-free mode” is more attractive compared to labelled detection methods, as it allows: i) to reduce number of chemicals involved in detection, ii) to reduce number of manipulations requested for analysis, iii) to measure analytical signal in “real-time” (Warsinke, 2000). Usually, in label-

free detection the amperometric signals are low because any auxiliary redox processes are registered. In this case, just small changes in charge densities and/or conductivity of electrochemical system are exploited for transduction of analytical signal. It seems that the mechanism of the Ag-Ag interaction at CP based electrodes involves the variation in the capacitive properties of the polymer modified with materials exhibiting biological recognition (Shin and Kim, 1996). Reduction of the number of additional reagents including very reactive redox mediators is a key issue for the development of bioanalytical systems that can operate *in vivo*. Such systems are mainly devoted for detection of polypeptides, biologically active proteins and antibodies that exhibit selectivity towards compounds that are applied in this case. However, direct detection based on electrochemical affinity sensors is less sensitive compared to methods based on indirect analyte detection (Skladal, 1997).

Regarding electrochemical techniques employed for detection of analytical signals, affinity sensors might be traditionally divided into conductometric, amperometric, capacitive and potentiometric sensors.

Conductivity measurements are most simple from the technical point of view and have been adapted in immunoassays where secondary antibodies labelled by enzymes were applied. The major requirement for such enzymes is the ability to catalyse reactions generating a high number of ions. Enzyme -urease- is sometimes applied in this kind of conductometric sensors.

Traditional constant potential amperometric techniques have been used mainly for the indirect detection of DNA and proteins. Redox mediators as well as redox active enzymes are usually applied in this case for the generation of amperometric signal. To monitor binding events without using any labelled compounds usually alternating potential amperometric techniques are applied. Sinusoidal alteration of potential at different frequencies is applied where so-called electrochemical impedance spectroscopy is used for detection of interaction between immobilized biologically selective compound and analyte of interest (e.g. target DNA or proteins). The potential steps are applied in the case where so-called *pulsed amperometric detection* is used for the detection. In pulsed amperometric detection as well as in electrochemical impedance spectroscopy based methods, potentials are applied between working electrode modified with biological recognition layer and the reference electrode. Registered current peaks, if potential pulses are applied, and changes in electrochemical impedance spectra might be directly related to the concentration of the analyte in solution (Bender and Sadik, 1998). A conducting polymer modified electrode has been used in affinity sensors for DNA and protein detection. Such sensors are mainly based on electrochemical impedance measurements or based on pulsed amperometric detection (Ramanaviciene and Ramanavicius, 2004b, Ramanaviciene and Ramanavicius, 2004c). Besides the amperometric detection methods, methods based on electrochemical capacitance measurements can be used for the real-time and label-free measurement of the interaction between affinity reagents. The capacitance of such system depends on the thickness and dielectric properties of biological recognition layer before and after interaction with analyte (Moreno-Hagelsieb et al., 2007).

Potentiometric detection usually exploits relationship described by the Nernst equation: $E=E^0+RT/zFlna_m$ where E is the electromotive force, E^0 is the electrode standard potential, R the gas constant, T the absolute temperature, z the ion charge, F the Faraday constant and a_m the activity of the measured ion m^{Z+}. The altered surface potential, generated at an ion-selective electrode, provides a route to detect the ionic product formed by an enzymatic reaction. Regarding to Nernst equation, the change of electrode potential is proportional to the logarithm of ion activity, if the other cell potentials of the overall electrochemical cell remain constant.

Typically, potentiometric detection is less sensitive than amperometric detection. The detection limit usually is in the order of micromoles in potentiometric detection. Moreover, this method lacks selectivity, and a complex relation between ion activity and ion concentration results in some uncertainties in applying the Nernst equation precisely to detect analytes in real samples. In addition, potentiometric electrodes are dramatically influenced by pH and ionic strength (Kuann and Guilbault, 1987). To reduce the here mentioned disadvantages, specialized ion-selective electrodes are applied for more precise measurement of analyte concentrations (Oesch et al., 1986). Such electrodes might be directly integrated into Ion-Selective Field Effect Transistors (ISFET) (Janata, 1994). Here, the ISFET gate is modified by a special polymeric layer, which is sensitive to certain ions. In such a structure, surface potential is generated in the gate region by surface ions from a solution. This locally generated potential then modulates current flow across the silicon semiconductor present in the ISFET structure.

Theoretically, conductometric measurements might be applied for indirect electrochemical detection of

DNA and proteins. However, conductivity measurements are difficult due to the variable ionic background of clinical samples and the relatively small conductivity changes that are observed in such high ionic strength solutions. A solution in this case is application of a second comparative 'blank' electrochemical system (Ghindilis et al., 1998). Future potentiometric immunosensor may employ the technique of ion-channel switches. The basis of this innovative technique is that recognition events, e.g. antibody-antigen binding, change the number of properly aligned and properly functioning ion channels across a lipid bilayer membrane and thus ionic conductance change is achieved. This technique is applicable to receptor-, antibody- and nucleotide-based applications to detect picomolar analyte concentrations in complex media, not lower because of the amplification due to the ionic flows (Cornell et al., 1997).

In indirect electrochemical sensors for detection of DNA and proteins, the binding reaction is visualized indirectly via an auxiliary reaction by an electrochemically active compound. Amperometric transducers in indirect electrochemical immunoassays are used much more frequently than others (Livache et al., 1998). As antibodies and antigens are usually not electrochemically active within the desired potential range, redox-active compounds can be applied as labels for indication (Schuhmann et al., 1997). For the amperometric detection at constant potential, electrochemical labels should have the following properties: they should be reversibly electroactive, should not cause electrode fouling, and chemical groups for covalent-attachment should be available (Treloar et al, 1994). Electrochemically detectable species, such as nitrophenol, H_2O_2, and NH_3 that are substrates or products of enzymatic reactions catalysed by alkaline phosphatase, horseradish peroxidase, and urease, are important for electrochemical detection. Nitrophenol and H_2O_2 mostly are detected amperometrically at constant potential, while ammonia is electrochemically inactive at low potentials and can only be detected potentiometrically by an ammonia gas-sensing electrode (Liu et al., 2000).

Indirect electrochemical detection of DNA and proteins can be divided into two major subtypes: non-amplified and amplified electrochemical detection. In non-amplified electrochemical detection, the presence of one molecule of analyte will generate one electrochemical equivalent of registered signal, such detection is usually based on application of basic redox molecules like ferocene derivatives. Since the sensitivities of amperometric sensors for the redox compounds usually are in the micromolar range, non-amplified assays make only sense if the concentrations of analytes are in such a range (Yershov et al., 1996).

For more sensitive detection of DNA and proteins, the amplification of electroactive equivalents is required. A relatively basic way to amplify any kind of analytical signal is a pre-concentration step. However, the so-called "redox recycling" allows amplification of analytical signal without any pre-concentration (Deore et al., 1999). In this case, the redox compound is oxidized and reduced in a cyclic manner so that the detection of one labelled antigen or antibody molecule will generate multiple signal equivalents. Redox recycling can be performed in different ways by using electrode-electrode, electrode-enzyme or enzyme-enzyme couples (Schuhmann et al., 1997).

Recent developments and conclusions

During the last four years, we have developed an electrochemical system for detection of target DNA, which was based on pulsed amperometric detection (Ramanaviciene and Ramanavicius, 2004c). Pulsed amperometric detection allowed to simplify electrochemical equipment required for detection of target DNA binding. The same system was applied for detection of protein binding towards molecularly imprinted polypyrrole (Ramanaviciene and Ramanavicius, 2004b). In all studies related to DNA and protein detection, we have applied differently modified layers of conducting polymer, i.e. polypyrrole. According to our experience such conducting polymer layers are very useful for development of such electrochemical systems, as signal changes registered by pulsed amperometric detection method are several orders of magnitude higher than when such semiconducting layers are not applied. Nowadays, in detection of DNA and proteins, electrochemistry is often combined with surface plasmon resonance (Zhang et al., 2007, Kausaite et al., 2007), quartz crystal microgravimetric measurements (Wei et al., 2006, Ramanaviciene et al., 2004) and fluorescence measurements (Willner et al., 2007, Ramanavicius et al., 2007) to enhance sensitivity and selectivity of combined methods.

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Protein chip applications

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Abstract

The aim of this work was the testing of a diagnostic tool for the detection of allergenic proteins from stored product mites/cockroaches and house dust mites. Species-specific antibodies were tested for specificity and cross-reactivity, and detection limit by ELISA method. In this work, protein chips were manufactured either manually, using a MicroCaster, or robotically, using a SpotArray. The proteins tested were six recombinant Kunitz-type protease inhibitors (PI), two of group A (with anti-cathepsin D and anti-trypsin activity), two of group B (with anti-trypsin and anti-chymotrypsin activity) and two of group C (with anti-cathepsin B activity), and the soybean Bowman-Birk inhibitor. These proteins were immobilized onto epoxy activated glass slides. Protein chips were hybridised with serial dilutions of extracts from adult mites/insects and from spent growth media (SGM) in order to capture the allergenic proteases. Protein A was labelled with Alexa-555 and used for detection of polyclonal antibodies directed against the proteases. The detection limit was 0.5µg protein extract for *Aleuroglyphus ovatus* and 0.75µg for *Tyrophagus putrescentiae* specimens and protein extracts from the faeces fraction (SGM). By comparison, the detection limit of ELISA method was 2.5µg. The protease inhibitor-chips can allow rapid screening of proteases and their specificity by analysing the binding to each class of inhibitors. Results of protease binding to each KPI and recognition of allergenic proteases showed the practicability of protein microarrays as a diagnostic tool for detection of mite/insect contaminants in foods and work environment.

Introduction

In the past few years, protein chips have become a powerful tool for diagnostics and analysis of protein functions and protein-protein interactions. This technology allows fast, easy and parallel detection of multiple addressable elements from a minute amount of sample in a single experiment. It has been applied to analyse protein and protein-protein interaction as well as antibody-antigen, enzyme-substrate and enzyme-inhibitor interaction (Mc Beath and Schreiber, 2000; Templin et al., 2003; Cretich et al., 2006). In the recent years, the European Community and ESF supported the research on protein chips and the collaboration of institutes with main focus on these technologies (Schellenberg and Stribel, 2004). To this aim, the COST853 network activity was established to develop tools for Array technology dedicated to the detection of agricultural biomarkers.

Assay systems based on chip technology are currently applied for the identification, quantification and functional analysis of proteins. Protein chip technology is of major interest for proteomic research as well as for diagnostic applications since this miniaturized and parallelized assay systems can be used for the identification of biomarkers and the validation of potential target molecules. However such technology shows some problems in terms of protein stability. These problems are due to the unstable structure and functionality of protein. Protein-protein interaction takes place by different means such as electrostatic forces, hydrogen bonds and/or weak hydrophobic van der Waals interactions (Templin et al., 2003). Moreover protein interaction does not only depend on their primary structure, but also on their tertiary structure. For these reasons, an improvement in the efficiency of protein immobilization on the slide surface,

in terms of functional conservation and of the reproducibility of the amount of immobilized protein is needed.

Proteins can be immobilized onto substrates in a non-covalent interaction by adsorption on glass slides coated with poly-L-lysine (Haab et al., 2001), agarose (Afanassiev et al., 2000), polyacrylamide, hydrogels (Arenkov et al., 2000), on membranes made with polystyrene, poly-vinylidene fluoride (PVDF), on thin nitrocellulose film (FAST slides, Schleicher-and-Schuell), either on hydrophobic or hydrophilic surfaces (Angenendt et al., 2003); or in a covalent manner on glass surface functionalized by aldehyde residues, epoxy groups (Tam et al., 2002), or even by using cross-linking agents. A drawback of random covalent immobilization is a partial block of the active sites of the proteins that become then less accessible to the interacting molecules. The covalent binding enhances the stability of immobilized proteins but could cause changes in the protein conformation. Structural modification of the protein can result from the immobilization through interaction between the support and the protein, or from changes in the protein microenvironment. The non-covalent adhesion of antibodies to nitrocellulose has been extensively used in ELISA method. However, an oriented immobilization could provide better spatial accessibilities of active binding sites than non-oriented immobilization. Therefore the greatest difficulty in the realization of a protein-chip is the preservation of functionality of the immobilized proteins.

An application of protein chips is the activity-based detection of enzymes on a microarray, such as the screening of anti-protease specificity using fluorescently-labelled inhibitors. In this approach, commercial enzymes are immobilized onto epoxy-activated slides and incubated with labelled inhibitors: the formation of inhibitor-bound enzyme complexes is detected by a fluorescence-based microarray scanner. The screening of both enzyme activity and specificity in a chip format has been also successfully performed. A series of peptide substrates conjugated with fluorogenic coumarine bound to aldehyde-activated surfaces (Salisbury et al., 2002) were used to screen enzymatic cleavage and release of coumarin and to find substrate specificity of proteases. In a similar study, different proteases and phosphatases were incubated with fluorogenic substrates spotted onto poly-L-lysine or amino activated glass slides, to screen for their activities (Uttamchandani et al., 2005).

Using protein chip technology, we report in this paper the use of a protein microarray system for detection of insect proteases, known as major allergens in food and environment, using recombinant Kunitz-type protease inhibitors (KPI) immobilized onto a glass slide. Previously, we have validated the KPI chip applied to the characterisation of protease specificity towards potato KPI inhibitors induced by fungal infection, and in characterisation of new KPIs cloned in wild varieties of the *Solanum* genus (Speransky et al., 2007). Kunitz-type inhibitors (22-24 kDa) are a polymorphic class of plant protease inhibitors found in seeds, storage organs and vegetative tissues, and play a role in plant defence against pathogens and insects (Ryan, 1990). The Kunitz-type inhibitors found in potato are represented by three major homology groups A, B and C. KPI-A group includes inhibitors of aspartic proteinases such as cathepsin D. The KPI group B contains inhibitors of serine proteinases grouped in the chymotrypsin clan, as trypsin, chymotrypsin, and elastase. The KPI group C includes proteins that inhibit bacterial subtilisins and plant cysteine proteinases as well as some non-proteinase enzymes such as invertase and α-amylase. Considering the role of Kunitz-type inhibitors as components of plant defence mechanisms against insects and pathogens (Linthorst, 1991; Boulter, 1993; Schuler et al., 1998; Turrà, 2006), their polymorphism may be the result of adaptive evolution of the plant in response to insect and microbial or fungal pests. It is known that insects and microbial pathogens are able to adapt to these inhibitors and to render them ineffective (Marchetti et al., 2000). This can be counteracted by a large repertoire of functional inhibitor variants and the evolution of new variants (Lison et al., 2006). Therefore, the development of a microarray strategy to perform high-throughput screenings of protease inhibitors could be a valid tool either for evolutionary studies or for discovering new inhibitors for applicative use. The Kunitz-type inhibitors were chosen as model to study the interaction between protease and protease inhibitors on a glass slide and to set up an on-chip inhibitory assay protocol since the inhibitory activity against specific proteases are usually assayed by biochemical tests. The proteases were chosen from the results of protease inhibition specificity using colorimetric assays, and from protease binding assays using the protein chip format.

Stored product and house dust arthropods include many species affecting human health. Mites as *Acarus siro*, *Dermatophagoides farinae*, *Tyrophagus putrescentiae,* and beetles as *Tribolium castaneum* are often found in contaminated stored products and farming environments. The bodies and metabolites of these arthropods can contaminate stored foods and grain, thus introducing health-threats through the food chain.

At least twenty classes of major allergens are known to be present in house dust and stored product mites. Six of these protein classes are proteases/hydrolases (Table 1). The proteases are serine-type (chymotrypsin, trypsin-like) and cysteine-type (Stewart and Robinson, 2003). In addition, other enzymes with hydrolase activity can also be targeted by the Kunitz-type inhibitors with unknown function (as the anti-invertase activity present in some KPI group C protein). A further level of selectivity was introduced in the detection step, performed using species-specific polyclonal antibodies. The method described in this paper, based on binding and purification of mite or beetle enzymes, and detection with species-specific antibodies, can allow high-throughput, rapid screening and content of proteases, addressing also the enzyme specificity by its binding to each KPI.

Table 1: The major allergen classes in house dust mites, storage product mites and storage products insect.

Class	Biochemical function	Stored product species				Dust species		
		Ac. siro	*Gly. domesticus*	*Lep. destructor*	*Tyr. putrescentiae*	*Blo. tropicalis*	*Dermatoph. farinae*	*Dermatoph. pteronyssinus*
1	Cysteine prot.	Aca s 1	Gly d 1	Lep d 1	Tyr p 1	Blo t 1	Der f 1	Der p 1
2	Aspartic protease							
3	Trypsin	Aca s 3		Lep d 3	Tyr p 3	Blo t 3	Der f 3	Der p 3
4	Amylase					Blo t 4		Der p 4
5								
6	Chymotrypsin			Lep d 6		Blo t 6	Der f 6	Der p 6
7								
8	Glutathionate-S-transferase							
9	Collagenolytic serine protease			Lep d 9				Der p 9
10	Tropomyosin							
11	Paramyosin							
12								
13	Fatty acid binding prot							
14	Vitellogenin/Apolipophorin							
15	Chitinase			Lep d 15				
16	Gelsolin/villin							
17	Ca binding protein							
18	Lysozyme-like							
19	Anti-microbial peptide							
20	Arginine kinase							

Material and Methods

Kunitz-type inhibitor expression purification and inhibition assays

Trypsin, chymotrypsin, cathepsin B, papain, subtilisin, pancreatic elastase, protease inhibitors as the soybean Bowman-Birk inhibitor, and BSA were provided commercially (Sigma, St. Louis, US). The sequences corresponding to the mature inhibitor polypeptides from *Solanum stoloniferum* (KPI-A), *S. palustre* (KPI-B) and *S. tuberosum* (KPI-C) were cloned in the pQE30 expression vector, to transform *Escherichia coli* BL21 DE3 strain. The KPI sequences were expressed in *E. coli* cultures (100 ml) grown at 37°C, transferred to 25° C and induced with 1 mM IPTG for 24 h. Cells were harvested by centrifugation at 5000 g and resuspended with 5 mL of Bugbuster (Novagen, Merck, Darmstadt, DE) supplied in 50 mM Tris-HCl buffer, pH 8.0 containing benzonase. After incubation for 30 min at room temperature, the homogenates were centrifuged at 16000 g for 20 min at 4 °C and the clear supernatants applied to a His select cartridge column (1.25 ml, Sigma). Elution was carried out according to the manufacturer's instructions. The KPI proteins, purified by Ni+ affinity column, were not further HPLC processed, due to the good purity of recovered proteins. Protein identity was checked by western blot using a polyclonal antibody raised against potato PKPI B10 protein (Speransky et al. 2007).

Recombinant Kunitz-type inhibitor proteins were tested for their ability to inhibit the serine proteases trypsin, α-chymotrypsin, elastase, subtilisin and the cysteine proteases papain and cathepsin B at different molar ratios of inhibitor and protease by using N-benzoyl-arginine-nitroanilide as substrate according to previous reports (Santino et al. 1998). Group B KPI proteins were biochemically characterised and

differentiated (determination of Ki using bovine trypsin). In further experiments KPI-B1 and KPI-B4 were used among the group B cluster, as their Ki values (345 nM and 84 nM, respectively) indicated high bovine trypsin inhibition even at low substrate concentrations. The KPI-B proteins were also found to inhibit trypsin activity in mite extracts.

Chip production

We used epoxy-group surface activation of glass slides for the covalent binding of proteins. Glass slides were cleaned in piranha solution (H_2SO_4 : H_2O_2, 7 : 3) for 2 hours. The slides were washed thoroughly three times with deionised water and ethanol (95% w/v), and dried with flushing nitrogen. For the functionalization with epoxy-groups, cleaned slides were then incubated for 2 hours with a solution containing ethanol, acetic acid (0,1 % w/v) and 3-glycidoxypropyldimethoxymethylsilane (1% w/v) in deionised water. After incubation the slides were cleaned with ethanol 3 times and incubated at 150 °C overnight. The slides were then washed with ethanol three times and dried under flushing nitrogen.

Protein immobilisation and hybridisation

Six recombinant His-tagged Kunitz-type protease inhibitors (KPI-A1 and, KPI-A2 for KPI group A, KPI-B1 and KPI-B4 for group B, KPI-C3 and KPI-C4 for group C) from *Solanaceae* species were chosen for immobilization onto the epoxy-activated slides. All proteins were desalted with Zeba desalt spin columns (Pierce, Rockford, US). The recombinant Kunitz-type protease inhibitors, bovine serum albumin (BSA), as negative control, and the commercial soybean Bowman-Birk inhibitor were diluted at different concentration (0.1, 0.2, and 0.4 mg/ml) in $NaHCO_3$ (0.1M, pH 9) / glycerol (40% w/v) buffer and arrayed on epoxy- slides. Other spotting buffers were also tested: trehalose, saccharose and polyethylene glycol were used as hydrating agents and compared to glycerol for homogeneity and reproducibility of spot sizes. Printing was performed in triplicate for each protease at 4°C and about 60% humidity. A MicroCaster (Schleicher-and-Schuell, Whatman, Brentford, UK) was used to print manually the slides, delivering 60-80 nl drops. The robotic printing using the SpotArray 24 (Perkin Elmer, Waltham, USA) allowed deposition of 1 nl drops with miniaturisation of the sub-arrays, good reduction of spot variability and higher reproducibility of results compared to the manually printed spots. The slides were incubated at room temperature (RT) for 2 hours in a humidity chamber (70% humidity). The resulting protein chips were sealed in a box and kept in dark for storage at 4 ºC. Experiments with slides stored for periods of two to three months performed as well as the freshly printed slides. Before use, the slides were incubated for 1 h on a shaker at RT in a solution containing 0.5M glycine and 1% w/v BSA in phosphate buffer saline (PBS) to block the free epoxy groups, then washed with PBS + BSA (0.1% w/v) and dried by centrifugation at 500 g x 2 min.

Control of the protein chip

Hybridisation control was performed to evaluate the result of KPI printing: 1 mg of protease was labelled with Alexa-555 protein labelling kit (Molecular Probes, Invitrogen, Carlsbad, US) and purified to remove free dyes with gel-filtration biogel P-6 (Bio-Rad, Richmond, US) according to the manufacturer's protocols. 50 ul of protease in Tris buffer (0.1M pH 7.5 for trypsin and chymotrypsin and 0.1M pH 6.5 for cathepsin B) containing the labelled proteases (5 µl of stock solutions, serially diluted to 0.01, 0.1 and 1mg/ml final concentrations) was applied to the printed slide using the coverslip method and incubated for 1 h in the dark at RT in a sealed chamber (70% humidity). After incubation the slides were subsequently washed with PBS containing 0.2% Tween-20 and 0.1% BSA, with shaking at 4°C during 15 min, then washed twice in PBS + BSA (0.05% w/v) with shaking at 4°C during 5 min and dried by centrifugation at 500 g during 2 min. The slide was scanned with Array Scanner 428 (Affymetrix, S. Clara, US).

Detection of mite/insect allergenic proteases by capture on the protease inhibitor chip

Mite cultures (*Acarus siro*, *Dermatophagoides farinae*, *Aleuroglyphus ovatus, Lepidoglyphus destructor*, *Tyrophagus putrescentiae*) and rabbit immunization to produce polyclonal antibodies were done at VURV in Prague. Protein extracts of frozen adults or from spent growth media (SGM) were weighted, washed with water and homogenised in water or PBS. Protein concentration was determined using the Bradford method (Bio-Rad). Further experiments were made using 5-10 µl of total protein extract (1 to 1.5 µg/µl) diluted 1:5

or 1:10 in PBS, of which 20 µl aliquot was used in hybridization assays (final concentration of total proteins between 0.5 and 1 µg). The Protease inhibitor chips were hybridised with this protein fraction, washed with Tween-20/Tris buffer saline (TBS-T), as in Western blot protocols, then incubated with the polyclonal antibody, diluted 1:20 (0.5 µg/ml). After removal of aspecifically bound antibodies by washing in TBS-T; the slides were incubated with the protein A fluorescently labelled for the detection of signals (Figure 1). Slides were scanned with an Affymetrix 428 dual-laser scanner, and images saved as TIFF and Bitmap files using ScanAnalyse. The sensibility of antigen detection on protein chips were compared to results obtained using dot blot and ELISA assays, detected with the same polyclonal antibodies (Kudlikova et al 2004).

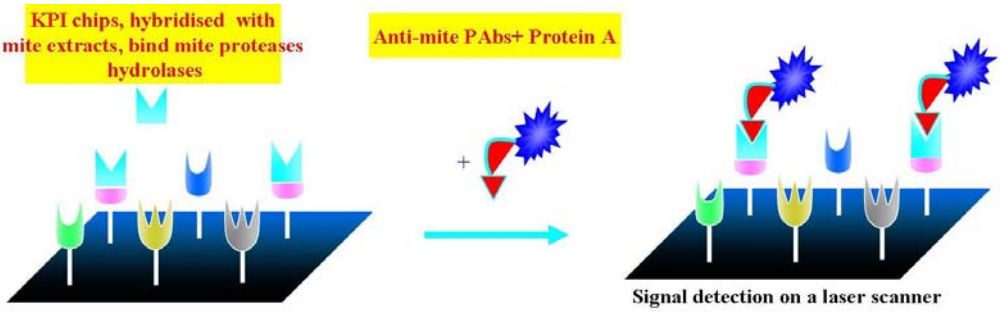
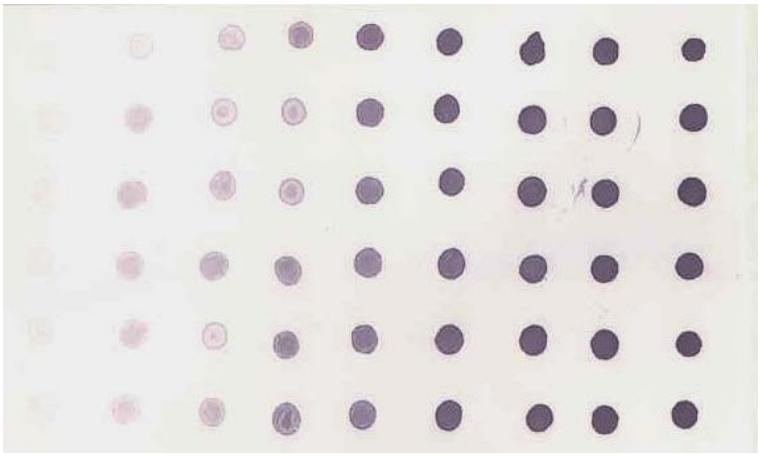


Figure 1: Scheme of the experimental protocol for mite proteins hybridisation and detection with polyclonal antibody/ protein A complex. Total mite extracts were incubated with the protease inhibitor chips (left). Then, the protease-protease inhibitor interaction was visualised by the binding of polyclonal antibodies specific to that mite species.

Results

A series of His-tagged potato Kunitz-type protease inhibitors (KPI), two of group B (KPI-B1 and KPI-B4); two of group A (KPI-A1 and KPI-A2); and two of group C (KPI-C3 and KPI-C4) were purified from recombinant *E. coli* cell cultures and used for biochemical characterization of proteases. In a colorimetric assay using protease substrates (Santino et al. 1998), papain, elastase, cathepsin B, cathepsin D, chymotrypsin and trypsin were tested for inhibition of their activity. These assays showed that trypsin was inhibited by recombinant KPIs of groups A and B but not by group C; chymotrypsin was inhibited only by group B while cathepsin B was inhibited to some extent by all groups (data not shown).
Then, we tested the anti-mite antibodies produced in-house for the detection of mite proteins using dot blot hybridisation as an established method for detecting small amount of contaminating proteins in a complex protein mixture (Figure 2).



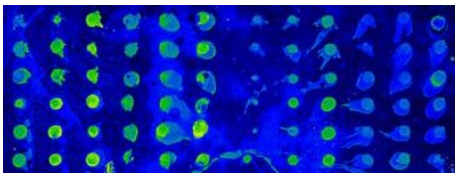
Concentrations: 1 2.5 5 10 25 50 75 100 µg/ml

Figure 2: Example of detection of mite proteins using dot blot method. This biochemical assay gave results similar to those obtained by sandwich-ELISA, using anti-mite polyclonal antibodies and secondary antibodies labelled with horse-radish peroxidase. Sensibility of the assay was good, starting from 1 µg/µl.

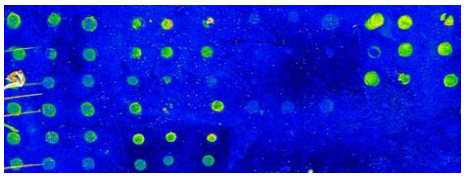
Then, we shifted to KPI protein chip combined with the protein A-polyclonal antibody for mite protease allergen detection. The first challenge of the protein chip was in chip manufacturing. In this work the partial denaturation of proteins after binding to the hydrophobic surface in silanized glass was studied. The proteins used for spotting required the addition of protective substances in the spotting buffer, and we tested the addition of glycerol (MacBeath and Schreiber, 2000), trehalose (Kusnezow et al., 2003), saccharose (Avseenko et al., 2001) and polyethylene glycol (PEG) of low molecular weight (Lee and Kim, 2002).The recombinant Kunitz-type inhibitors and the positive controls were reconstituted at increasing concentration (0.1, 0.2, and 0.4 mg/ml) in $NaHCO_3$ buffer with 40% protective agent and spotted in triplicate. The array was designed to contain the soybean Bowman-Birk inhibitor (SBBI) as trypsin /chymotrypsin binding control. Glycerol in these tests was found to outperform all the other additives. Glycerol enhanced the binding and stability of KPIs resulting in higher fluorescence signals, which could be related to prolonged hydration of proteins (Olle et al. 2005). In this case, the proteins remained hydrated for longer time during the incubation step after the printing process, probably because water evaporated more slowly in the glycerol-containing spots. This allowed the completion of reaction and covalent binding between epoxy functional groups on the slide surface and the functional groups (amine, hydroxyl and thiol group) in the proteins (McBeath and Schreiber 2000).

Kunitz-type protease inhibitors were further characterised towards their affinity for protease after binding to the glass surface to compare the proportion of native form and evaluate the level of denaturation using lowest concentrations of trypsin. We compared the immobilization of trypsin and chymotrypsin inhibitors printed in replicate onto epoxy slides in presence of glycerol (40% w/v), and visualised the hybridization with trypsin labelled with Alexa-555. Serial dilutions of the protease were tested. Protein activity on the array surface was studied for each protease-protease inhibitor interaction to evaluate binding independently of the amount and density of protein bound in each spot (Gordus and Mc Beath, 2006). The binding and interaction between trypsin and SBBI, KPI-B and KPI-A inhibitors at decreasing trypsin concentrations (10-100 ng/µl) was analyzed. High sensitivity of detection was found at the lowest trypsin dilutions for KPI-A inhibitors, and was put in relation to highest stability of KPI-A bound to the glass. Conversely, group B KPIs showed to have lost 50% of trypsin binding activity compared to their protease-binding activity in solution. This could have been caused by the steric hindrance, lower rotation freedom after covalent attachment, or protein denaturation caused by the hydrophobic glass surface. However, due to high trypsin affinity, the decrease of native KPI-B in respect to total KPI-B spotted did not affect the ability to capture trypsin and chymotrypsin at 100 ng/µl or higher concentration levels. Therefore, the protein chip provided a simple, qualitative confirmation of protease inhibitor-protease binding and specificity for qualitative analysis. In quantitative analysis of inhibitory activity, to compare two or more inhibitors on the chip, various concentration points of the probe/substrate/protease partner were tested. Overall, the results showed that the immobilized inhibitors interacting with the proteases confirm the protease specificity results found using biochemical tests.

Biochemical assays showed that Kunitz-type PIs possessed anti-trypsin activity in mite and insect extracts. Our data allowed us to use our KPI chip as a new diagnostic tool for detection of mite allergens and as a protease capture system using the binding properties of the KPI toward such enzymes.

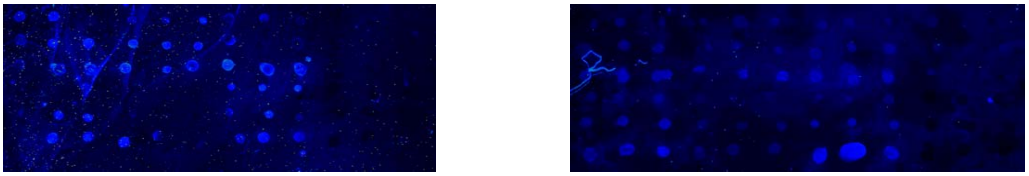


KPI-A2 KPI-B4 KPI-C4 SBBI
KPI-A1 KPI-B1 KPI-C3 BSA
***A. ovatus* (adults) 0.35 µg/µl**



KPI-A2 KPI-B4 KPI-C4 SBBI
KPI-A1 KPI-B1 KPI-C3 BSA
***A. ovatus* (SGM extracts) 0.1 µg/µl**

Figure 3: Comparison of detection of *Aleuroglyphus ovatus* proteases in adults and SGM extracts using protein chips.



KPI-A2 KPI-B4 KPI-C4 SBBI
KPI-A1 KPI-B1 KPI-C3 BSA
***T. castaneum* (adults) 0.2 µg/µl**

KPI-A2 KPI-B4 KPI-C4 SBBI
KPI-A1 KPI-B1 KPI-C3 BSA
***T. castaneum* (SGM) 0.1 µg/µl**

Figure 4: Comparison of detection of *Tribolium castaneum* proteases in adults and SGM extracts using protein chips.

Detection of allergen proteases and other hydrolytic enzymes was observed using Alexa-555 labelled protein-A and polyclonal Abs specific to mite species *A. siro*, *L. s destructor*, *T. putrescentiae, A. ovatus*, and to the beetle species *T. castaneum,* respectively. Positive results were obtained with extract dilutions as low as 1:20 (a high sensitivity limit, corresponding to 500 ng of total proteins), and good resolution at 1:5 dilution (corresponding to 7.5 µg of total proteins) (Figures 3 and 4, showing the detection of *A. ovatus* and *T. castaneum*, respectively). For most of the species, the total protein extract (1.5 mg/ml) did not produce good signals, whereas clear and better signals were observed starting from the 1:2 and 1:4 dilutions. It is known that these extracts contain lipids, chitin and polysaccharides that may block the protein interactions on the chip and could bind to the glass surface and to KPI probes, thus inhibiting the detection. We performed all the protein chip studies using serial dilutions of the total extracts starting from 1:4, 1:10 and 1:20. A good differentiation between SGM, rich in digestive proteases (Krizkova-Kudlikova et al., 2006), and arthropod adults, containing a different pool of proteases, was observed (Table 2). This differentiation was also made possible by the availability of different polyclonal antibodies specifically produced against different antigen sources (SGM, larvae, and adults). A possible contribution of the growth stage-specific antibody in the selective recognition of a particular class of protease cannot be excluded.

Table 2: Differential detection of proteases from various storage product pests.

	Trypsin/ chymotrypsin	Serine proteases	Aspartic proteases	Cysteine proteases or hydrolases
Aleuroglyphus ovatus adults	intense	intense	strong	none
Aleuroglyphus ovatus SGM	intense	strong	strong	intense
Tribolium castaneum adults	none	intense	strong	good
Tribolium castaneum SGM	none	weak	good	good
Lepidoglyphus adults	good	good	good	weak
Thyrophagus putresc. adults	intense	good	intense	none
Acarus siro adults	weak	intense	none	none

An other advantage of the protein chip method, compared to the dot blot method, is the reduction of hybridisation volumes with the concentration of the probe on a very small surface, so that the total protein amount is reduced between 10 to 20 times. Furthermore, the sensibility of the protein chip method is higher: concentrations useful for detection are only 0.2 µg/µl or higher, corresponding to a 5 times increase in sensibility compared to the dot blot method.

Discussion

The KPI protein chip experiments were made possible by the availability of a wide set of species-specific polyclonal antibodies, raised in rabbit against each mite or beetle species. This set of polyclonal antibodies, possessing low cross-reactivity and good discrimination at species or family level, were characterized in a previous work (Kudlikova et al. 2004). Specificity of antibodies allowed the differentiation of these food/ house contaminating pests using both the ELISA method and the protein chip experiments. In comparison to the performance of ELISA method, the resolution was good even with lower amounts of sample used, and sensibility was increased of 1 order of magnitude in respect to dot blot hybridization (500 ng in protein chips, compared to 5 μg of proteins in dot blot, Figure 2). This can be explained by the higher background caused by antibody binding to membranes, whereas the glass slide showed low signal-to-noise ratios.
Detection and differentiation of protease allergens was made possible using the protease inhibitor chips. In this work, the results of protein chip and dot blot techniques were compared to find the unique protease profiles for each of the species studied. Proteases are considered a threat because they can trigger allergy through their interaction with the immune system cells (Shakib and Gough, 2000). A diagnostic system able to recognise and detect low levels of allergen proteases could be very useful in food safety applications as well as in analysis of agricultural biomarkers. This protein chip tool could allow the detection and differentiation of insects and their excrements in contaminated food, as well as the possibility to detect specific classes of protease allergens. Further work is needed to assess the feasibility of protein extraction from environmental samples, but the results obtained using the spent growth media are encouraging and show the feasibility of detection from media where mite fed on, and which represents a prototype of the stored grain environment.

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Luminex technology:
Multiplex detection using xMAP technology.

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Introduction

Detection of pathogens in the food and feed production chain needs to be fast, reliable and preferably at low cost. Annually, millions of tests are run by inspection services and companies using a double antibody sandwich ELISA (DAS-ELISA). In each ELISA test, generally only one virus or bacteria is detected, requiring running a number of assays in parallel and this results in additional costs in terms of labour, disposables, chemicals and waste as compared to an assay in which all viruses or bacteria can be targeted simultaneously in one sample, namely a multiplex assay.

Several multiplex assays for plant viruses have been developed in the last few years, based on the detection of nucleic acids, but these methods have never been adopted by the inspection services for high throughput (HTP) screening. These technologies require laborious and expensive RNA purification procedures. Moreover, nucleic acid based amplification procedures are prone to cross-contamination and inhibition, resulting in false-positive and false-negative reactions, respectively.

For multiplex detection of plant pathogens, the Luminex xMAP technology may be a true cost-effective alternative for ELISA. This technology has proven its value already for high throughput multiplex screening in a number of fields such as human diagnostics (Kellar and Douglass, 2003) and food microbiology (Dunbar et al., 2003) by using protein and nucleic acid based techniques.

In the xMAP microsphere immunoassays (MIA), antibody-coated beads are added to the samples after transfer to a microtiter plate and this mixture is incubated. Subsequently the secondary antibodies conjugated with a reporter fluorochrome are added and measured on a Luminex 100 ST. Up to 100 different bead sets are available which can be analyzed simultaneously.

The workflow of this technology is comparable to ELISA; after sample preparation (Figure 1.1) the samples are transferred to a microtiter plate (Figure 1.2) and the pre-mixed beads coated with the antibodies are added (Figure 1.3) and incubated. The specific bead-antibody combination will recognize the antigens(s) (Figure 1.4). After washing the secondary antibodies are added and these will bind also to the antigens. After incubation the samples are measured (Figure 1.5) and the obtained results are graphically displayed (Figure 1.6). The signals of each bead set are shown in a separate histogram. In general the whole procedure can be performed in less than 2 hours.

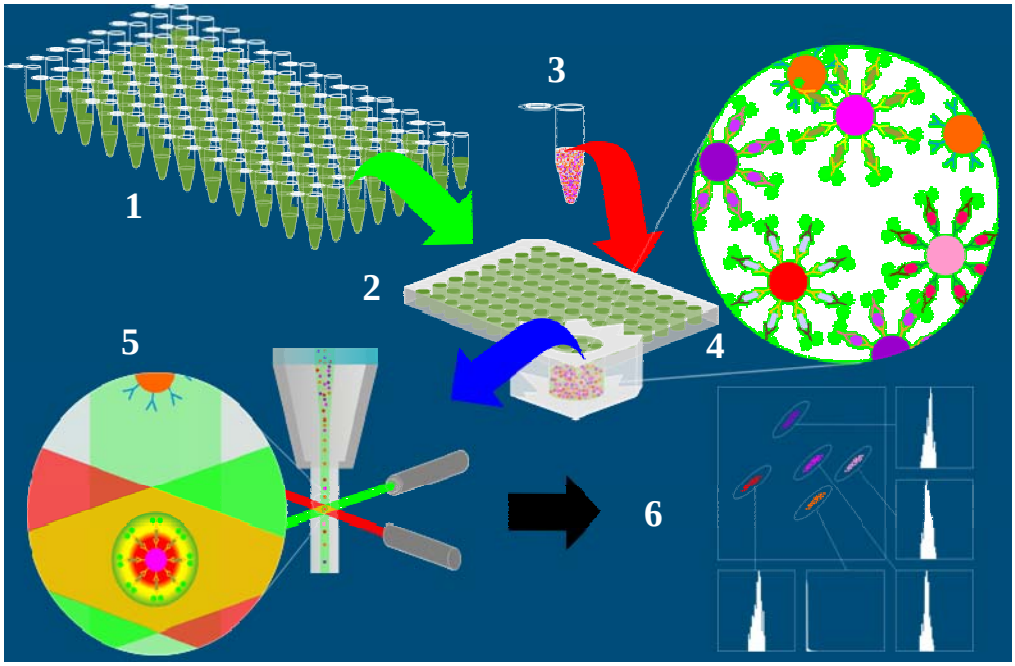

Figure 1: Schematic overview of the MIA workflow: 1. Sample preparation, 2. Sample transfer, 3. Addition of premixed bead-antibody combination to the samples, 4. Antibodies binding the antigens, 5. Performing of the measurement, 6. Display of the results.

Material and methods

Bead coupling was performed as described by (Peters et al., 2006). Sample extraction and wash, no-wash assays were performed as described by (Bergervoet et al., 2007). The major difference between a wash and no-wash procedure is the bead type used; for the no wash assay the standard beads are used, whereas for the wash assay paramagnetic beads are used. These paramagnetic beads allow the use of wash steps at different

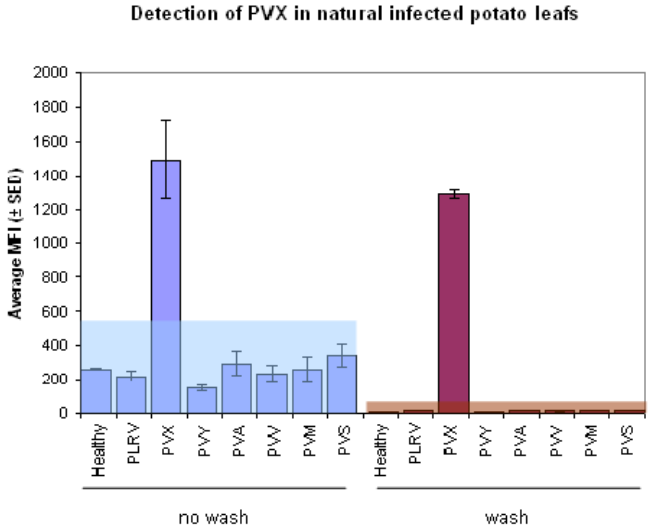

Figure 2: Detection of Potato virus X (PVX) in a simplex microsphere immunoassay. Naturally infected leaf extracts of PVY, PVX, PLRV, PVM: Potato Virus M; PVV: Potato Virus V; PVA: Potato Virus A and PBRSV (Potato Black RingSpot Virus were tested). Results obtained with a standard bead set are shown in the left panel; results obtained with paramagnetic beads are shown in the right panel. Detection signals exceeding twice the background values (shaded areas) are considered as a positive signal.

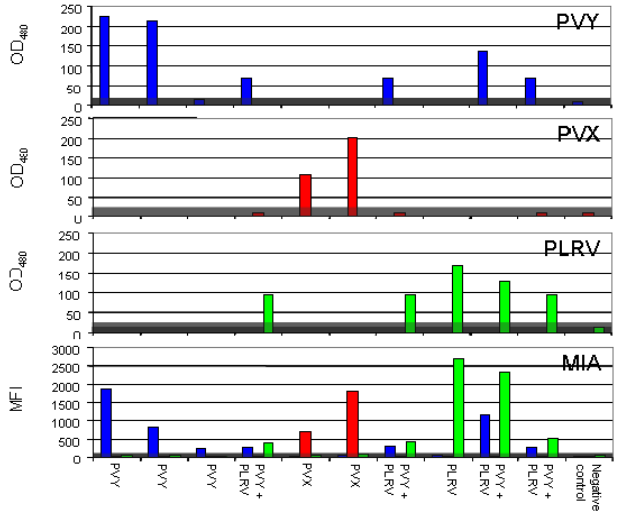

Figure 3: DAS–ELISA detection of Potato Virus Y (PVY ■), Potato Virus X (PVX ■) and Ppotato LeafRoll Virus (PLRV ■). Naturally infected leaf extracts and positive controls containing partially purified virus material were tested. Detection signals exceeding three times the background value (shaded areas) are considered as a positive signal (n=3).

points in the assay and in this way material which could obstruct the sample probe, or could interfere with the assay otherwise, can be removed.

Results

Some types of plant material are prone to cause problems using the Luminex analyzer because of probe clogging. When this happens, the use of paramagnetic beads could avoid this problem as washing steps could be included. Comparison of the standard beads versus the magnetic beads revealed that the signal to noise ratio improved. The average non specific signal strength which was of approx. 200 MFI using standard beads (Figure 2, left panel) decreased to approx. 50 MFI using paramagnetic beads (Figure 2, right panel).
When compared to the standard DAS – ELISA as performed by the Dutch inspection services, the MIA assay performed comparably (Figure 3). Results of the ELISA (Figure 3, panel PVY, PVX and PLRV) show that all viruses were detected correctly and that the negative control is below the background level. For the MIA, the results were comparable, all viruses were correctly detected and the negative control was below the background level (Figure 3, MIA panel). Furthermore, no false positive or negative were observed.

Conclusions

MIA offers a number of advantages when compared to ELISA. In MIA, a large number of beads (replicates) are measured per sample (typically 100–200), whereas for ELISA, only a few replicates per sample are used at maximum. This higher number results in an improved precision compared to ELISA. Another advantage of MIA method is speed; a complete MIA can be finished in approximately 2 hours, including the time used by the Luminex analyzer (approx. 30 min per 96-well plate), while the standard procedure for detection of potato viruses by DAS-ELISA is completed in 16 hours. Furthermore, the viruses can be detected in a multiplex assay, which reduces sample preparation time and requires less sample material, reagents and disposables. The multiplex MIA can also be extended to other potato viruses, thus increasing the benefit of MIA compared to ELISA.
For the comparison of standard beads versus paramagnetic beads, it can be concluded that in general the paramagnetic beads are superior to standard beads (used in a no wash assay), in respect to an improved signal to noise ratio and the avoidance of probe clogging. However, if probe clogging is not a problem, the no-wash assay has still an advantage above the paramagnetic beads because fewer steps are involved.

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Plant, animal and food pathogens analyzed using genome-wide microarrays: towards improved diagnostics and control of bacterial pathogens

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Abstract

The aim of the study was to utilize whole-genome sequence data of the important bacterial pathogens for designing microarrays and use them to compare genomic differences of the biologically different strains of bacterial pathogens of potato, *E. coli* infecting cattle and humans, and *Clostridium botulinum* causing food poisoning. All assays resulted in data that are useful in bacterial diagnostics.

Introduction

There are many bacterial pathogens that cause severe yield losses in cultivated crops and the harvested yield, are transmitted from animals to humans, or cause food poisoning and endanger human and animal health. Potatoes are affected by many bacterial pathogens that are widely distributed in potato production areas (Lehtonen et al., 2004; Van der Wolf and De Boer, 2007). *Clostridium botulinum* is a dangerous food pathogen that produces a potentially lethal, paralytic neurotoxin during its growth. Based on their physiology, *C. botulinum* strains are divided into four distinct groups I-IV, with groups I (proteolytic) and II (nonproteolytic) being human pathogens and producing neurotoxin types A, B, E, or F (Lindström and Korkeala, 2006; Sebaihia et al., 2007). Enterohaemorrhagic *Escherichia coli* (EHEC) is a food and waterborne pathogen with a very low infective dose. EHEC infection poses a significant risk of two serious complications, hemorrhagic colitis and haemolytic uremic syndrome. Production of Shiga toxins (*stx*) and adherence-mediating factor intimin (*eae*A) are considered important in the pathogenesis (Karch et al., 1999; O'Connel, 2007). Most EHEC outbreaks have been caused by *E. coli* O157 strains, and their main reservoir is considered to be cattle (Lahti et al., 2002).

The whole genomes of many bacterial pathogens have been recently resolved. Owing to the continuously improving efficiency of the DNA sequencing technologies, genomes of bacterial species and strains will be sequenced in increasing numbers and the accumulation of whole-genome data will accelerate. Hence, the whole-genome sequences of bacteria will become the most important basis for development of molecular diagnostic tools. The aim of this study was to utilize the currently available whole-genome sequence data of important bacterial pathogens in plants, animals and food for comparison of the biologically differing strains to obtain data that would be useful in bacterial diagnostics.

Materials and methods

DNA isolation and labelling

Total DNA was extracted with the appropriate methods optimised for the different bacterial species studied. DNA was quantified and fluorescently labelled with Cy3 or Cy5 (GE Healthcare).

Potato pathogens

Whole-genome sequences of *Erwinia carotovora* subsp. *atroseptica* (*Eca*) (recently reclassified as *Pectobacterium atrosepticum*), *Streptomyces scabies* (*Ss*) and *Clavibacter michiganensis* ssp. *sepedonicus* (*Cms*) were used. Probes were designed for all genes of *Eca*, ca. 50 % of the genes of *Ss*, and ca. 30 % of the genes of *Cms*. Probes were also designed for the 16S-23S intergenic region sequences, the *nip* genes of *Erwinia* species encoding a necrosis-inducing protein (Mattinen et al., 2004), and the pathogenicity island of *Streptomyces turgidiscabies* (*St*) (Kers et al., 2005). All sequence data except those for the *nip* genes and those for *Ss* and *Cms* whole-genome sequences were obtained from Genbank. Probes were designed using OligoArray 2.0 software (Rouillard et al. 2003). Melting temperature (Tm) range was calculated for 40-nucleotide (nt) probes whose GC content was 45-55 %. The resulting *Tm* range (82-90°C) was used as a design criterion for all probes on the array. Subsequently, a poly(T) linker was added to each probe to reach the final length of 60 nt. The resultant 60-mer probes were synthesized *in situ* on Agilent 8*15K custom arrays (Agilent). Each array contained 9676 unique probes. Several strains of each bacterial species were tested.

EHEC bacteria

The OciChip *E. coli* O157 Arrays (Ocimum Biosolutions) were used for the comparison of gene content of pathogenic and non-pathogenic *E. coli* strains. Twenty-one *E. coli* strains were selected for the studies, of which 19 belonged to serogroup O157.

C. botulinum

A total of 60 strains of *C. botulinum* from various sources were compared on the DNA microarray (Institute of Food Research, IFR; Norwich, UK) based on the genome of the group I, type A strain of *C. botulinum* (ATCC 3502) (Sebaihia et al., 2007). The array contains 3433 probes for chromosomal DNA, 18 probes to plasmid genes and also probes for genes associated with neurotoxicity.

Data analysis

Hybridizations were carried out using the microarray manufacturers' protocols and repeated two or more times. Microarray slides were scanned with the GenePix 4200 AL scanner (Axon Instruments). Image analysis and spot segmentation were done with GenePix Pro 6.0 software. Spot intensity values were investigated using the kernel density method for the data on potato pathogens (see Results) since no reference samples were used. In the experiments on the EHEC bacteria and *C. botulinum*, normalized logarithmic ratios were computed between the test and reference sample for each probe on the array.

Results and discussion

Comparison of bacterial pathogens of potato

The whole-genome information was utilized in probe design to minimize the possibility that a probe would hybridize to more than a single locus in the bacterial species. When investigating the shape of the histogram based on logarithmic signal intensities from an array, usually three peaks could be clearly distinguished. It allowed classifying the probes into three categories: I) no signal, II) non-specific probe (weak signal), and III) specific probe (strong signal). Analysis and classification of the unknown sample was based on the probes belonging to the third category. An array-specific threshold to identify probes belonging to this group was determined for each array/hybridization separately (Figure 1). The closely related species, e.g., *Eca*, *E. carotovora* subsp. *carotovora* (*Ecc*) (currently *Pectobacterium carotovorum*) and *E. chrysanthemi* (*Ech*) (recently reclassified to *Dickey* spp.), and on the other hand *Ss* and *St*, were detected with many same probes. However, for each species and subspecies tested, a characteristic pattern of signals was observed which identified the bacterium. Furthermore, the probes for ITS and *nip* gene provided species and subspecies-specific signals, which further confirmed identification (Figure 1).

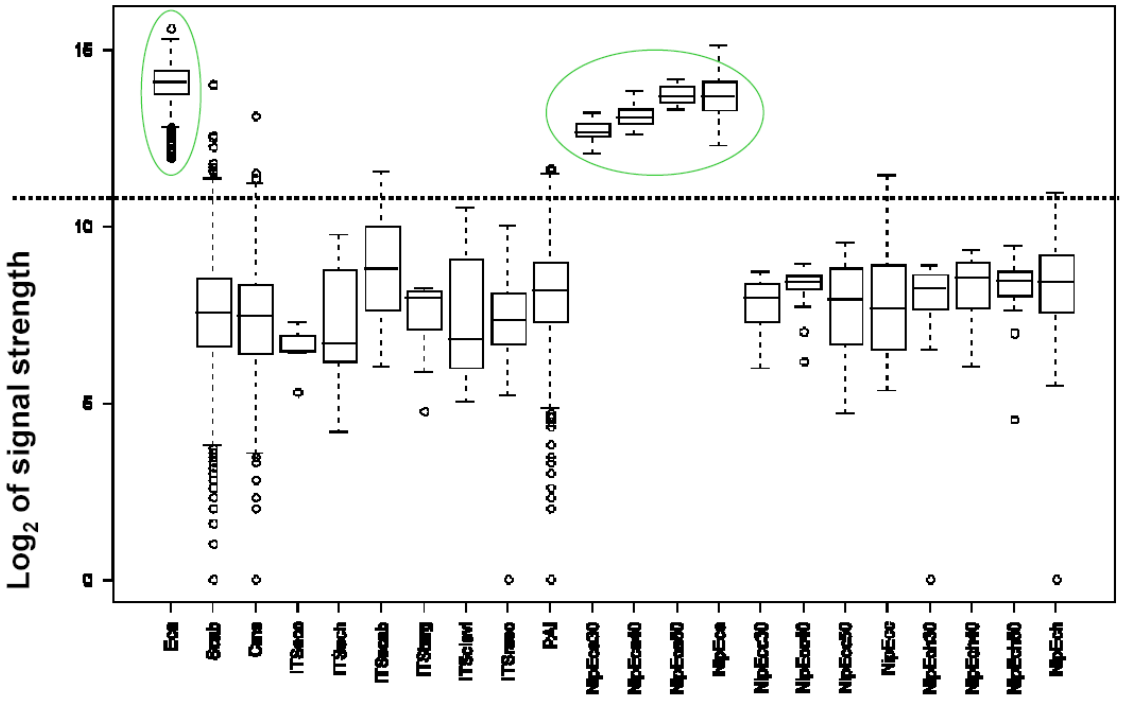

Figure 1: Logarithmic (log_2) signal intensities obtained following hybridization with the genomic DNA of E*rwinia carotovora* subsp. *atroseptica* (Eca). The signals of the 9676 probes on the array are grouped according to the species and specific genomic regions or genes used for probe design. The array-specific threshold signal intensity to identify probes belonging to category III ('specific' signals) which were used for comparison with data obtained with other species is shown by the dotted line. The highest and most specific signals for Eca were obtained with probes targeting the *Nip* genes (four different lengths of probes tested) and probes targeting various other genes of Eca (circled signals).

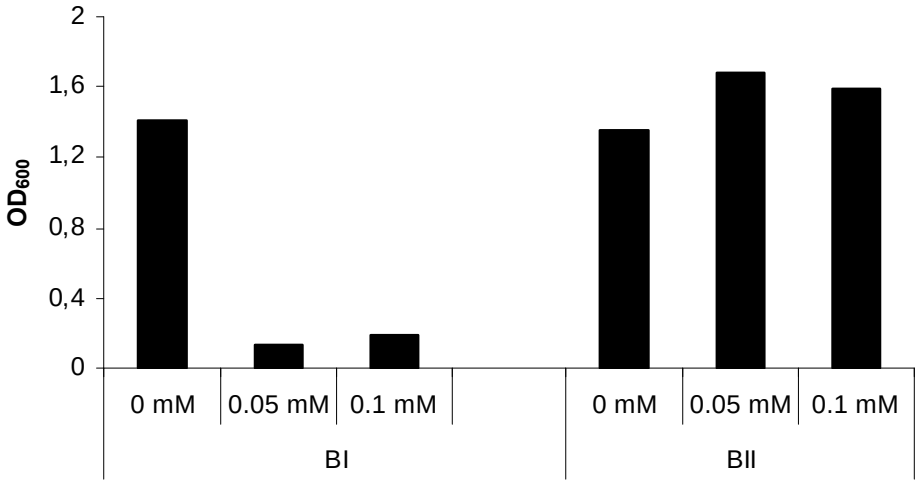

Figure 2: Optical density measurement after overnight growth of two genetically different strains (BI and BII) of *C. botulinum* type B (Group I) in liquid medium with different concentrations of sodium arsenite.

Genomic comparison of Clostridium botulinum with DNA microarrays

A total of 60 *C. botulinum* strains from various sources were compared. The preliminary analysis of data showed significant differences between groups I and II of *C. botulinum*, confirming the previous phylogenetic studies suggesting that the two groups have distinct genetic backgrounds. The data enabled the development of a straightforward PCR assay to distinguish between the two groups (Dahlsten et al. 2006), which was a major improvement in the diagnostics of *C. botulinum*. Within *C. botulinum* group I, strains

producing the same toxin type were mainly grouped together. Moreover, two large clusters of nearly identical group I type B strains of Nordic origin were observed. These two clusters differed by their resistance to arsenic (Figure 2) and cadmium. Corresponding differences were observed in physiological tests in nutrient broth (Lindström et al., 2007). A more detailed analysis of these results is ongoing.

Microarray-based studies on gene content of Escherichia coli O157

Prior to microarray experiments, the tested strains were divided into four groups: I) six strains associated with human disease, II) six strains with *eae* and *stx* genes, III) five strains with *eae* but no *stx* genes, and IV) four non-EHEC strains. The microarray profile-based similarities between strains were compared with the existing classification of strains described above. Group IV differed from all other groups, which was explained by the absence of *ca.* 100 genes. Differences between groups I, II, and III were smaller and there was variation within each group. A set of genes, which best explained the differences between pathogenic (group I) and possibly pathogenic (group III) strains, was selected using statistical analysis. Based on this set of genes, the strains belonging to group II were computed against the strains of group I and III, in order to categorize the group II strains as pathogenic or possibly pathogenic (Figure 3).

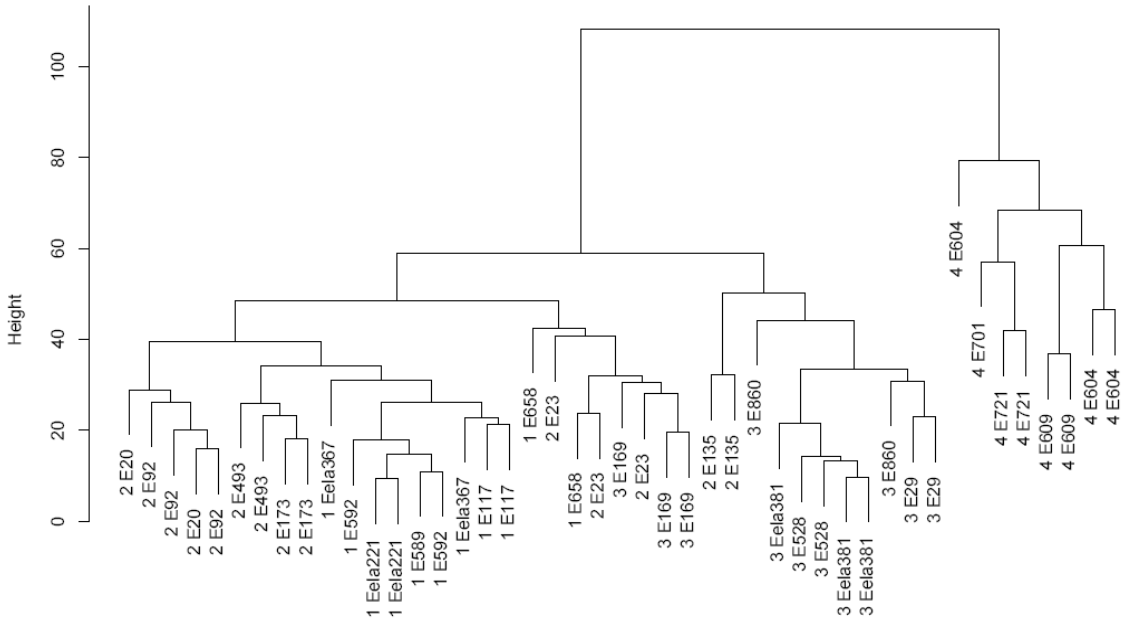

Figure 3: Dendrogram showing relations between the studied *E. coli* strains based on a selected set of genes (692 genes; Wilcoxon Mann-Whitney Test, $p < 0.05$). The first number of each sample refers to the original classification of strains in four groups (1-4 = I-IV, respectively). Slight differences of the results from repeated experiments are reflected by the same strain appearing multiple times in the dendrogram; however, the placement of the strains within the main groups was stable over repeated experiments. On the right side, strains of group IV clustered together. The strains of groups I and III mainly formed their own clusters, but the strains of group II were distributed within these two main clusters. In the middle of the tree, three strains from the three groups (strains E658, E23, and E169) presented similar hybridisation patterns for the selected set of genes and therefore clustered together.

Conclusions

The genome-wide microarray analysis was found to be a powerful tool for detecting genetic differences between the biologically different strains of bacterial pathogens. A large amount of scientifically novel information was obtained. The data from all pathogen groups had also diagnostic value. The array and data obtained on potato pathogens were of direct diagnostic use. The results obtained on *C. botulinum* were used for making a novel PCR-based test. The microarray analysis of EHEC bacteria revealed 100 candidate genes associated with pathogenicity. In future studies, the results and experience will be used to design new microarrays with higher resolution on genetic differences. The methods for data analysis will also be further developed taking into consideration the particular challenges of efficient and cost-effective diagnostic use of microarrays.

Acknowledgements

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Analysis of methanotroph community structure by a functional oligonucleotide MDM : potential and shortcomings

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Introduction

Microbial diagnostic microarrays (MDMs) based on functional genes (also termed functional gene arrays, FGAs) consist of nucleic acid probes targeted against functional genes – i.e., genes conferring a specific function to the microorganisms carrying them, such as nitrogen metabolism or methane oxidation (Wu et al., 2001; Taroncher-Oldenburget al., 2003; Bodrossy et al., 2003). The probes applied may be short (typically 15-30mer) (Bodrossy et al., 2003; Stralis-Pavese et al., 2004) and long (typically 40-70mer) (Denef et al., 2003; Taroncher-Oldenburg et al., 2003; Tiquia et al., 2004) oligonucleotides as well as PCR amplified gene fragments (Wu et al., 2001; Cho and Tiedje, 2001; Dennis et al., 2003). Microarrays based on short oligonucleotides are bound to be applied in combination with a consensus PCR amplifying the target gene from a wide range of (optimally all) microbes (Loy et al., 2002; Wilson et al., 2002; Bodrossy et al., 2003; Bonch-Osmolovskaya et al., 2003). The PCR amplification is required to narrow down the complexity of the labelled target allowing detectable signal.
Functional oligonucleotide MDMs employing short oligos enable a phylogenetic investigation of a functionally defined microbial community. The phylogeny in this case is that of the functional gene, the validity of which needs to be investigated in advance (i.e. compared to that based on 16S or other established phylogenetic markers). The advantage of this type of MDM is that it focuses the labelling onto this defined group of bacteria, enabling the detection of otherwise minor microbial groups. Furthermore, the presence and existence of so far unknown members of a functional group may also be indicated (as opposed to rRNA based microarrays).
Long oligonucleotide and gene fragment MDMs enable a more limited resolution, thus signals arising from bacteria carrying closely related proteins are not readily differentiated (Li, et al., 2005). On the other hand, a range of genes, encoding proteins with related functions, can easily be targeted on a single array which in turn can deliver information on the metabolic potential of the entire microbial community.

Material and Method

Oligonucleotide probe design

Database and phylogenetic trees were constructed and oligonucleotide probes were designed using the phylogenetic software package ARB (Strunk et al., 2000). A comprehensive database containing all published *pmoA*/*amoA* and related sequences, as well as many unpublished ones was established. Alignments were made using Old Aligner function in ARB_EDIT. Neighbor joining DNA and protein trees were constructed and used to guide the probe design process. Probes were designed using the Probe Design and Probe Match functions, accessing a PT-server database created from the ARB database. Outputs of the Probe Match function were imported into CalcOligo 2.03 (www.calcoligo.org). CalcOligo was used to create an Excel table indicating predicted melting temperatures (based on the nearest neighbor model and SantaLucia parameters), length and GC content of the probes and the number of weighted mismatches

between each probe-target pair. Nearest neighbor T_m values were calculated with concentration settings of 250 nM for oligonucleotide and 50 mM for Na^+. Factors for weighing mismatches in CalcOligo were as follows: position 5' 1st: 0.3; 5' 2nd: 0.6; 5' 3rd: 1.0; 3' 1st: 0.3; 3' 2nd: 0.8; 3' 3rd: 1.1; all other positions: 1.2; basepairs: dArC: 1.2; dTrC: 1.2; dGrU: 0.7; dTrG: 0.4; all other mismatched basepairs 1.0. Probe-target pairs with weighted mismatch values of up to 1.5 were expected to yield positive hybridisation under the conditions applied.

Microarray preparation

Oligonucleotides for immobilization were custom synthesized (VBC Genomics, Vienna, Austria) with a 5' NH_2 group, followed by a C_{12} spacer and five thymidines residues preceding the probe sequence. A 384 well flat bottom plate was prepared with 30 µl of 50 µM oligonucleotide solutions in 50% (v/v) DMSO. Samples were spotted with an OmniGrid spotter (1 TeleChem SMP3 pin) at 50% relative humidity (using the humidity controller of the spotter) and 22°C, onto silylated slides (with aldehyde chemistry, Cel Associates, Houston). Arrays were always spotted in triplicate to enable a statistical correction for errors. Spotted slides were incubated overnight at room temperature (22 °C) at <30% relative humidity, rinsed twice in 0.2% (w/v) SDS for 2 min at room temperature with vigorous agitation to remove the unbound DNA. Slides were then rinsed twice in distilled water (dH_2O) for 2 min at room temperature with vigorous agitation, transferred into dH_2O, preheated to 95-100°C for 2 min, and allowed to cool at room temperature (~5 min). Slides were treated in a freshly (immediately before use) prepared sodium borohydride solution for 5 min at room temperature to reduce free aldehydes. Preparation of the sodium borohydride solution: 0.5 g $NaBH_4$ was dissolved in 150 ml phosphate buffered saline (PBS; 8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4, in 1000 ml H_2O, pH 7.4, autoclaved) then 44 ml of 100% ethanol was added to reduce bubbling. Slides were rinsed three times in 0.2% (w/v) SDS and once in dH_2O for 1 min each at room temperature. Finally, slides were dried individually using an airgun fitted with a cotton wool filter inside (to keep oil microdroplets away from the slide surface). Dried slides were stored at room temperature in the dark prior to use.

Environmental DNA preparation

DNA was prepared from soil samples using a method based on the FastDNA spin kit for soil (QBiogene). 0.3 g soil and 780 µl lysis buffer (100 mM $NaPO_4$ pH 7.0; 1% CTAB; 1.5 M NaCl; 5 mg/ml lysozyme (added right before use)) was added into a Multimix FastPrep tube and incubated at 37°C for 30 minutes. MT buffer (122 µl) was added and tubes were shaken in the FastPrep instrument for 30 seconds at 5.5 m/s. Samples were centrifuged for 15 min at 12,000 x g and 700 µl supernatant were collected. 500 µl lysis buffer and 50 µl MT buffer were added to the FastPrep tubes, extraction was repeated and a second 700 µl of supernatant was transferred into separate Eppendorf tubes. At this step, 2 x 700 µl supernatant was obtained from each sample. 5 µl of 10 mg/ml freshly made proteinase K was added to each tube. Tubes were incubated at 65 °C for 30 min. Samples were extracted with phenol-chloroform-isoamyl alcohol (25:24:1), followed by a chloroform-isoamyl alcohol (24:1) extraction. 125 µl of 7.5 M potassium acetate was added, samples were incubated on ice for 5 min and then centrifuged at 12,000 x g for 10 min. Supernatants (2x600 µl per soil sample) were transferred to new tubes, 700 µl Binding Matrix was added and tubes were mixed for 5 min in an Eppendorf shaker. Binding Matrix with bound DNA was pelleted by 1 min centrifugation at 12,000 x g, supernatant was discarded and pellet was resuspended in 500 µl Wash Buffer. The resulting suspension was added into a Spinfilter, and centrifuged for 1 min at 10000 rpm. Eluate was discarded and pellet was washed again in 500 µl Wash Buffer. After discarding the second eluate, the Spinfilter was centrifuged for another 10 seconds to dry the pellet. The filter was taken into a new tube, 200 µl of TE pH 8.0 was added, and after 1 min incubation at room temperature centrifuged for 1 min at 12,000 x g. The eluate collected in the catch tube contained the purified DNA which was stored at -20 °C until use.

Target preparation

The *pmoA*/*amoA* genes were amplified using the forward primer *pmoA*189 (5'-GGBGACTGGGACTTCTGG-3') and either one of the reverse primers T7-mb661 (5'-TAATACGACTCACTATAGCCGGMGCAACGTCYTTACC-3') or T7-A682 (5'-TAATACGACTCACTATAGGAASGCNGAGAAGAASGC-3') where B=(C/G/T), M=(A/C), Y=(C/T), S=(C/G) and N=(A/C/G/T). Primers T7-mb661 and T7-A682 were specific for methanotrophs and for

methanotrophs / AOBs / homologous genes from environmental libraries, respectively. The reverse primers contained the T7 promoter site (5'-TAATACGACTCACTATAG-3') at their 5' end, which enabled T7 RNA polymerase mediated *in vitro* transcription using the PCR products as templates. For each target, three PCR reactions of 50 µl volume each, consisting of 1x PCR buffer, 1.5mM $MgCl_2$, 50nM for each four dNTPs, 15 pmol of both primers, 1 ng genomic/environmental DNA or 0.1 ng cloned PCR product as template, and 1U of Taq polymerase (Invitrogen), were performed in a Hybaid Combi Thermal Reactor TR2 using *Taq* DNA polymerase in accordance with the manufacturer's instructions. Amplification conditions were: 95°C for 5 min before template was added; then 32 cycles of: 1 min at 95°C; 1 min at 58 °C for mb661 or at 56 °C for *pmoA*682; 1 min at 72°C ; followed by a final elongation step of 10 min at 72°C. PCR products were pooled and purified using the HighPure PCR purification kit (Roche Diagnostics GmbH, Mannheim, Germany), according to manufacturer's instructions. Purified DNA was dissolved in ultrapure water to a DNA concentration of 50 ng/µl and stored at -20°C.

Alternatively, a new, two-step PCR was applied to amplify the *pmoA* and related genes from environmental DNA samples. In the first step, 15 cycles were carried out with composite primers composed of *pmoA*-specific 3' regions and non-related 5' head regions (T3c-*pmoA*189 - CAGAGATGCAAATTAACCCTCACTAAAGGGNGACTGGGACTTCTGG, and T7c-mb661 - CCAAGCCTTCTAATACGACTCACTATAGCCGGMGCAACGTCYTTACC). The second step consisted of an extra 25 cycles carried out with primers consisting of only the head regions (T3c - CAGAGATGCAAATTAACCCTCACTAAAG and T7c - CCAAGCCTTCTAATACGACTCACTATAG). Exact PCR conditions were as above with the following modifications. Only 1.5 pmoles of primers T3c-*pmoA*189 and T7c-mb661 were added per 50 µl reaction initially. After the completion of the first 15 cycles, 15 pmoles of each primer T3c and T7c were added with an extra 1 U of *Taq* DNA polymerase and a further 25 cycles were carried out. Annealing temperature was 58 °C throughout the entire PCR protocol. This two-step PCR was found to yield community composition results comparable to those obtained with traditional PCR using the *pmoA* specific primers only. Further, it was possible to amplify *pmoA* and related genes from samples which did not give rise to PCR products with the traditional method.

Working under RNAse-free conditions, *in vitro* transcription was carried out as follows: 8 µl purified PCR product (50 ng/µl), (50 ng/µl), 4 µl 5x T7 RNA polymerase buffer, 2 µl DTT (100 mM), 0.5 µl RNAsin (40 U/µl) (Promega), 1 µl each of ATP, CTP, GTP (10 mM), 0.5 µl UTP (10 mM), 1 µl T7 RNA polymerase (40 U/µl) (Gibco BRL) and 1 µl Cy3 or Cy5-UTP (5 mM) were added into a 1.5 ml microcentrifuge tube and incubated at 37 °C for 4 hours. RNA was purified immediately using the Qiagen RNeasy kit according to manufacturer's instructions. Purified RNA was eluted into 50 µl dH_2O. RNA yields and dye incorporation rates were measured by spectrophotometry.

Purified RNA was fragmented by incubating with 10 mM $ZnCl_2$ and 20 mM Tris.Cl (pH 7.4) at 60 °C for 30 min. Fragmentation was stopped by the addition of 10 mM EDTA pH 8.0 to the reaction and putting it on ice. RNAsin (1 µl 40 U/µl) was added to the fragmented target. Fragmented, labelled RNA targets were stored at -20 °C. Length of the fragmented RNA target was measured by running the sample on an ABI capillary sequencer as well as running on a thin, 2% agarose gel applied onto a standard microscope slide and subsequent scanning in a GenePix 4000A scanner.

Reference targets and artificial target mixtures for testing the quantification potential were synthesized by mixing known amounts of purified PCR products and carrying out *in vitro* transcription and target fragmentation as described above.

Hybridisation

No preHybridisation was done. Hybridisation was carried out in a custom tailored aluminum block used as an insert for a temperature controlled Belly Dancer (Stovall Life Sciences Inc., Greensboro, NC, USA) set at maximum bending (about 10°). The Hybridisation block was preheated to 55 °C for at least 30 min to allow the temperature to stabilize. An Eppendorf incubator was also preheated to 65 °C. HybriWell (Grace BioLabs) stick-on Hybridisation chambers (200 µl in volume) were applied onto the slides containing the arrays. Assembled slides were preheated on top of the Hybridisation block. For each Hybridisation, 124 µl DEPC-treated water, 2 µl 10% SDS, 4 µl of 50x Denhardt's reagent (Sigma), 60 µl 20x SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7.0) and 10 µl target RNA (corresponding to about 400 ng RNA) were added into a 1.5 ml Eppendorf tube and incubated at 65 °C for 1-15 min. Preheated Hybridisation mixtures were applied onto assembled slides via the port in the lower positions (to minimize risk of air bubbles being

trapped within the chamber). Chambers were sealed with seal spots (Grace BioLabs) and incubated overnight at 55 °C at 30-40 rpm circulation and maximum bending.
Following Hybridisation, HybriWell chambers were removed individually and slides were immersed immediately into 2xSSC, 0.1% (w/v) SDS at room temperature (22 °C). Slides were washed by shaking at room temperature for 5 min in 2xSSC, 0.1% (w/v) SDS; twice for 5 min in 0.2x SSC and finally for 5 min in 0.1x SSC. Slides were dried individually using an airgun with a cotton wool filter inside. Slides were stored at room temperature in the dark and scanned the same day.

Scanning and data analysis

Hybridized slides were scanned at 3 lines to average, at 10 μm resolution with a GenePix 4000A laser scanner (Axon, Foster City, Calif., USA) at wavelengths of 532 nm and 635 nm for Cy3 and Cy5, respectively. Fluorescent images were captured as multi-layer tiff images and analyzed with the GenePix Pro 3.0 software (Axon). Microsoft Excel was used for statistical analysis and presentation of results.
Results were normalized to a positive control. Hybridisation signal for each probe was expressed as percentage of the signal (median of signal minus background) of the positive control probe mtrof173 on the same array. As each slide contained triplicate arrays, normalized signal intensities of the triplicate spots on a slide were used to determine average results and standard deviations. Several probes produced non-specific background signal up to 3% of their maximum signal (obtained with perfect match targets). Hybridisation between a probe and a target was thus considered positive if the signal was at least 5% of the strongest signal obtained for that probe with the validation set of reference strains / clones. For probes, where no perfect match reference target was available or the strongest signal was less, than 60 (% of the signal obtained for mtrof173), this reference value was arbitrarily set to 60. This was found to minimize false positive calls while not creating any false negative call.
Even though no dedicated negative controls were applied, for each individual hybridisation over 70% of all probes present on the array were negative controls.

Results and discussion

An example – the pmoA microarray for methanotrophs

The gene encoding the particulate methane monooxygenase gene (*pmoA*) – the key enzyme in methane oxidation – was chosen for the development of a microarray (Bodrossy et al., 2003) to identify methanotrophs, bacteria which are capable of utilizing methane as their sole source of carbon and energy (Murrell et al. 1998; Hanson and Hanson 1996). These bacteria therefore play an essential role in mitigating the greenhouse effect by metabolizing most of the methane produced in diverse soil, sediment and aqueous environments, from landfill site cover soils to upland forest soils, from freshwater lake to deep sea sediments, from arctic seawater to hot springs. Understanding the factors influencing their diversity and activity is of high importance in order to adapt human activities to maintain and protect optimal microbial methane oxidation.
The particulate membrane bound MMO (pMMO) is present in all known methanotrophs (Hanson and Hanson, 1996) except for *Methylocella* (Dedysh et al. 2000). The sequence of *pmoA* encoding the 27 kDa subunit of pMMO reflects evolutionary relationships amongst *pmoA* containing bacteria (Holmes et al., 1995). The ammonia monooxygenase (AMO) of autotrophic ammonia oxidizing bacteria (AOB) is evolutionarily related to pMMO and *pmoA* has a high degree of identity with *amoA*, the gene encoding for the corresponding subunit of the AMO (McDonald and Murrell 1997). Both *pmoA* and *amoA* genes can be present in one to three, generally highly similar copies in the genomes of methane and ammonia oxidizers (McTavish et al. 1993; Semrau et al. 1995; Stolyar, et al. 2001; Purkhold et al. 2000;Bourne et al. 2001). There are genes cloned from environmental samples, distantly related to *pmoA*/*amoA*, where it is not clear whether the encoded enzyme is oxidizing methane and/or ammonia, or neither of them (Bourne et al., 2001; Henckel et al., 2000; Holmes et al., 1999). Our current database, consisting of published sequences, as well as unpublished sequences from collaborators and ourselves, consists of over 2300 nearly full-length (470 bp or longer) sequences and a further 1000 partial sequences (320-470 bp in length) (Figure 1).
The *pmoA* microarray has been updated and extended several times (i.e. (Stralis-Pavese et al., 2004)) to include the novel sequence information accumulated in public databases.
The current (unpublished) version, *pmoA* array #5, contains 138 (17-27 oligomer) probes targeting all

methanotrophs (including uncultivated members) as well as the related ammonium monooxygenase (*amoA*) genes of ammonium oxidizing bacteria, and the phylogenetic resolution achieved was below the species level – as known in January 2006. The array contains multiple, hierarchically nested probes wherever this was possible, to increase the confidence of the results and to enable the detection of novel groups. The method relies on previous PCR amplification as reasoned above. PCR amplicons are converted into Cy3-labelled, fragmented, single-stranded RNA targets via *in vitro* transcription and subsequent chemical fragmentation. Each array contains three subarrays, the signals from which are always averaged. Average probe signals are then normalised against a positive control (targeting the forward PCR primer applied to amplify the *pmoA* gene). As the hybridisation capacity of the probes may vary by up to two orders of magnitude, the (normalised) signal of each probe has still to be compared to a reference value. These reference values are obtained during validation, where the array is hybridised with targets from pure strains or environmental clones. The set of these validation (reference) targets provides at least one perfect match hybridisation to almost all probes on the array. The reference value for each probe is then the maximal hybridisation signal obtained with a perfect match target during the validation process. Raw hybridisation data are extracted via a spot segmentation software (GenePix 6.0 in our case), followed by analysis using custom-recorded macros in Excel. The best visualisation of complex community data is achieved via a gradual colour coded representation, as generated by the software Genespring (and also offered by the conditional formatting option in Excel 2007) (Stralis-Pavese et al., 2004). (Figure 2)

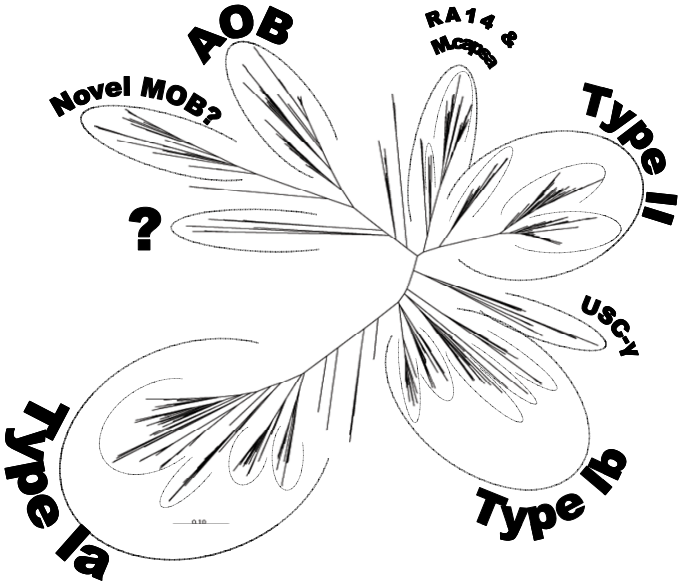

Figure 1: *pmoA*/*amoA* based radial phylogenetic tree of methanotrophs, nitrifyers and functionally related bacteria. Major clades are indicated. "?" refers to a group of sequences known exclusively from environmental clones, where the function of the encoded protein is uncertain. "Novel MOB?" refers to a clade where increasing indirect evidence as well as a few cultivated members strongly suggest that the encoded protein is responsible for methane oxidation. AOB – Ammonia oxidisers. Type Ia, Type Ib, and Type II refers to characterised groups of aerobic methanotrophs, with many cultivated members. RA14 and USC-γ refer to two groups of (uncultivated) atmospheric methane oxidisers.

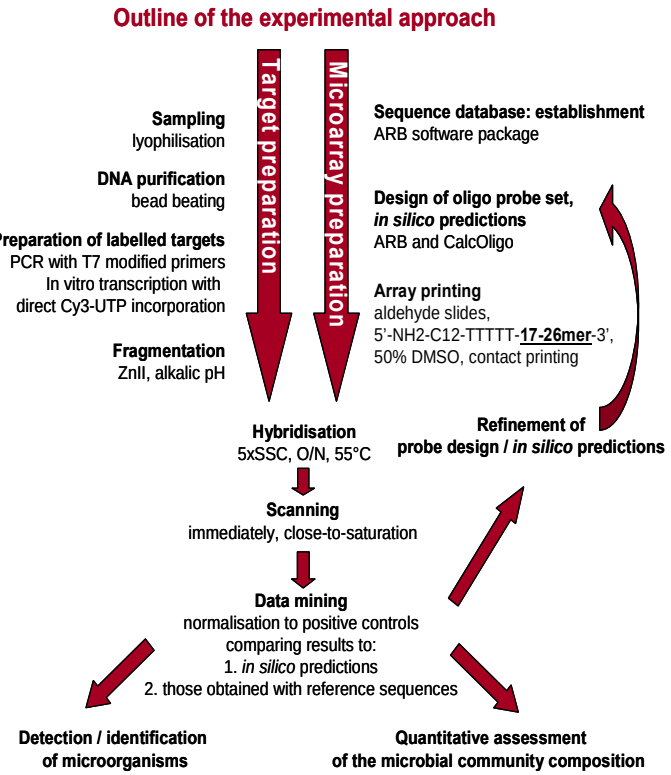

Figure 2: Experimental scheme of microbial diagnostic microarray development.

Application examples – potential and shortcomings

The *pmoA* microarray has been applied in a number of projects and environments. We will use examples from these projects to illustrate the potential and limitations of, primarily, short functional oligonucleotide microarrays.

The currently applied methodology enables one user to analyse about 80 samples during an ordinary working week day, including all steps from DNA purification to analysis. Consumables prices are between 22 and 27 € per sample (not including the price for spotting the microarray itself, which may vary widely depending on the service provider). The target created thus is sufficient for five hybridisations and can be stored at -20°C for at least two years without noticeable decrease in quality (as judged by a repeated hybridisation following such storage). Sensitivity of the assay is determined by the relative abundance of the targeted sequence (targeted species, genus, subspecies or any other phylogenetic level) within the entire community that is amplified via the PCR applied. In this sense, the sensitivity is 5% - that is, a population that is at least 5% of the total community is detected with very high certainty, while those below this limit may or may not be detected, depending on the probes targeting them (Stralis-Pavese et al., 2004).

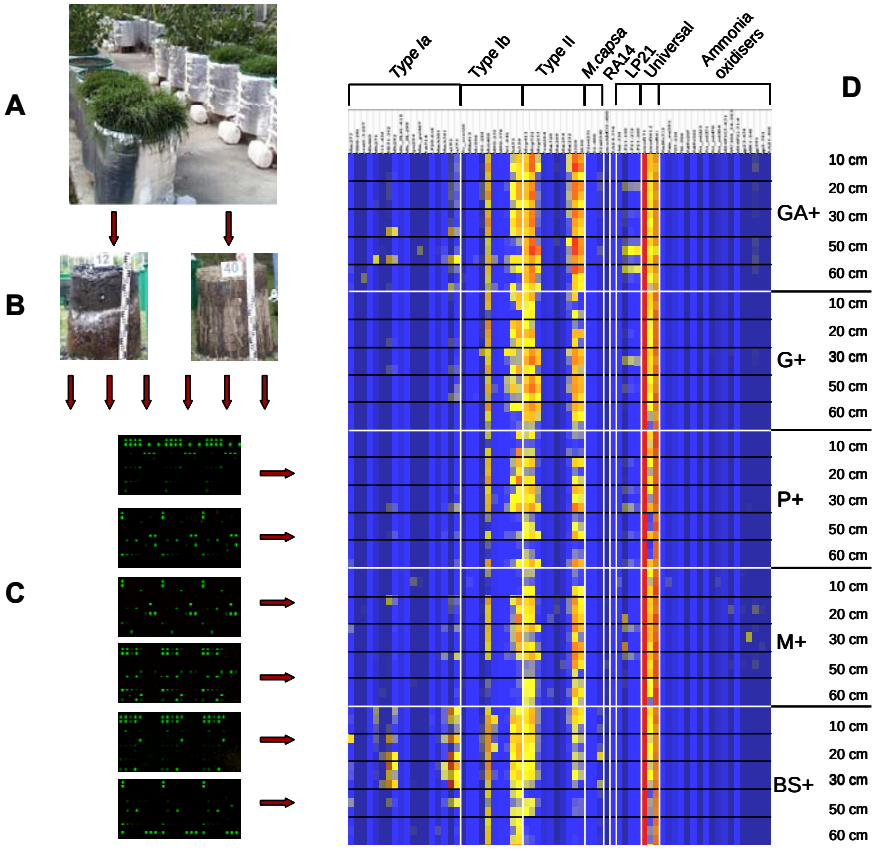

Figure 3: Analysis of methanotroph communities in soils of a lysimeter experiment simulating landfill conditions. A. Lysimeter experiment testing the effect of different vegetation regimes on methane oxidation. B. Sampling soils at different depths of lysimeter pots. C. Microarray analysis by using a *pmoA* oligonucleotide microarray (Stralis-Pavese, Sessitsch, Weilharter, Reichenauer, Riesing, Csontos, Murrell, and Bodrossy 2004). D. Summarised results of the microarray analysis. Results of individual microarray experiments were first normalized to positive control probe mtrof173, then to the reference values determined individually for each probe, averaged between parallels and displayed using the GeneSpring software. In essence, a value of 1.0 indicates maximum achievable signal for an individual probe, while a value of 0.1 indicates that about 10% of the total PCR product hybridized to that probe. Colour coding is indicated on the side bar. GA+ = Grass-alfalfa mixture, G+ = Grass, P+ = Poplar, M+ = *Miscanthus*, BS+ = Bulk soil. For each vegetation cover and each depth layer, results from three parallel lysimeters are shown.

The high throughput, moderate costs and high resolution of the method make it especially suited for the analysis and visualisation of spatial heterogeneity (Stralis-Pavese et al., 2004) (Figure 3).

Hierarchically nested probe sets enable the detection of hitherto unknown taxa. This was shown by the analysis of methanotroph communities from upland forest soil samples (Knief et al., 2005) (microarray data unpublished) (Figure 4). Similarly, the array enables a rapid screening for environments or environmental niches harbouring particular groups.

By purifying environmental RNA, followed by RT-PCR, the microarray can be applied to analyse the active community as opposed to the total (active, less active, dormant, etc.) community (Bodrossy et al., 2006) (Figure 5).

The potential of the microarray technology to diagnose environmental changes was demonstrated by an accidental result. A lysimeter (GA+ 20) within a large-scale experiment (Stralis-Pavese et al., 2004) displayed a most unusual pattern of methanotroph diversity. Besides the diversity found in other, parallel (GA+) lysimeters, significant diversity of type Ia and type Ib methanotrophs was detected. Checking the gas profile revealed that there had been a major fault in the lysimeter, most likely a gas leakage near to the bottom (70 cm), where biogas was added. Type Ia methanotroph populations were previously shown to

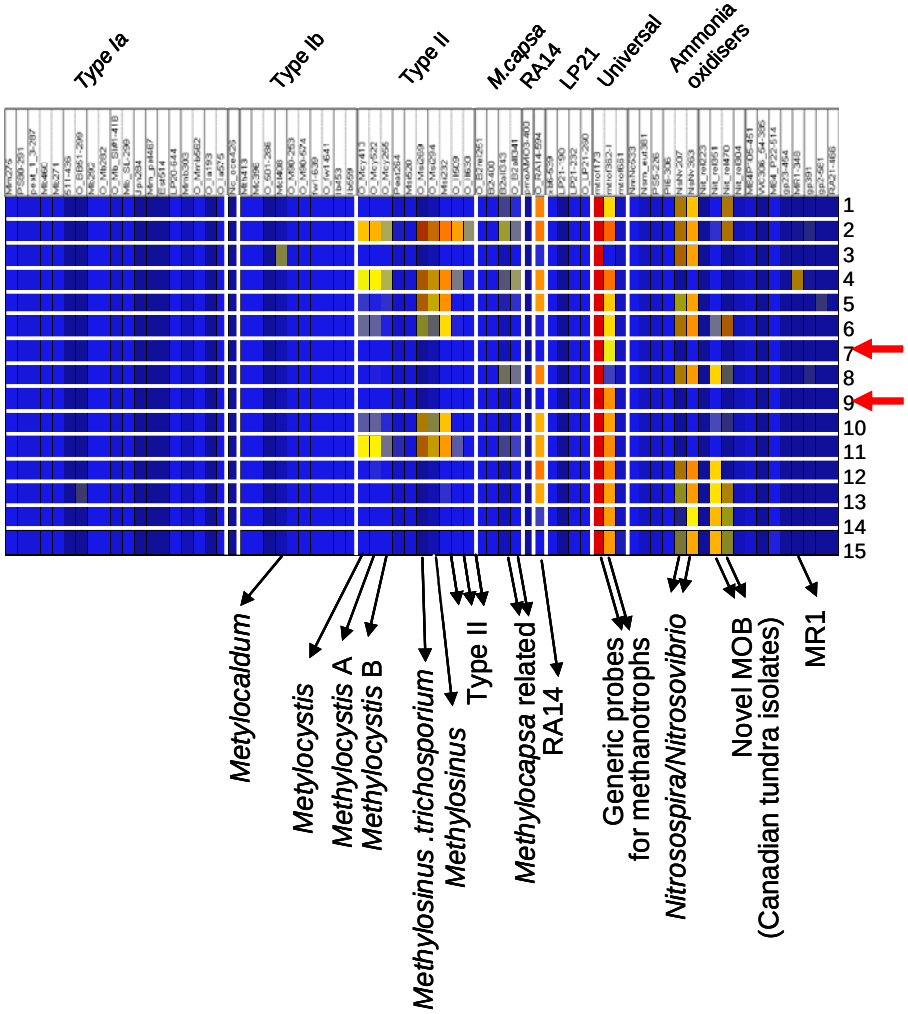

Figure 4: Analysis of methanotroph communities inhabiting different upland soils by using the *pmoA* microarray. Samples #7 and #9 indicate the presence of novel methanotrophs as hybridisation signals were only obtained with generic probes for methanotrophs. Confirmatory results were obtained by analysis of *pmoA* clone libraries (Knief et al., 2005).

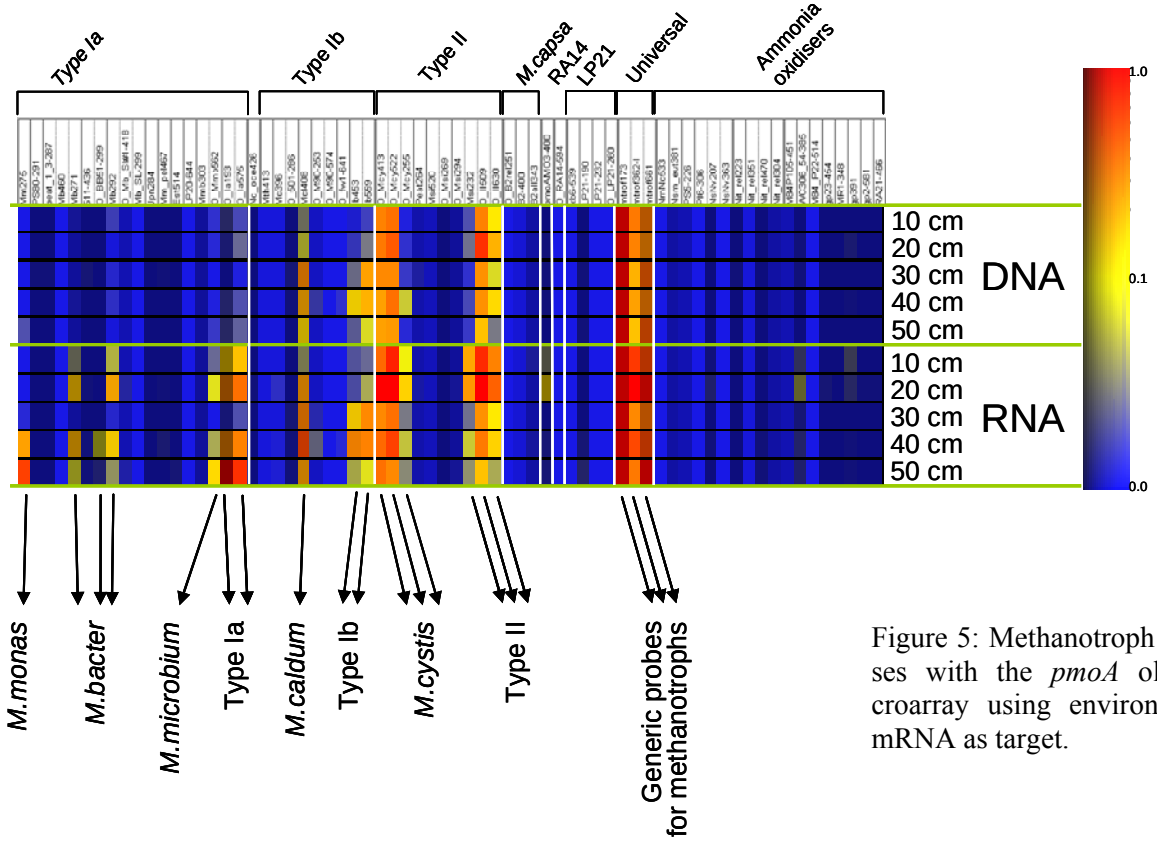

Figure 5: Methanotroph community analyses with the *pmoA* oligonucleotide microarray using environmental DNA and mRNA as target.

compete more efficiently with type II methanotrophs under relatively high oxygen and low methane partial pressures (Amaral and Knowles 1995; Graham et al., 1993; Henckel et al., 2000; Macalady et al., 2002).

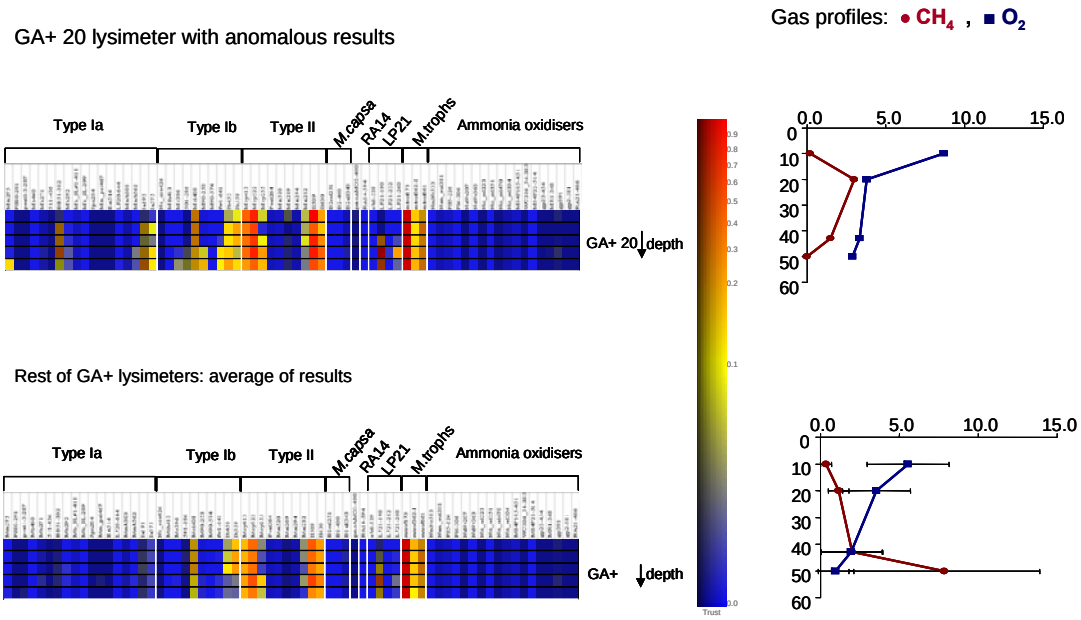

Figure 6: Methanotroph community profile of lysimeter GA+ 20 compared to the average profile of three other GA+ lysimeters (data from Figure 3). Gas depth profiles are also shown.

These conditions were fulfilled by the bottom layers of this particular lysimeter, whereby gas concentration ratios were shifted towards low methane - high oxygen (Figure 6).
Microarrays also lend themselves as tools to follow, visualise and evaluate various enrichment and isolation strategies and their various biases. Figure 7 shows the results of such an experiment. Without going into any details, it is striking, how heavily even small differences change the community composition. Not less striking is the temporal change of the community composition over time – populations become dominant and then disappear again. When looking at the results, the 5% detection limit of the array should be kept in mind – that is, absent signals don't necessarily indicate the absence of a group, but rather that its relative abundance within methanotrophs is below this threshold.

Conclusions

As the fine technical details and correspondingly the exact potential and limitation of MDMs vary from method to method, we attempted to illustrate the general applicability of these tools by focusing on the one we know best, our own.
Key characteristics of the presented method are:

- requirement for previous PCR amplification of the target gene
- detection threshold at 5% relative abundance
- phylogenetic resolution at or below the species level
- hierarchically nested probe sets (genus, family level)
- 80 samples per week per user, from sample to results
- 22-27€/sample consumables costs (excl. microarray spotting)
- focuses onto a functional group of bacteria
- enables the identification and detection of uncultivated members of this functional group

By combining high resolution, high throughput and affordable overall costs, functional oligonucleotide MDMs are a powerful tool for mapping the spatial and temporal variability and dynamics of microbial community structure in the environment.

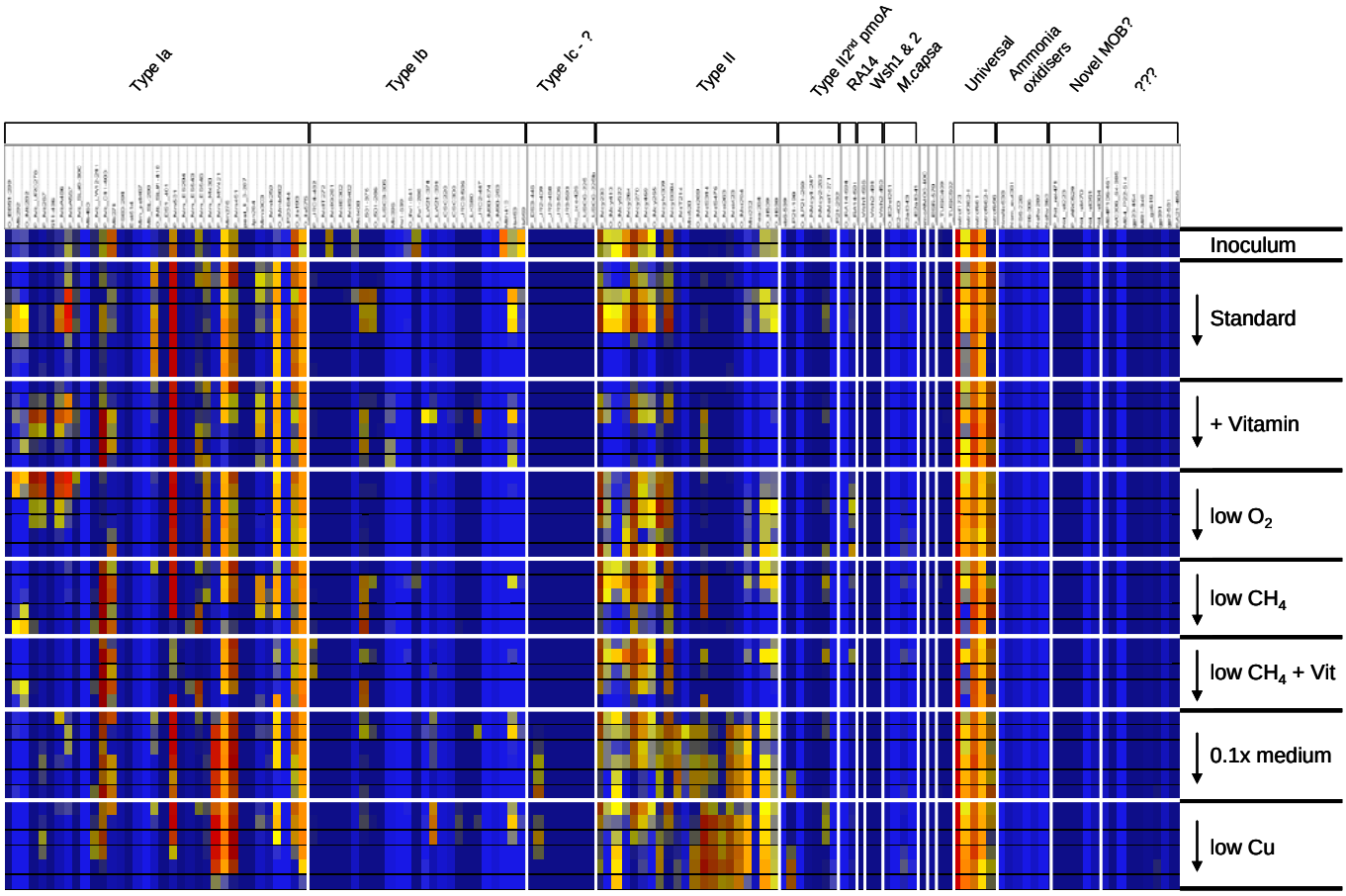

Figure 7: Visualisation of enrichment bias. An environmental sample containing a broad diversity of methanotrophs was used to inoculate liquid enrichments under "standard" and slightly varying conditions. Low O_2 / CH_4 : ~1% concentration of the corresponding gases. 0.1x medium: standard medium in 10x dilution. Low Cu: same diluted medium, without the addition of copper (an essential cofactor for one of the forms of the methane monooxygenase). Each block is organised into a time series, from the top towards the bottom.

Acknowledgements

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Diagnostic Application of Padlock Probes – Multiplex Detection of Plant Pathogens using Universal Microarrays

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Abstract

Padlock probes (PLP) are long oligonucleotides, whose ends are complementary to adjacent target sequences. Upon hybridisation to the target, the two ends are brought into contact, allowing PLP circularisation by ligation. PLPs provide extremely specific target recognition that is followed by universal amplification and microarray detection. Since target recognition is separated from downstream processing, PLPs enable the development of flexible and extendable diagnostic systems, targeting diverse organisms. To adapt padlock technology for diagnostic purposes, we optimised PLP design to ensure high specificity, eliminating ligation on non-target sequences under real-world assay conditions. We designed and tested different PLPs to target various plant pathogens at the genus, species and sub-species levels, and developed a prototype PLP-based plant health chip. Excellent specificity was demonstrated towards the target organisms. The developed multiplex diagnostic system was validated using genomic DNAs of characterized isolates and artificial mixtures thereof, but also of practical samples.

Introduction

The accurate identification and detection of pathogenic microorganisms or other targets of interest has become increasingly important in clinical diagnostics and other pest management strategies. Traditionally, the predominant techniques aimed at this goal target only a single pathogen per assay, making comprehensive screening of samples laborious and time-consuming.

To increase efficiency, it is desirable to develop multiplex assays which can detect several pathogens simultaneously. Microarrays may enable highly parallel detection of diverse organisms (Bodrossy and Sessitsch, 2004). Padlock probes (PLP) offer a means of combining pathogen-specific molecular recognition and universal amplification, thereby increasing sensitivity and multiplexing capabilities without limiting the range of potential target organisms. PLPs are long oligonucleotides of approximately 100 bases containing target complementary regions at both their 5' and 3' ends (Figure 1). These regions recognise adjacent sequences on the target DNA (Nilsson *et al*., 1994) and universal primer sites and a unique sequence identifier, the so-called ZipCode, lie between these segments. Upon hybridisation the ends of the probes get into adjacent position and can be joined by enzymatic ligation. This ligation and the resulting circular molecule can only take place when both end segments recognise their target sequences correctly. Non-circularised probes are removed by exonuclease treatment, while the circularised ones may be amplified with universal primers. Subsequently, the target-specific products are detected by a universal cZipCode microarray (Shoemaker *et al*., 1996). PLPs have been shown to have good specificity and very high multiplexing capabilities in genotyping assays (Hardenbol *et al*., 2003). They are promising for multiplex diagnostic analyses, since one can expect limited bias in the universal amplification step because of the use of non-degenerate, universal primers and the uniform size of the amplicons. Furthermore, universal microarrays may provide less non-specific hybridisation allowing a higher dynamic range of pathogen detection.

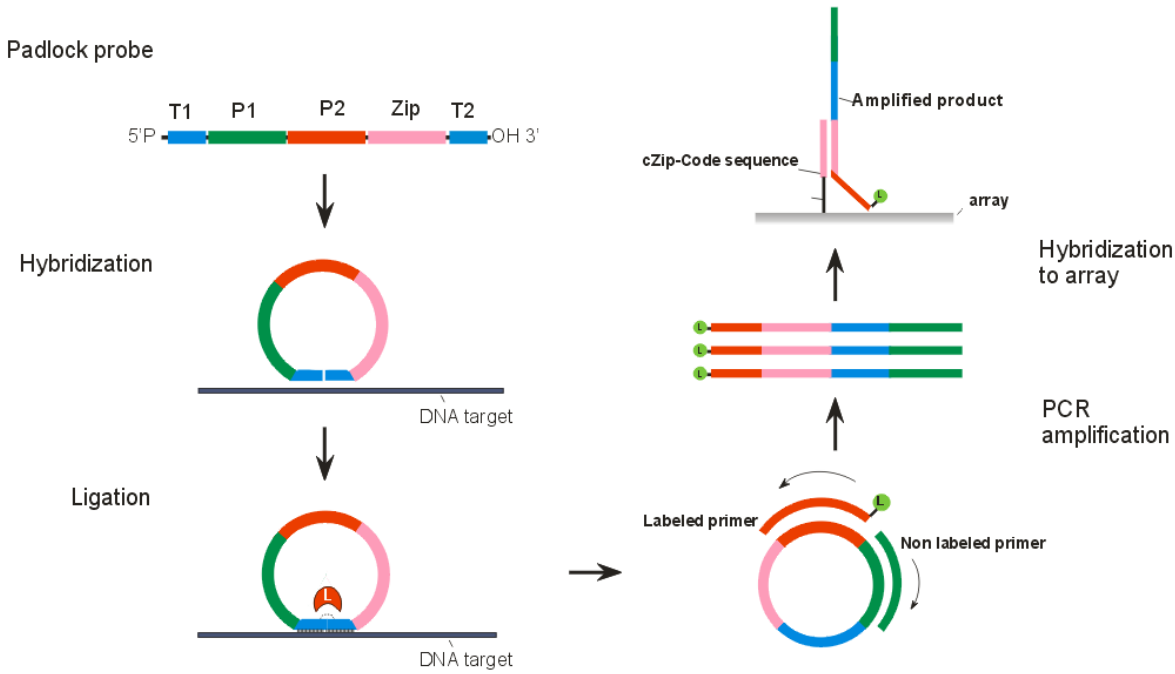

Figure 1: Scheme of the padlock probe (PLP) composition, ligation, PCR amplification and hybridization on a microarray.

Materials and methods

Nucleic acids used in the study

The pathogenic organisms were derived from the culture collection of Plant Research International B.V. Genomic DNAs were extracted as previously described (Bonants *et al.*, 1997). PLPs and target oligonucleotides, listed in Tables 1 and 2, were synthesised by Eurogentec S.A. (Seraing, Belgium).

Padlock probe design

Relevant nucleic acid sequences derived from Genbank and from independent sequencing studies were aligned by using ClustalW. Diagnostic sequences were identified for each target group. Potential PLP target complementary regions were selected in a way that the discriminatory nucleotides would bind to the 3'arm region. Further design rules are described in (Szemes *et al.*, 2005). The PLP arm sequences were combined with the universal primer sites (P1: 5' CTCGACCGTTAGCAGCATGA 3'; P2: 5' CCGAGATGTACCGCTATCGT 3') and a ZipCode sequence. The unique identifier was chosen from GeneFlex^{TM} TagArray set (Affymetrix) in a way to minimize PLP secondary structures.

Ligation, capturing and exonuclease treatment

Genomic DNA was fragmented by digestion using EcoRI, HindIII and BamHI (New England Biolabs) for 30 min.Cycled ligation, capturing of ligated probes and exonuclease treatment were performed as previously described (Szemes *et al.*, 2005; Van Doorn *et al.*, 2007).

Real-time PCR

Amplification of ligated PLPs was followed in real-time using an ABI Prism 7700 Sequence Detector System (Applied Biosystems) and the qPCR kit (Eurogentec) (Szemes *et al.*, 2005).

LATE-PCR

For microarray hybridisation, circularised PLP probes were amplified in LATE-PCR (linear-after-the-exponential PCR) (Sanchez *et al.*, 2004) to produce a large amount of ssDNA amplicons (Szemes *et al.*, 2005).

Table 1: Target-complementary regions and ZipCode sequences of the developed diagnostic PLPs. Nucleotides or gaps due to deletions used to discriminate targets from most similar non-target sequences are underlined. Bold characters indicate polymorphism within the target group.

Targeted species/group	5' target complementary sequence (5'- 3')	3' target complementary sequence (5'- 3')	ZipCode sequence (5'-3')
Phytophthora spp.	TATCTAGTTAAAAGCA**GA**GACTTTCGTC	CTGCTGAAAGTTGC	GTCACGTATGGTTCGCTGCT
Phytophthora cactorum	GACTTTCGTCCCCACAGTATAATCAGTATTAAGGAAT	TATCTAGTTAAAAGCAAG	ACTCCAGTGCCAAGTACGAT
Verticillium dahliae	TTTATACCAACGATACTTCTGAGTGTT	CATCAGTCTCTCTG	CGTTCCTAAAGCTGAGTCTG
Phytophthora infestans	TCGATTCGTGGTATGGTTGGCTTCGGCT	CGTTAATGGAGAAATGC	GCACTAACTGGTCTGGGTCA
Fusarium oxysporum	GCGAGTCCCAACACCAAGCTGTGCTTG	GGAACGCGAATTAAC_	ATGCAGCGTAGGTATCGACT
Myrothecium roridum	CGGTGGTGGCCATGCCGTAAAACACC	ACTCGCATTGGAGCT	AATGCTCACATCGCAGGTAC
Phytophthora nicotianae	TAGTAGTCTTTTTTTCTTTTAAACCCATTCCTTAAT	GCTTCGGCCTGATT	TCCCGAATGACAAGGCACGA
Rhizoctonia solani AG 4-2	GACTTCTGTCTACTTAATTCATATAAACTCAATT	CTT_CTACTCCCCCTT_	TGTGATAATTTCGACGAGGC
Rhizoctonia solani AG 4-1	GGTCCAATAAAGT**T**CCTT**C**CCCCCTAGAAAA	AGTCCAA_**G**GAGAGTA___	ATTAACTCGACTGCCGCGTG
Pythium ultimum	CGAAAAAACGAACGCAACCATGTGAGACACTT	CGACAGATTCTCGAT	TCGCCGTTGGTCTGTATGCA
Meloidogyne hapla	GTTTATCGTTGTGAATGGCTGTCGCTGGTG	ATTCGAATAGTCTCAAC	CTTCGTGGCTAGTCTGTGAC

Table 2: Design and experimental characteristics of the PLP set. Probes were named after the targeted species/ subgroup. Lengths and melting temperatures (T_m) of PLP target-complementary regions are indicated. The number of nucleotides discriminating the targeted sequence from that of the known most similar non-target organisms is shown for each PLP. Sensitivity was defined as the lowest concentration of perfectly matching oligonucleotide that could be detected under standard assay conditions.

Name	5' arm		3' arm		Closest non-target relative	nbr of discrimi-nating nucleotides	Sensitivity (fM)
	Length (nt)	T_m (°C)	Length (nt)	T_m (°C)			
PLP P-spp	29	60.0	14	39.5	*Pythium splendens*	1	2
PLP P-cac	37	65.8	18	39.6	*Phytophthora nicotianae*	2	20
PLP V-dahl	28	58.3	14	35.4	*Verticillium alboatrum*	2	2
PLP P-inf	28	68.9	17	44.0	*Phytophthora sojae*	3	2
PLP F-oxy	27	68.9	15	41.5	*Fusarium equiseti*	3	2
PLP Myr-ror	27	69.0	15	46.5	*Myrothecium verrucaria*	5	0.2
PLP P-nic	36	61.0	14	43.6	*Phytophthora cactorum*	7	2
PLP Rhiz-4-2	36	59.2	15	41.0	*Rhizoctonia solani* AG 4-1	7	2
PLP Rhiz-4-1	32	66.2	15	39.5	*Rhizoctonia solani* AG 4-2	8	2
PLP Pyt-u	32	66.2	15	40.6	*Pythium splendens*	10	0.2
PLP Mel-h	30	65.7	17	41.1	*Meloidogyne incognita*	15	2

Microarray preparation and hybridisation

Complementary ZipCode (cZipCode) oligonucleotides carrying a C12 linker and a 5' NH_2 group were synthesised and spotted on Nexterion MPX-E16 epoxy-coated slides by Isogen B.V. (Utrecht, The Netherlands) according to manufacturer's instructions (Schott Nexterion). Hybridisation, washing and analysis of microarray data were performed as described before (Szemes et al., 2005).

Results

Design and testing of diagnostic padlock probes specificity

For diagnostic applications, the high discriminatory power of the ligation is of prime importance, since very similar non-target DNA molecules can be present potentially in much higher concentration than the target DNA. Therefore, we aimed to optimise the reaction conditions and PLP design for maximum discrimination of single mismatches, which subsequently could be extrapolated to diagnostic assay design.

Based on the principles described before (Szemes et al., 2005), we designed PLPs targeting ten economically important plant pathogens (Table 1). In each case we selected discriminatory areas within the ITS regions of rRNA operons because of their high copy number (Atkins and Clark, 2004), which could significantly increase the sensitivity of the assay. Furthermore, ITS regions have been extensively used in phylogenetic studies (Cooke *et al*., 2000) and a large number of sequences are available for plant pathogenic organisms, which may ensure reliable assay design. Sequences available in Genbank and those obtained from independent sequencing studies (Bonants, unpublished results) were aligned, and diagnostic regions for each target organism were selected. Preferably, we chose regions containing more than one discriminatory nucleotides and very few polymorphic positions within the targeted species/subgroups. The 3' arm sequences were selected to be 14-18 nucleotide-long and had a T_m around 40°C (Table 2). In general, the 3' arm sequence hybridised to the discriminatory region and contained a highly destabilizing mismatch or a gap at the 3' end when bound to the non-target sequence. The 5' arm sequences were 27-37 nucleotide-long. In an attempt of hierarchical diagnostic analysis, we also designed a genus-specific PLP to target all *Phytophthora* species and discriminate them from related *oomycetes*. After selecting the target-complementary regions, they were combined with the universal primer site sequences and a unique ZipCode sequence was selected for each probe.

The developed probes were tested for sensitivity and discriminatory power using synthetic oligonucleotides representing target nucleic acids and the most similar non-target sequences (Szemes *et al.*, 2005). As the final analysis was to be performed on array, we chose the LATE-PCR protocol (Sanchez *et al*., 2004) to achieve efficient amplification and produce large amount of ssDNA in one step, which is ideal for microarray hybridisation. In all the subsequent experiments, this method was used to amplify ligated PLPs. Fixed amounts of PLPs were ligated on their respective target and on the related, but non-target oligonucleotides, present in a wide concentration range.

Validation – PLP-based multiplex detection of plant pathogenic organisms

A mix of the developed 11 PLPs was ligated on various genomic DNAs, treated with exonucleases and subjected to LATE-PCR using Cy3-labeled forward primer. The labelled PLP amplicons were analysed on multi-chamber, low-density universal microarray, which enabled the simultaneous assay of 16 samples on a single slide. The tag array used in our experiments contained 30 probes in 9 replicates, together with 90 hybridisation control probes distributed over the deposition area. This layout allows for the future extension of the PLP set to target other pathogens and enables high-throughput screening.

Using the developed PLP set, we tested genomic DNAs from a panel of well-characterized isolates of plant pathogenic organisms (Figures 2 a-g). In each case, 1 ng genomic DNA could be specifically and reliably detected without any cross-reaction. All the *Phytophthora* species were correctly recognized by PLP Phyt-spp, including *P. cactorum*, which contained two adjacent mismatches with the 5' arm sequence of the probe (Table 1). This polymorphism was apparently well tolerated, resulting in a positive signal. For four probes (PLPs P-cac, P-nic, P-inf and V-dahl) analysis was also performed with DNA of a very closely related organism (Table 2), but no cross-reaction was observed, indicating excellent specificity.

Next, we evaluated the ability of the developed diagnostic system to detect several pathogens in parallel. Mixtures of equal amounts of genomic DNAs representing three targeted organisms were tested (Figures 2 h-j). In two out of three cases, the pathogens were correctly and unambiguously identified by all four cognate probes, resulting in detection at the genus and species/subgroup level. The components of the third mixture, *P. cactorum, R. solani AG 4-1 and V. dahliae* were also correctly identified by using the species/ subgroup-specific probes. The PLP Phyt-spp signal, however, was below the threshold, most probably due to the two adjacent mismatches with *P. cactorum* DNA.

To explore the sensitivity of the system in a multiplexed setting, we tested the detection threshold for *F. oxysporum* and *M. roridum* in the presence of a large excess of the other target DNA (Figure 2 k-l). As little

as 0.5 pg of *F. oxysporum* DNA could be detected in the presence of 500 pg of *M. roridum* DNA, corresponding to a dynamic range of 1000. In a reverse situation, the detection threshold was 5 pg for *M. roridum* in the presence of 500 pg *Fusarium* DNA, indicating that a reciprocal dynamic range of 100 is achievable using this system.

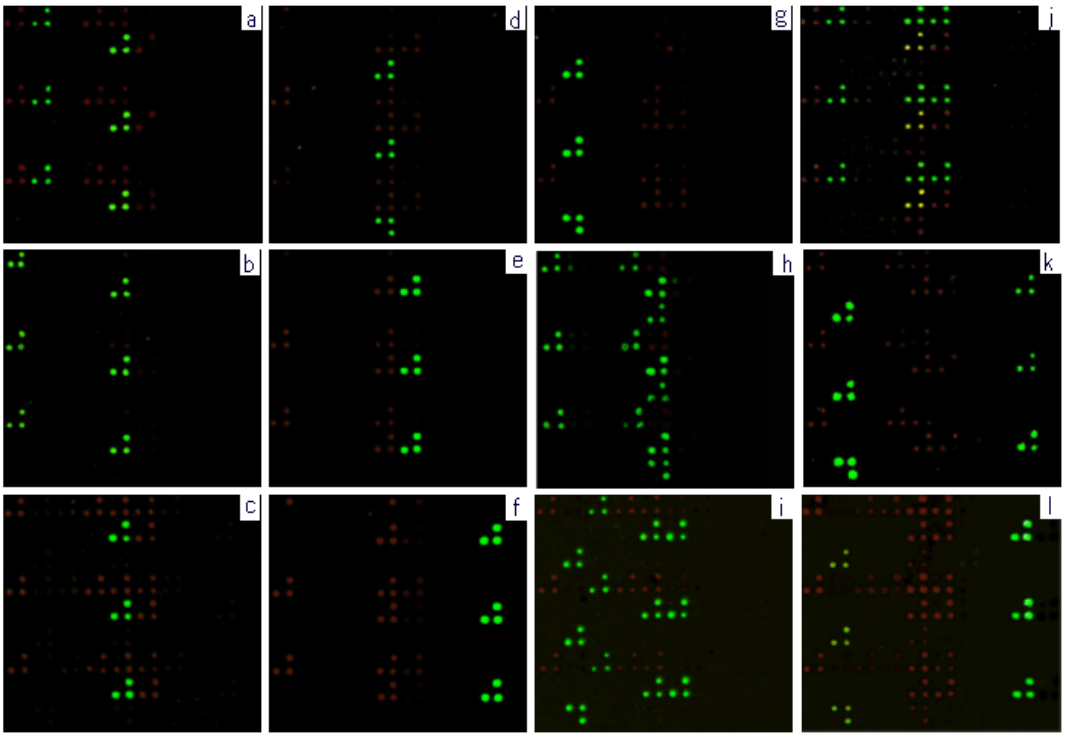

Figure 2: Detection of genomic DNAs corresponding to individual (a - g) and complex pathogen samples (h - l) on a universal microarray. The analyzed targets were: (**a)** *P. cactorum*; 1 ng (**b)** *P. nicotianae*, 1 ng; (**c)** *P. sojae*, 1 ng; (**d)** *R. solani AG 4-2*, 1 ng; (**e)** *M. hapla*, 1 ng; (**f)** *F. oxysporum*, 1 ng; **(g)** *M. roridum*, 1 ng; (**h**) *Pyt. ultimum*, 500 pg; *M. hapla,* 500 pg and *P. nicotianae*, 500 pg; **(i)** *P. infestans*, 500 pg; *R. solani AG 4-2*, 500 pg and *M. roridum*, 500 pg; **(j)** *P. cactorum*, 500 pg; *R. solani AG 4-1*, 500 pg and *V . dahliae*, 500 pg. **(k)** *F. oxysporum*, 0.5 pg and *M. roridum*, 500 pg; **(l)** *F. oxysporum,* 500 pg and *M. roridum* 5 pg.

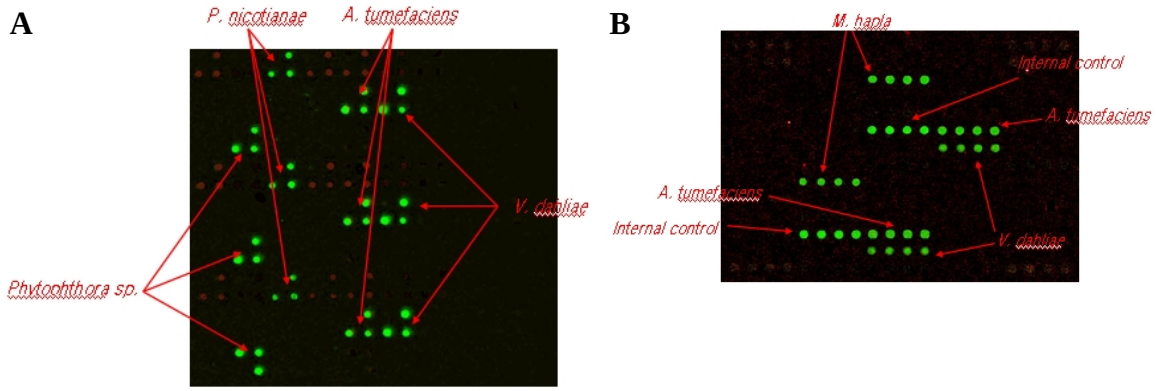

Figure 3: (A) Detection of DNA isolated from filters. Water was spiked with different pathogens and filtrated through a Millipore filter (0.22 µ). DNA was isolated and tested with padlock probesmixtures. The analysed pathogens were *Phytophthora nicotianae*, *Verticillium dahliae* and *Agrobacterium tumefaciens*. (B) Detection of DNA isolated from soil samples. Soil was spiked with different pathogens and DNA was isolated and tested with padlock probes mixtures. The analysed pathogens were *Meloïdogyne hapla*, *Verticillium dahliae* and *Agrobacterium tumefaciens*

Validation: PLP-based multiplex detection of plant pathogenic organisms spiked to water and soil

To analyse practical samples in a multiplexed setting, we spiked water and soil respectively with different pathogens. Water was spiked with propagules of *Phytophthora nicotianae*, *Verticillium dahliae* and *Agrobacterium tumefaciens*. Soil samples were spiked with *Meloïdogyne hapla*, *Verticillium dahliae* and *Agrobacterium tumefaciens*. DNA was extracted as described and isolated. DNA samples were tested with a mixture of PLPs. The spiked organisms could easily be detected in water (Figure 3A) and soil (Figure 3B).

Discussion

In this study we investigated the diagnostic application of padlock probes in a multiplex setting for the first time. Based upon our findings, we developed a PLP- and universal microarray-based assay for the detection of several important plant pathogens.

The possibility to detect a mutant allele in 500-times excess wild type sequence was previously demonstrated (Thomas *et al*., 1999). No multiplex PLP-based diagnostic system was, however, developed and characterized to-date. For genotyping versatile and highly-multiplexed PLP-based assays were developed (Hardenbol *et al*., 2003; Baner *et al*., 2003; Alsmadi *et al*., 2003). In these assays, however, the discrimination power of PLPs was found to be much lower. Hardenbol reported an average ratio of maximum allele signal vs. maximum non-allele signal of only 17 (Hardenbol *et al*., 2003). The striking discrepancy probably arises from the fact that in a highly-multiplexed reaction positive signals are decreased due to competition, while background signals increase cumulatively. Although the reported discrimination is sufficient for reliable allele calls in a highly multiplexed setting, diagnostic assays require a much higher discriminatory range.

Therefore, to adapt PLP technology for multiplexed pathogen detection, we aimed to increase PLP discriminatory power. To these ends, we tested different PLP design strategies and determined the discriminatory power by real-time PCR. Since the circularised ligation products were in the linear quantification range of the applied method, the obtained numbers reflected the discriminatory power of ligation without amplification bias. We found that asymmetric PLP design, in which a long 5' arm serves as an anchor sequence and the binding of a short 3' arm is an equilibrium process, could increase mismatch discrimination by almost one order of magnitude. Faruqi and colleagues also demonstrated the superiority of asymmetric PLP design (Baner *et al*., 2003). Their assay conditions and evaluation method, however, were very different from those used in this study. We set our conditions according to the planned multiplex detection system, so that the results could be directly extrapolated. A further advantage of the asymmetric design is that while the 3' arm may ensure excellent specificity, the binding of the long 5' arm is quite stable and might tolerate potential mismatches caused by polymorphisms within the target group.

We targeted the ITS region of rRNA operons for pathogen detection because of their high copy number and the large database of sequences for this region to aid assay design. Based on alignments of several hundred sequences, eleven PLPs were designed to detect economically important plant pathogens at genus, species and subspecies level. Since many of the isolates whose sequences were used in the design process were not available, we carried out the initial PLP testing by using complementary oligonucleotides. These tests showed that the PLPs had comparable sensitivity and a discriminatory range of 10^5-10^7 and above. PLP P-cac, one of the probes with the lowest discriminatory range, was further tested for specificity using genomic DNAs. The probe proved to be perfectly specific: no cross-reaction was observed with 10^5 times higher amount of non-cognate DNA than the demonstrated sensitivity towards its target. This amount was much higher than what one may expect in real-world samples, suggesting that no false ligation is likely to occur in a real assay.

The PLP design and the assay procedures were streamlined to provide reliable performance. New probes are incorporated into the multiplex set only after they were proven to have sufficient sensitivity and specificity in the oligonucleotide test. The ligated PLPs were amplified in LATE-PCR, a one-tube method to produce large amount ssDNA amplicons, which are ideal for microarray hybridization. Furthermore, since there is linear amplification after the exponential phase in LATE-PCR, it better reflects the initial template ratios even at end point detection (Szemes *et al.*, 2005). We believe this property could be exploited in the future for semi-quantitative pathogen detection. The labelled PLP amplicons were analyzed on 16-well microarrays, enabling sufficient through-put. In the near future, 48- and later 96-well formats are expected to

become available, thereby fulfilling the need for high-throughput sample analysis on low-density arrays (Schott Nexterion).
The developed multiplex detection system was validated with characterized isolates representing target organisms as well as close non-target relatives. In addition, artificial mixtures of pathogen genomic DNAs were tested. In all cases, the targeted pathogens were correctly identified and discriminated from related non-target organisms.
The sensitivity and dynamic range of detection was determined using *F. oxysporum* and *M. roridum* genomic DNAs. Target DNAs of 0.5-5 pg could be detected in the presence of a large excess of the other target DNA, with a dynamic range of 100 to 1000. It is important to note that in all the assays an even larger amount of non-target DNA (20 ng) was always present, which did not interfere with detection. The sensitivity and dynamic range of the developed method compare favourably with those of other diagnostic microarrays (Alsmadi *et al*., 2003; Faruqi *et al*., 2001; Bodrossy *et al*., 2003; Castiglioni *et al*., 2004).
In this study we described a PLP-based multiplex diagnostic system. The presented method offers numerous advantages over other approaches ((Alsmadi *et al*., 2003; Faruqi *et al*., 2001; Bodrossy *et al*., 2003; Castiglioni *et al*., 2004; Denef *et al*., 2003; Lievens *et al*., 2003). There is no practical restrain on the selection of the targeted pathogens. One may enlarge or reduce the PLP set at any time to increase the range of detected organisms or to focus on a particular group. Using PLPs and universal arrays, target recognition becomes independent of the downstream processes, including amplification and array analysis, reducing cost and allowing multipurpose applications.
By changing the design of the padlock probes quantitative analyses can also be achieved by using a new platform of real time PCR, the OpenArray™ system of BioTrove (Van Doorn *et al.*, 2007). Other amplification strategies of the circularised padlock probes such as rolling circle amplification (RCA) are being explored as well as other detection platforms (e.g. nanoparticles and Luminex).

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Quantitative multiplex detection of plant pathogens using a novel ligation probe-based system coupled with universal, high-throughput real-time PCR on OpenArrays™

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Abstract

Diagnostics and disease-management strategies require technologies enabling the simultaneous detection and quantification of a wide range of pathogenic microorganisms. Most multiplex quantitative detection methods available suffer from compromises between the level of multiplexing, throughput and accuracy of quantification. Here, we demonstrate the efficacy of a novel high-throughput ligation-based assay for simultaneous quantitative detection of multiple plant pathogens. The ligation probes, designated PRI-lock probes, are long oligonucleotides with target complementary regions at their 5' and 3' ends. Upon perfect target hybridization, the PRI-lock probes are circularized via enzymatic ligation, subsequently serving as template for individual, standardized amplification via unique probe-specific primers. Adaptation to OpenArrays™, which can accommodate up to 3072 PCR amplifications, performed in 33 nl, allowed high-throughput real-time quantification. The assay combines the multiplex capabilities and specificity of ligation reactions with high-throughput real-time PCR in the OpenArray™, resulting in a flexible, quantitative multiplex diagnostic system. The performance of the PRI-lock detection system was demonstrated using 13 probes targeting several significant plant pathogens at different taxonomic levels. All probes specifically detected their corresponding targets and provided perfect discrimination against non-target organisms with very similar ligation target sites. The nucleic acid targets could be reliably quantified over 5-6 orders of magnitude with a dynamic detection range of more than 10^4. Pathogen quantification was equally robust in single target versus mixed target assays. This novel assay enables very specific, high-throughput, quantitative detection of multiple pathogens over a wide range of target concentrations and should be easily adaptable for versatile diagnostic purposes.

Introduction

Current technologies for multiplex, quantitative analyses frequently suffer from compromises between the level of multiplexing, throughput and accuracy of quantification. In general, for the detection of nucleic acids, microarrays and macroarrays can provide very high levels of multiplexing (Sholberg et al., 2005; Tambong *et al*. 2006), but yield a limited range of accurate quantitative information (Lievens *et al*., 2005a) and relatively low sample throughput compared to real-time, quantitative PCR (qPCR) (Call, 2005; Ding

and Cantor, 2004). At present, qPCR provides the most reliable means of target quantification, and it is suitable for the analysis of relatively large sample numbers (Klein, 2002; Schena *et al.*, 2004). Nowadays, successful multiplex qPCR-based pathogen detection methodologies have been realized (Lievens *et al.*, 2006; Schena *et al.*, 2006; Tooley *et al.*, 2006), however, due to a limited number of potential dyes, the attainable level of multiplexing is low (Ding and Cantor, 2004; Mackay, 2004).

Recently, a conceptually new, high-throughput platform has become available for real-time PCR, capable of accommodating more than 3000 reactions per array (Morrison *et al.*, 2006). The OpenArray™ has 48 subarrays, allowing parallel testing of up to 48 samples, and each subarray contains 64 microscopic through-holes of 33 nl volumes (Figure 1A). Primers are pre-loaded and dried down in user specified holes. The holes function as capillaries, accurately self metering sample and master mix added by an automated loader. Hydrophilic holes and hydrophobic array-surface coatings ensure the sample remains isolated through surface tension.

The assay system described in this report incorporates the most attractive features of several approaches to provide a unique detection platform that combines multiplex technology with stringent diagnostic standards: (1) high specificity is achieved via the use of ligation-based circularization probes, (2) real-time PCR methodology provides excellent sensitivity and accurate quantification, and (3) adaptation to the newly developed OpenArray™ technology facilitates high-throughput sample screening.

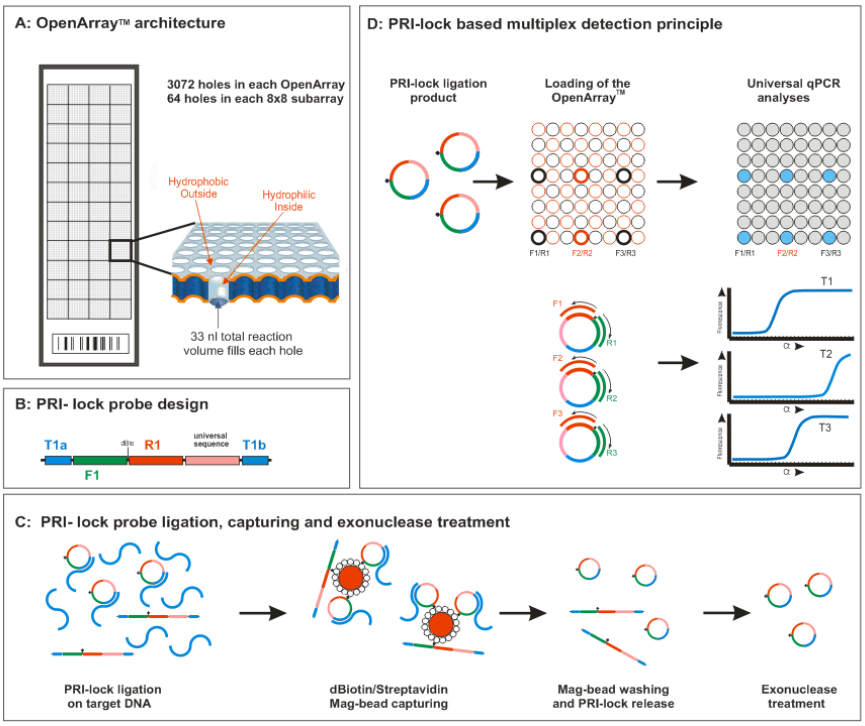

Figure 1: Schematic overview of the proposed assay. (**A**) OpenArray™ architecture. The OpenArray™ has 48 subarrays and each subarray contains 64 microscopic through-holes of 33 nl volume. The primers are pre-loaded into the holes. The sample combined with the reaction mix is auto-loaded due to the surface tension, provided by the hydrophilic coating of the holes and the hydrophobic surface of the array. (**B**) PRI-lock probe design. T1a and T1b indicate target complementary regions. Unique primer sites ensure specific amplification (forward: F1 and reverse: R1) and each PRI-lock contains a universal sequence and a desthiobiotin moiety (dBio). (**C**) Multiple target specific PRI-lock probes are ligated on fragmented DNA samples. T1a and T1b bind to adjacent sequences of the target and in case of a perfect match, the probe is circularized by a ligase. The probes are captured via the desthiobiotin moiety using magnetic streptavidin-coated beads. The PRI-lock probes are washed and quantitatively eluted from the beads. Unreacted probes are removed by exonuclease treatment. (**D**) Circularized probes are loaded and independently amplified on the Biotrove OpenArray™ platform using PRI-lock probe specific primers. The amplification is monitored using SYBR-Green and the ligated PRI-lock probes are quantified based on the threshold cycle number (C_T) of the

Here, we propose that a new, ligation based probe assay can bridge the gap between highly specific target recognition and high-throughput, multiplex pathogen quantification. Circularizable ligation probes (Padlock probes) (Nilsson *et al.*, 1994) have previously been applied successfully for multiplex pathogen detection (Szemes *et al.*, 2005), but do not allow quantification. In the currently developed assay, the circularized probes are amplified by using probe-unique primer pairs via real-time PCR, enabling accurate target quantification in a highly multiplex format. The utilized probes have been termed PRI-lock probes and they consist of two target complementary regions, one at each terminus of the probe (Figure 1B). The target complementary arms are connected via a compound linker sequence, containing unique primer binding sites, a generic sequence and a desthiobiotin moiety for specific capture and release (Hirsch *et al.*, 2002) (Figure

1B). The primer binding sites were selected to ensure optimal amplification under universal conditions and lack of interaction during the ligation and PCR steps.
In the implemented strategy, mixtures of multiple PRI-lock probes are ligated on fragmented DNA (Figure 1C). Target recognition is achieved by specific hybridization of both arm sequences, and efficient ligation occurs only if the end nucleotides perfectly match the target (Nilsson *et al*., 2006). Therefore, the probes confer superior specificity (Szemes *et al*., 2005; Nilsson *et al*., 2006). After ligation, the probes are reversibly captured via the desthiobiotin moiety, treated with exonuclease (Figure 1C) and finally individually assayed via real-time PCR on OpenArray™ plates (Figure 1D).
In this study, we characterize the quantification power of circularizable ligation probes over a range of target concentrations and multiple target ratios and report the development of a high-throughput, quantitative multiplex diagnostic assay. The specificity, sensitivity, linear quantification range, and the dynamic detection range of the developed assay were demonstrated using 13 pathogen specific PRI-lock probes, ligated on individual and mixed target DNAs, followed by real-time PCR on OpenArrays™.

Methods

Nucleic acids used in the study

Microorganisms were derived from the culture collection of Plant Research International BV (Table 1). Genomic DNAs from all microorganisms were isolated using the Puregene Genomic DNA isolation kit (Gentra/Biozym, Landgraaf, the Netherlands) according to the manufacturer's instructions. Ligation targets for assay optimization were generated using 500 pg extracted genomic DNA as PCR template. Generic primers were used to amplify selected genes as described previously (Van Doorn *et al*., 2007). The PRI-lock probes listed in Table 1 and all the other oligonucleotides used in this study were synthesized by Eurogentec SA (Seraing, Belgium).

PRI-lock probe design

The PRI-lock probe target complementary regions were engineered according to previously described design criteria (Szemes *et al*., 2005). Relevant nucleic acid sequences derived from Genbank and from independent sequencing studies were aligned by using ClustalW. Diagnostic sequences were identified for each target group. Potential PRI-lock probe target complementary regions were selected in a way that the discriminatory nucleotides would bind to the 3'arm region. Further design rules are described elsewhere (Szemes *et al*., 2005; Van Doorn *et al*., 2007).

Ligation, capturing and exonuclease treatment

Genomic DNA was fragmented by digestion using EcoRI, HindIII and BamHI (New England Biolabs) for 30 min, and used as template. Cycled ligation, capturing of ligated probes and exonucleae treatment were performed as previously described (Szemes *et al*., 2005; Van Doorn *et al*., 2007). PCR fragments and genomic DNAs were used as templates for ligation. To monitor the ligation efficiency and provide a reference for normalization, an Internal Ligation Control (ILC) PRI-lock probe was constructed (Van Doorn *et al*., 2007).

Real-time PCR (ABI) and Biotrove OpenArray™ real-time PCR

Amplification of ligated PRI-lock probes was monitored in real-time using the 7500 Real-Time PCR system (Applied Biosystems, Foster City, USA). Amplification of ligated PRI-lock probes was followed in real-time using an OpenArray™ NT Cycler (BioTrove Inc., Woburn, USA). OpenArray™ subarrays were pre-loaded by Biotrove with the selected primer pairs. Each primer pair was spotted in duplicate. Samples were loaded into OpenArray™ plates using the OpenArray™ NT Autoloader according to the manufacturer's protocols. The PCR OpenArray™ thermal cycling protocol consisted of 90°C for 10 min, followed by cycles of 28 s at 95°C, 1 min at 55°C and 70 s at 72°C (imaging step).

Table 1: Target complementary regions and unique primer sequences for the PRI-lock probes.

Targeted species/ Group	5' Target complementary sequence (5'-3')	3' Target complementary sequence (5'-3')	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Phytophthora spp.	TATCTAGTTAAAAGCA**GA**GACTTTCGTC	CTGCTGAAAGTTGC	TACGAACGTCTTAGCACTCC	GGTGTTGATTCGCGTCTACT
P. infestans	TCGATTCGTGGTATGGTTGGCTTCGGCT	CGTTAATGGAGAAATGC	AGAGTCGGTAGGCACTATGG	CGTATGTCGAATGCAGCTGA
R. solani AG 2-2	TCTGCCTCACAGGTTCACAGGTGTGTGTGG	TTCCCGTCCATGTC	GAGTTCCCGTGCGTTAGATC	TACGGCGCTTGGGACATGAT
R. solani AG 4-1	GGTCCAATAAAGT**T**CCTT**C**CCCCCTAGAAAA	AGTCCAA_**G**GAGAGTA___	CGTGTCCATCGAGCTGCATA	GACGGCATTCAGAGTACGCT
R. solani AG 4-2	GACTTCTGTCTACTTAATTCATATAAACTCAATT	CTT_CTACTCCCCCTT_	CTGCCTGTGACTCGTGTATC	AGACCGTATCGTCCACAGTG
F. oxysporum	GCGAGTCCCAACACCAAGCTGTGCTTG	GGAACGCGAATTAAC_	CTGGTGCATGTACTCGACTG	ATCAGATCGACTCGGTAGCT
M. roridum	CGGTGGTGGCCATGCCGTAAAACACC	ACTCGCATTGGAGCT	CATCCAGCTCAACGTATCCA	CCTACTGTGACGCTGTGATG
V. dahliae	TTTATACCAACGATACTTCTGAGTGTT	CATCAGTCTCTCTG	ATCTGGATCAACGTCGCGCT	ATACAGTCGTCGGGGTCGAA
V. alboatrum / V. tricorpus	TTTATACCAACGATACTTCTGAGTGTTCTTAGTGAAC	GTACATCAGTCTCTTTA	GCATCGGGTTCACGCCTATA	TGAAGCACTGACACGCGAAG
M. hapla	GTTTATCGTTGTGAATGGCTGTCGCTGGTG	ATTCGAATAGTCTCAAC	TATGGGTCTTGCTGATACGC	TCCGTCTGTTGAGTTAGGCC
E. carotovora carotovora	AAAACCTGTGCGTTCATCGATGCTGAACAT	TCAACGCGAAGGA	AGAATCGTACACGCTGCTGG	AATACGACTGACACGAGCTG
A. tumefaciens	TCCGGTTGA**T**AGTTGAGGACA**G**CATTGGAC	GTTGGTCGTCCGCT	CAATACCTGTGACGAGCTGG	ACCCGGTCACTCAGCATATA
G. Proteo bacterial spp.	GGCCTTCTTCACACACGCGGCATGGCTGCA	GCTTTACAACCCGAA	ACAGGTCATCGAACTCTCAC	AGAACACGTCAGAGGTCCGT
Internal Ligation Control (ILC)	GGGAGAACACTGCGTGGTTTTCACATAC	GCTTGTGCCTCTCGA	CTATCGCGTGCTAGTCGTCT	ATTCTAATCAATCGTCGCGG

Bold characters indicate polymorphism within the target group. Nucleotides or gaps owing to deletions used to discriminate from most similar, non-target sequences, are underlined. (No such sequence was found for the PRI-lock probes *G. Proteo* bacterial spp. and *A. tumefaciens*)

Results

PRI-lock probe design and evaluation of assay performance

The newly designed probing system was experimentally tested using 13 PRI-lock probes engineered to detect several economically important plant pathogens at different taxonomic levels (Table 1). The target complementary regions were selected as previously described, and the specificity of the probes was verified (Szemes *et al*., 2005). Unlike padlock probes, each of the PRI-lock probes was designed with unique primer binding sites allowing quantitative detection. The PRI-lock probes were also engineered with a desthiobiotin moiety between the primers sites for reversible PRI-lock probe capture, washing and release using streptavidin-coated magnetic beads. The introduction of this additional purification step removes excess non-target DNAs and possible enzyme-inhibiting compounds, resulting in increased efficiency of exonuclease treatment and reduced assay background (data not shown).

The developed PRI-lock probe system was validated in several steps. To evaluate the specificity of the designed PRI-lock probes, a mixture of 14 probes (13 target probes plus one internal ligation control) was ligated on various individual DNA targets. The samples were screened for amplification with all the individual PRI-lock probe specific primer pairs in conventional real-time PCR. All the target sequences were specifically detected without exception (Table 2) and no amplification of ligated PRI-lock probes with other, non-cognate primer pairs occurred (data not shown). In this and all subsequent experiments, the amplification efficiency of the PCR control was found to be uniform (data not shown) and the C_T values were normalized using the ILC. To further ensure specificity towards the targeted pathogens, 3 non-target organisms with very similar ligation sites (1, 3 and 7 mismatches compared to the perfect ligation sequence) for the PRI-lock mixture were tested. The rest of the probes discriminated their targets from the most similar non-target sequence based on more than 7 mismatches and were not tested. In agreement with previous results (Szemes *et al*., 2005), no signal was observed in the presence of the non-target organisms with very similar ligation sites (Van Doorn *et al*., 2007), demonstrating the specific target recognition by the designed PRI-lock probes.

To evaluate the performance of the PRI-lock system in a multiplex setting, the probe mix was ligated on several target DNA mixtures and analyzed in conventional real-time PCR. Multiple target DNA sequences were detected with no statistically significant change in the observed C_T values compared with the C_T values observed for single-target detection (Table 2).

In diagnostic applications, we expect to quantify several target organisms over large concentration differences. To investigate the sensitivity and linear quantification range of the PRI-lock probe system in conventional real-time PCR, a 10-fold dilution series of all selected targets were detected and quantified. A linear quantification range of 6 orders of magnitude was achieved with a sensitivity of 10^3 -10^5 target copies/ µl initial ligation mixture for all targets and corresponding PRI-lock probes.

Application of the PRI-lock based multiplex quantitative detection on the Biotrove OpenArray™ platform

On a conventional real-time PCR machine, analysis of a single sample however, would typically require 14 separate reactions, one for each probe, making large scale screenings time consuming, laborious and expensive. A solution was offered by the Biotrove OpenArray™ system, which provides a platform for the parallel analysis of 48 samples against the 14 PRI-lock probes. To compare the performance of the PRI-lock system in the Biotrove OpenArray™ with the performance in conventional real-time PCR, samples containing single or multiple targets were ligated and analyzed on the Biotrove OpenArray™ platform. The obtained C_T values were found to be similar to the C_T values observed in the conventional real-time PCR (Table 2), although the number of target copies per PCR was lower in the OpenArray™ due to smaller volume.

Quantification and validation

To enable accurate pathogen quantification of unknown samples, calibration curves were constructed for each of the 13 PRI-lock probe targets. Because the ligation of different probes is independent, the calibration curves could be constructed based on C_T values measured in a multiplex setting. The PRI-lock probes were ligated on a 10-fold dilution series of all target DNA sequences and amplified in the Biotrove OpenArray™

platform. The obtained calibration curves showed that all the target pathogens were quantified over at least 5 orders of magnitude (Van Doorn *et al*., 2007). The correlation between the logarithmic target concentration and the observed C_T value was very high for all the detected pathogens (lowest $R^2 = 0.984$, average $R^2 = 0.993$).
The developed multiplex system was tested on individual practical samples from recirculation water systems. Pathogens, detected with traditional methods, were also perfectly detected in multiplex setting.

Table 2: Specificity and multiplexing of the PRI-lock probe system in conventional real-time PCR and in the Biotrove OpenArray™ for single and multiple targets.

	Single target		Multiplex mix 1		Multiplex mix 2		Multiplex mix 3	
PRI-lock Probe	C_T(AB)	C_T(BT)	C_T(AB)	C_T(BT)	C_T(AB)	C_T(BT)	C_T(AB)	C_T(BT)
Phytophthora spp.	15.5 (0.14)	16.4 (0.17)	15.7 (0.10)	16.7 (0.25)	16.1 (0.04)	16.8 (0.12)	16.3 (0.16)	15.9 (0.09)
P. infestans	14.8 (0.12)	15.4 (0.18)	--	--	15.2 (0.08)	15.7 (0.12)	15.1 (0.02)	15.0 (0.08)
A. tumefaciens	16.4 (0.24)	16.2 (0.04)	--	--	--	--	15.8 (0.28)	15.9 (0.06)
G. Proteo bacterial spp	17.0 (0.11)	17.7 (0.08)	--	--	--	--	17.7 (1.03)	17.1 (0.04)
M. roridum	16.2 (0.07)	--	16.4 (0.02)	17.2 (0.05)	--	--	16.7 (0.12)	17.0 (0.16)
F. oxysporum	17.4 (0.11)	--	--	--	--	--	17.2 (0.08)	17.3 (0.04)
E. carotovora carotovora	15.1 (0.15)	--	--	--	--	--	15.2 (0.24)	15.4 (0.05)
V. dahliae	16.9 (0.01)	--	--	--	--	--	16.1 (0.04)	15.8 (0.03)
V. alboatrum / V. tricorpus	19.6 (0.11)	--	--	--	--	--	19.9 (0.29)	18.0 (0.10)
M. hapla	17.0 (0.30)	--	--	--	--	--	18.2 (0.23)	16.9 (0.11)
R. solani AG 2-2	16.3 (0.03)	--	--	--	15.8 (0.10)	17.1 (0.12)	16.2 (0.03)	16.2 (0.04)
R. solani AG 4-1	14.0 (0.02)	--	--	--	14.6 (0.13)	15.0 (0.11)	14.5 (0.13)	14.4 (0.09)
R. solani AG 4-2	19.3 (0.10)	--	--	--	19.5 (0.14)	20.0 (0.18)	19.3 (0.07)	18.8 (0.03)

The PRI-lock probes were ligated on 10^6 targets/µl ligation mixture. C_T values were normalized using the ILC control PRI-lock probe. Data represent average C_T values of three PCR replicates in the conventional real-time platform (n=3) and of four PCR replicates in the Biotrove OpenArray™ (n=4). Standard deviations are indicated between brackets. AB: samples run on the conventional real-time PCR platform. BT: Samples tested on the Biotrove OpenArray™ platform. Multiplex mix 1: *P. soj.* and *M. ror.* DNA, Multiplex mix 2: *P. inf.*, *R. sol.* AG 2-2, *R. sol.* AG 4-1 and *R. sol.* AG 4-2 DNA, Multiplex mix 3: ligation mixture containing all the DNA targets indicated in table 1, --: not tested.

Discussion

In this study, we demonstrated a specific, multiplex, PRI-lock probe-based, high-throughput detection assay using the Biotrove OpenArray™ platform for the detection and quantification of plant pathogens. The described application serves as a model for the development of rapid, molecular detection systems that offer an unprecedented combination of specificity, high throughput capabilities and robust target quantification.
To evaluate the designed PRI-lock probes and corresponding primer pairs, the probes were ligated on single target sequences and tested using conventional real-time PCR. In all cases, the targets were correctly detected and no false positives were observed, indicating highly specific ligation of the PRI-lock probes on their respective targets and no cross reaction with closely related targets. In addition, the influence of multiple targets on PRI-lock probe based detection was tested, and it was demonstrated that the presence of multiple targets had no statistically significant influence. Our multiplex detection system provided truly independent detection of the different pathogens, with no evidence of inhibition due to possible ligation competition.

Quantitative diagnostic assays require a linear range of quantification of several orders of magnitude. We showed that ligation of PRI-lock probes can reflect well the target quantity over at least 5 orders of magnitude.
The sensitivity of detection was determined by testing a 10-fold dilution series of ligation targets for all the PRI-lock probes. Sensitivities of 10^3 and 10^4 target copies/µl initial ligation mixture were achieved, depending on the PRI-lock probe.
Both, the inter-array and assay-to-assay variation of the PRI-lock system were very low, demonstrating the reproducibility and quantitative reliability of the PRI-lock detection assay. The developed quantitative multiplex detection assay was validated by testing various artificial mixtures of target DNAs. The observed dynamic range is an improvement over most previously developed multiplex pathogen detection assays, where the dynamic range was often limited to 100-1000 (Szemes *et al*., 2005; Denef *et al*., 2003). In the Biotrove OpenArray™ platform, target PCR amplifications are completely independent of each other, and, consequently, no PCR competition among the different ligated PRI-lock occurs. The dynamic range of detection in the PRI-lock detection assay is therefore, as long as the PRI-lock probes ligate on their respective targets, practically unlimited. Finally, artificial mixtures of genomic DNAs in different concentration ratios were tested. The components of the pathogen mixture were identified, and the original target input was calculated using the calibration formulas. Moreover, the ratios of the targets among the different ligation samples were correctly identified as well.
Circularization probes have previously been applied successfully for the detection of multiple plant pathogens in diagnostic samples (Szemes *et al*., 2005), but without the ability to quantify target numbers. To our knowledge, this report presents the first time that numerous plant pathogens could be simultaneously and accurately quantified using specific circularization probes in a single assay. For future applications, however, higher multiplexing is intended and therefore, the number of PRI-lock probes will be increased. Currently, assay background is considered as the biggest obstacle for increasing the level of multiplexing in traditional circularization probe-based diagnostic assays. Traditional circularization probes contain generic primer sites for PCR amplification (Szemes *et al*., 2005). Multiplex PCR via general primer sites carries the potential for competition during amplification, with a cumulative increase in background, which reduces assay sensitivity. In contrast, each PRI-lock probe carries a unique pair of primer binding sites, unrelated to the sequences of all the other probes. Circularized PRI-lock probes can therefore be amplified individually. Increasing the level of multiplexing would not be expected to increase the background signal. To further guarantee low background, even in highly multiplex settings, we are currently developing a universal TaqMan® probe which hybridizes to the generic sequence incorporated in all the PRI-lock probes. In addition, including a universal TaqMan® probe should speed up data analysis, and therefore sample throughput, since there is no need to conduct amplicon dissociation curve analysis. Given the independent PCR amplification of the ligated PRI-lock probes and the three slide capacity of the OpenArray™ NT Cycler, it should be feasible to engineer ultra-high throughput arrays for the quantitative detection of hundreds of targets simultaneously.

Conclusions

To date, most multiplex pathogen detection has been performed using traditional hybridization microarrays (Call, 2005; Lievens *et al*., 2005b). Although such platforms typically allow highly multiplex detection, they generally offer relatively low sample throughput and yield limited quantitative information compared to qPCR (Abruzzo *et al*., 2005). In this study, we described a high-throughput diagnostic system that combines very specific multiplex pathogen detection with accurate quantification over a range of target concentrations. The PRI-lock probes, combined with the OpenArray™ system, offer a flexible and adaptable design of high-throughput, quantitative multiplex detection assays, since the target recognition is separated from further downstream processing. It should be noted, although we have demonstrated that large quantities of non-target DNA do not influence the accuracy of PRI-lock probe-based detection, PRI-lock performance remains to be examined within field applications. The PRI-lock system described is readily modifiable and expandable to include an almost unlimited range of potential targets, providing an easily accessible platform for versatile diagnostic applications.

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A 16S rDNA-based microarray for high-throughput diversity analysis of complex bacterial communities

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Abstract

The improved understanding of the global functioning of microbial communities requires tools to rapidly describe spatiotemporal variations of their various taxon components. For this purpose, our group developed a 16S biochip for high throughput and simultaneous characterizations of bacterial taxa in complex environments, and especially agricultural soils and rhizospheres. The chip was tested with pure cultures as well as metagenomes of soil and rhizosphere communities. Complex community compositions obtained from statistical treatment of biochip data were verified by alternative methods such as cloning/sequencing and quantitative PCR performed with the same metagenomes. The project provided relevant information about the required probe characteristics and hybridization conditions, the level of probe specificity according to mismatches, the number of probes yielding false positive or negative results, and the biochip sensitivity, measured by the smallest fraction of a community that could be detected with enough confidence. The project *in fine* demonstrated the great potential of the high throughput microarray approach for aspects of environmental microbiology that need the simultaneous identification of huge numbers of taxa.

Introduction

Determining the taxonomic composition of bacterial communities is a constant request in microbial ecology. This is needed for a better knowledge of the taxon distribution in ecosystems or for more applied purposes such as the detection of pathogens, the monitoring of the fate of inoculated biocontrol agents or of the whole community in polluted soils. Moreover, because taxa are often present in huge number and because individual taxonomic identifications require a lot of expertise, taxonomic inventory of complex communities is yet poorly documented. As a result, the respective role of taxa in the global functioning of microbial communities is badly known. Our group is thus developing a general taxonomic microarray to analyse and monitor the diversity of complex bacterial community by targeting the 16S gene.

Microarrays have begun to be used for the detection and identification of microbial strains, species, genera or higher taxa in a single assay. The most obvious targets are *rrs* and *rrl* (i.e. 16S and 23S rRNA genes) and the feasibility of a microarray approach for bacterial identification has been tested by Bodrossy and Sessitsch (2004). Functional genes, e.g. *pmoA, nifH* have also been used to assess functional communities (Jenkins et al., 2004; Bodrossy et al., 2003). In contrast to functional genes, the high level of sequence conservation of the 16S rRNA gene and its ubiquity in the prokaryotic domain (Woese and Fox, 1977) allow access by PCR to almost all bacteria (Bruce et al., 1992). Consequently, one of the great advantages of the 16S rRNA gene is the availability of the largest databases for a single gene (Cole et al., 2005). These databases were used to construct the ARB 16S rRNA database, which contains correctly aligned sequences of high quality (Ludwig et al., 2004). The 16S rRNA gene can be used to design probes targeting bacteria at various taxonomic levels, from phyla and classes to species.

The present report reviewed results obtained during the project that described the development and the validation of a multi-purpose 16S microarray either with pure cultures (Sanguin et al., 2006a) or with natural bacterial communities from bulk or rhizosphere soils (Sanguin et al., 2006b), and its practical application

for analyzing bacterial communities differences between soils which are either safe, conducive or suppressive to take-all (Sanguin et al., 2006c).

Design and resulting characteristics of the 16S microarray

Probe characteristics

Probes complementary to *rrs* sequences (16S rDNA gene) were designed to allow hybridization with amplified ribosomal DNA or ribosomal RNA (Figure 1). Preliminary tests showed that 18–21 nt long probes exhibited the best compromise between sensitivity, specificity, and the necessity to homogenize probes with respect to their dissociation temperature. The chosen probe length was thus generally 20 nt with some modifications when length could not be adjusted to 20 nt as it affected specificity. Most probes were chosen with predicted melting temperatures of 65 ± 5 °C and a minimum GC content of 50%. In addition, probes having selective nucleotides (corresponding to mismatches) located in a central position were favoured to enhance specificity. Probes with strong hairpin structures ($\Delta G \leq -2$ kcal mol^{-1}, $Tm \geq 50$ °C) were removed from the probe set.

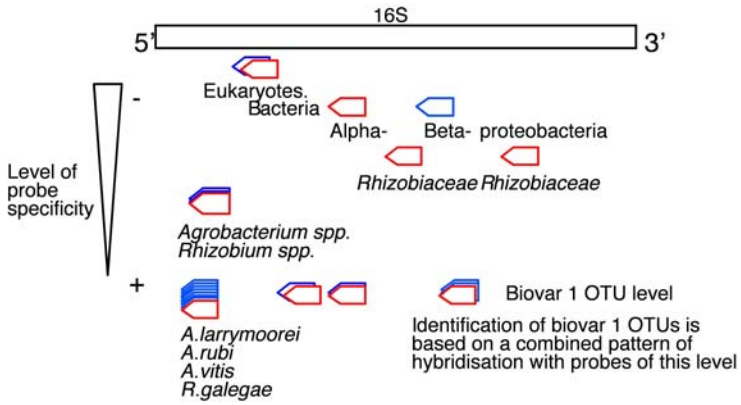

Figure 1: Nested phylogenetic strategy design and localization of *rrs* probes allowing identification of *Agrobacterium* members at various taxonomic levels from cluster of species (16S OTU) to phyla and domains. The probe quality was systematically tested with *Agrobacterium* pure cultures. The same design strategy was used for other taxa included in the biochip but the specificity of probe responses to hybridizations could be tested only when pure cultures were available. The unavailability of numerous bacteria in pure culture is a known limitation for the exhaustive testing of all 16S probes.

Taxonomic range allowed by 16S probes

The finest taxonomic level allowed by the *rrs* diversity is higher than the species because in most taxa, such as *Agrobacterium* (Mougel et al., 2002), readily different *bona fide* (i.e. genomic) species may actually have identical 16S rDNA. In the present report, the term 16S operational taxonomic unit (OTU) was thus used to designate bacteria with the same 16S rRNA gene sequence. In addition, a single nucleotide polymorphism (SNP) is often not enough to discriminate clusters of closely related species. For instance, in the species complex *Agrobacterium tumefaciens* (i.e. biovar 1), several probes are required to unambiguously distinguish one OTU from one another.

Conversely, *rrs* is known to allow the design of probes targeting bacteria at various taxonomic levels higher than 16S OTUs (Figure 1). The phylogenetic software package ARB (Ludwig et al., 2004) (http://www.arb-home.de) with the ARB 16S rRNA database (ssu02jun.arb) was used to design probes and predict their specificity. The selected probes target a wide range of taxonomic levels from superior taxa phyla, class, families, genera and 16S OTUs. As a result of the nested phylogenetic strategy, OTUs hybridized to several probes corresponding to various taxonomic levels. Thus, even if there is no 16S OTU specific probe for some bacteria in the present probe set, there will always be at least one probe designed for higher taxonomic levels that would hybridize to any new strain.

Microarray probe set

The present set of validated probes (ca. 170) is available in supplemental data of Sanguin's papers (2006 a, b, c). The number of probes currently tested reach however ca. 800 and is still increasing, and will be made available soon. The most common taxa in soil and rhizospheres, *Alpha-, Beta-, Delta-, Epsilonproteobacteria*, *Actinobacteria*, *Acidobacteria*, *Planctomycetes*, *Firmicutes, Bacteroidetes, Actinobacteria, Verrucomicrobia*, *Chloroflexi*, *Cyanobacteria*, were the more intensively investigated. With some families, such as *Bradyrhizobiaceae*, only few probes for identification below the family level could be obtained.

This failure illustrates the limit of the 16S rRNA approach to discriminate between closely related taxa in certain cases.

DNA targets and hybridization conditions

General and population specific DNA targets

The target DNA hybridized on the biochip was obtained by PCR amplification of *rrs*. For standard analyses with pure cultures or for analyzing whole communities, PCR were performed with "universal" primers PA and PH' (Bruce et al., 1992). We verified that it was also possible to have access to taxonomic subsets of the whole community, for instance agrobacteria or pseudomonads, by using more selective primers. This is a very important potential of our 16S biochip approach. The specificity of the PCR reaction is so high that subsets of the whole community, which are hardly accessible with universal primers, could be thoroughly analyzed using more selective primers (Sanguin et al., 2006 b, c).

Hybridization conditions

In preliminary studies, PCR products were directly labelled by using a Cy3 labelled PA primer. Although, this 5'-end-labeling procedure was found efficient with probes designed in the 5' end of *rrs*, for unknown reasons it failed to provide significant hybridization signals with probes designed in the 3' end of *rrs*. Target products were later labelled with Cy3-dCTP (Amersham Biosciences Europe GmbH, Saclay, France) during PCR in order to generate internally labelled double-stranded DNA which hybridized all probes whatever their localization in *rrs*. Alternatively, the 16S amplicons were labelled by *in vitro* transcription as described by Stralis-Pavese et al. (2004), using in this case Cy3-UTP (Amersham Biosciences Europe GmbH, Saclay) and PCR products obtained with primers containing a T7 (5'-TAATACGACTCACTATAG-3') terminus in 5' to allow processing of the T7 RNA polymerase.

Mismatch level and probe specificity

The expected specificity of probes was estimated by determining potentially matching 16S OTUs with the function Probe Match of ARB against the totality of the ARB 16S rRNA database. Then, intensities of signal hybridization were plotted against weighted mismatch (WMM) values. With the chosen labelling and hybridization conditions (overnight hybridization at 50°C, washing at room temperature by shaking for 5 min in 2X SSC containing 0.03% SDS and then twice for 5 min in 0.5X SSC), an optimal WMM cut-off of 2 was found to be the best compromise between unpredicted positive hybridizations and unpredicted negative hybridizations: 2.8% and 0.8% for false positive and false negative hybridizations, respectively. However, the latest results obtained with RNA instead of DNA for hybridizations, the cut-off is 1.5 WMM (Grundmann, personal communication). This has important implications for designing new probes as well as for the interpretation of microarray results.

Significant hybridization signals were never found with 16S OTU displaying more than 3.5 WMM. For subsequent data analyses, bacteria targeted by each probe with up to 3.5 WMM were listed with the Probe Match function. The Probe Match output files were then transferred to CalcOligo version 1.07 (Bodrossy et al., 2003). The CalcOligo output file was used to fill a spreadsheet with the WMM values of microarray probes with each 16S rRNA gene sequence of the ARB database. This spreadsheet was used hereafter to analyze hybridization data (Figure 2). As a follow up of CalcOligo, ARB outputs are now transferred to a web-interfaced database (Prestat et al., 2005) designed to store and query a higher number of probe/target relationships. This was a necessary development as we now have more than one thousand probes available. This database is also designed to store all other ecological data and experimental conditions as required by MIAME standards as well as microarray results and statistical analyses to facilitate integration of data. Collection of feedback information on the behaviour and quality of probe responses is an important objective of this project.

Data processing

An important milestone of microarray studies is the way by which raw data are processed to provide reliable and comparable results between slides.

The basic probe pattern on the microarrays consisted of two spots for each of the control (i.e. universal)

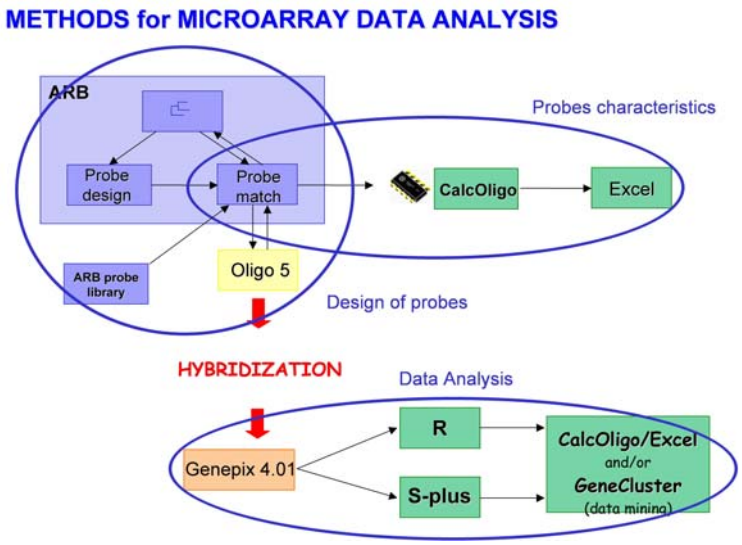

Figure 2: Flow charts of manual and bioinformatics methods used to select probes and to keep their characteristics in a spreadsheet for integration in subsequent analyses of microarray hybridization data. The most relevant characteristic of a probe is its expected level of specificity - expressed as the number of weighted mismatch (WMM between 0 and 3.5) - with 16S OTUs of the ARB database.

probes EUB342 and EUB338, four spots for the probe AntiPA (used as landmarks for image analysis) and one spot for each feature probe. The basic pattern was replicated six times on a microarray, leading thus to six spots per feature probe per microarray.

Filtration and normalisation

Pre-processing and subsequent statistical analyses use scripts developed from the R statistical software environment (R Development Core Team 2007, http://www.R-project.org).
Individual spots were considered positive if 80% of the spot pixels had an intensity higher than the median local background pixel intensity plus twice the standard deviation of the local background. This procedure decreases the probability of false positive signals to 2.5%. Moreover, a given feature probe was considered as truly hybridized when at least three of six replicate spots provided a significantly positive hybridization.
Using square root of intensity signals, statistics showed that best method of data normalization for reliable comparisons of different slides was given by expressing the intensity of each spot as the percentage of the total intensity signal of its local basic pattern.

Quantification

When microarrays are hybridized with DNA from pure cultures, all probes are thought to be in contact with an equal amount of targets. Intuitionally, similar hybridization signal intensities were expected. However, in spite of the great care taken for probe design and even in case of perfect match in stoichiometric conditions, hybridization intensity values span over a large range. Most probes with perfect mach displayed a relative hybridization intensity varying from 20% to 150 % of the intensity obtained with the universal probe EUB used as reference (with a median value at 96%). The median value was still 64% of the EUB intensity with probes having 0.1 to 1.9 WMM, but hybridization intensities dropped dramatically for 2 or more WMM. The underlying cause of intensity variations is not clear, but they are reproducible with the same relative ranking. The use of the 16S microarray for quantification needs thus the knowledge of the relative intensity obtained for each probe in case of perfect match. This was only possible for a limited number of probes, but, as indicated above, not reasonably feasible as the number of probes increased dramatically and because targeted OTUs are not available in pure culture. This is a major limitation of the present 16S microarray especially when it is used with environmental samples consisting in a mixture of 16S OTUs in variable amount.
A semi-quantitative scale was established for metagenomic DNAs based on the quartiles of the normalized hybridization intensities with four classes of relative fluorescence units (RFU) : > 3%, 1.5-3%, 0.9-1.5%, and < 0.9%. However, comparison of microarray results for environmental samples was carried out on the normalized intensity signals by principal component analysis (PCA) using the package ade4 (http://pbil.univ-lyon1.fr/ADE-4/) for R or by hierarchical cluster analysis and the K-Means test using R. The PCA was performed by using the covariance matrix, and the hierarchical cluster analysis was based on Ward's method.

Detection limit

Spiking experiments were done with 16S rRNA PCR products from bacteria not present in the tested soil. Microarray analyses showed that the detection limit was 0.03% of a DNA type in a complex metagenome. This means 0.45 ng of DNA or at least 10^8 amplicon copies. This detection limit should be extrapolated cautiously to estimate the detection threshold of community members, since DNA extraction and PCR from complex samples were not taken into account in the experiment. Nevertheless, the probes for *Agrobacterium* biovar 1 hybridized in the case of bulk soil, in which this taxon amounts to 10^3 CFU g/1 (Vogel et al., 2002), and this level is among the lowest detection levels published so far in microarray studies.

Microarray analysis of bacterial communities

The 16S microarray was used to analyze the taxonomic content of complex soil and rhizosphere communities. In all instances, results were satisfyingly validated by comparison with data obtained by the accurate but long and expensive cloning sequencing approach performed with the same metagenomic PCR products (Figure 3).

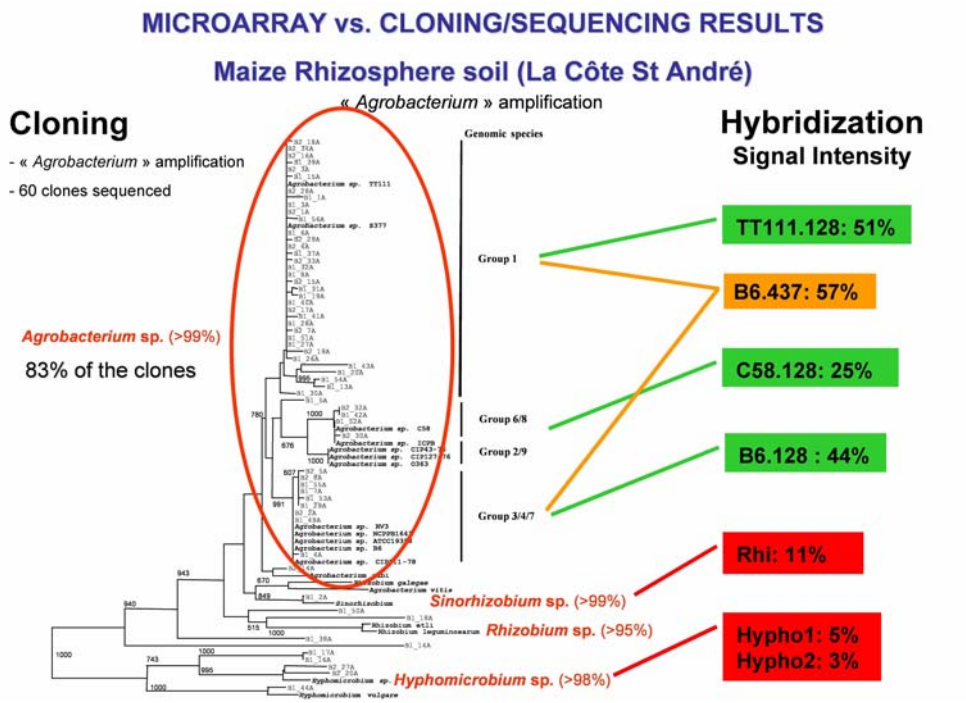

Figure 3: Comparison of the diversity and number of taxa inferred from the cloning/sequencing analysis to quantitative microarray data both obtained with the same PCR products obtained from a maize rhizosphere community (adapted from Sanguin et al., 2006a). Present results focused on the *Agrobacterium* sub-community obtained with *Agrobacterium* selective primers. More than 83% of the sequenced clones were identified as Agrobacterium members, among which a large majority had the same 16S sequences than TT111, B6or C58, and the reference strains of Agrobacterium sp. G1, G4 and G8, respectively. In parallel, probes TT111.128, B6.128 and C58.128 delivered the most intense hybridization signal of the microarray. Interestingly, the selectivity of the Agrobacterium primers was low and 16S from related taxa *Sinorhizobium, Rhizobium* as well as the more distant *Hyphomicrobium* were detected by both methodologies.

The 16S microarray was found to be efficient in rapidly providing a "snapshot" of the taxon composition of a given community for instance a maize rhizosphere (Sanguin et al., 2006a). It was also found to be particularly relevant to contrast taxon composition differences between related communities such as bulk soil and rhizosphere from the same location (Sanguin et al., 2006b) or soils indemn, conducive or suppressive to take all (Sanguin et al., 2006c).

Bacterial communities of a maize field

At the whole communities level, the studied maize rhizosphere revealed a high dominance of *Proteobacteria* (97%), mainly *Betaproteobacteria* (64%) and *Alphaproteobacteria* (28%), whereas only 3% were affiliated with *Acidobacteria*. Conversely, *Proteobacteria* (57%) and *Acidobacteria* (17%) dominated the related bulk soil, but several major phyla were also observed, i.e., *Planctomycetes*, *Bacteroidetes*, *Gemmatimonadetes*, *Chloroflexi*/ *Thermomicrobia*, *Verrucomicrobia*, *Actinobacteria*, and *Firmicutes*, as well as the TM7 division.

For taxonomic levels below that of the phylum, strong hybridization signals were observed for several *Alphaproteobacteria* probes, such as those targeting the three families *Rhizobiaceae*, *Bradyrhizobiaceae*, and *Brucellaceae* and *Sphingomonas* spp. For the *Agrobacterium* spp. taxon, probes yielded positive signals of various intensity levels, among which the three specific probes targeting the genomic species of *Agrobacterium* biovar 1 yielded strong hybridization signals (Figure 3).

Differential taxonomic content of related communities can be obtained by plotting directly the normalized hybridization intensities of probes. By this way, one can have a direct access to major taxa as well as to taxa specific of one community (Figure 4). As a result, by contrast to what was revealed by *Agrobacterium* probes of the specific and relative abundance of *Agrobacterium* members in the maize rhizopshere, Rhizo157 indicates the occurrence of a much large amount of *Rhizobium* sp. in the bulk soil than in the maize rhizosphere. This rapidly showed that related genera *Agrobacterium* and *Rhizobium*, both present in the studied soil, should have differential rhizospheric affinities for maize.

This is in agreement with our knowledge of the prevalence of *Agrobacterium* in this biotope. In addition, the detection of other taxa, e.g. *Sinorhizobium* and *Rhizobium* is in agreement with previous data from the same experimental site (Teyssier-Cuvelle et al., 1999).

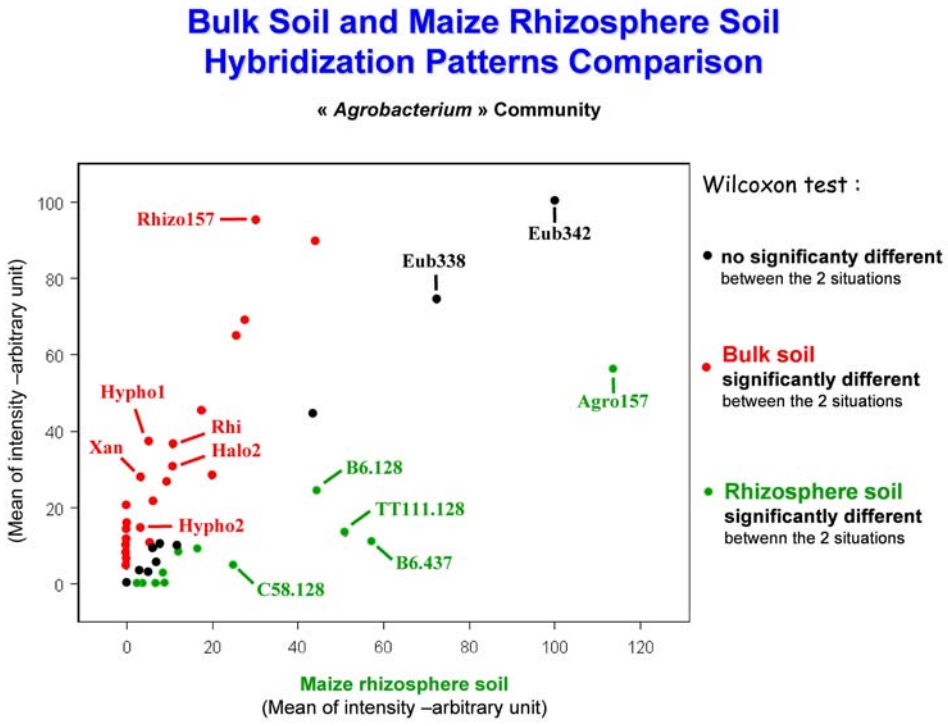

Figure 4: Comparison of microarray hybridization patterns obtained with bulk and maize rhizosphere soils from the same location (adapted from Sanguin et al., 2006b).

Microarray analysis of communities of soils safe, conducive or suppressive to take-all

Take-all is an important wheat disease caused by the soil-borne fungus *Gaeumannomyces graminis* var. *tritici*. Disease severity can be high, but a decline of take-all disease may take place in the following years in case of wheat monocropping. Microbial populations known to be associated to take-all decline (disease suppressiveness) include cultivable antagonistic fluorescent *Pseudomonas* spp. producing the antifungal compound 2,4-diacetylphloroglucinol. Changes in the diversity of rhizosphere pseudomonads linked with

take-all decline of wheat was monitored based on the use of a taxonomic microarray which contains ca. 700 probes targeting bacteria at various taxonomic levels. Some probes (ca. 70) target pseudomonads, including groups of biocontrol *Pseudomonas* strains (ca. 20 probes).
Rhizosphere samples were collected at the INRA station of La Gruche (Brittany, France) from plots grown with wheat for one year (treatment PI; low level of take-all disease), five years (treatment PV; high level of disease) or ten years (treatment PX; low level of disease, suppressiveness reached). This experimental set-up enabled comparison of treatments under same conditions of soil composition, microclimate, wheat cultivar and farming techniques.

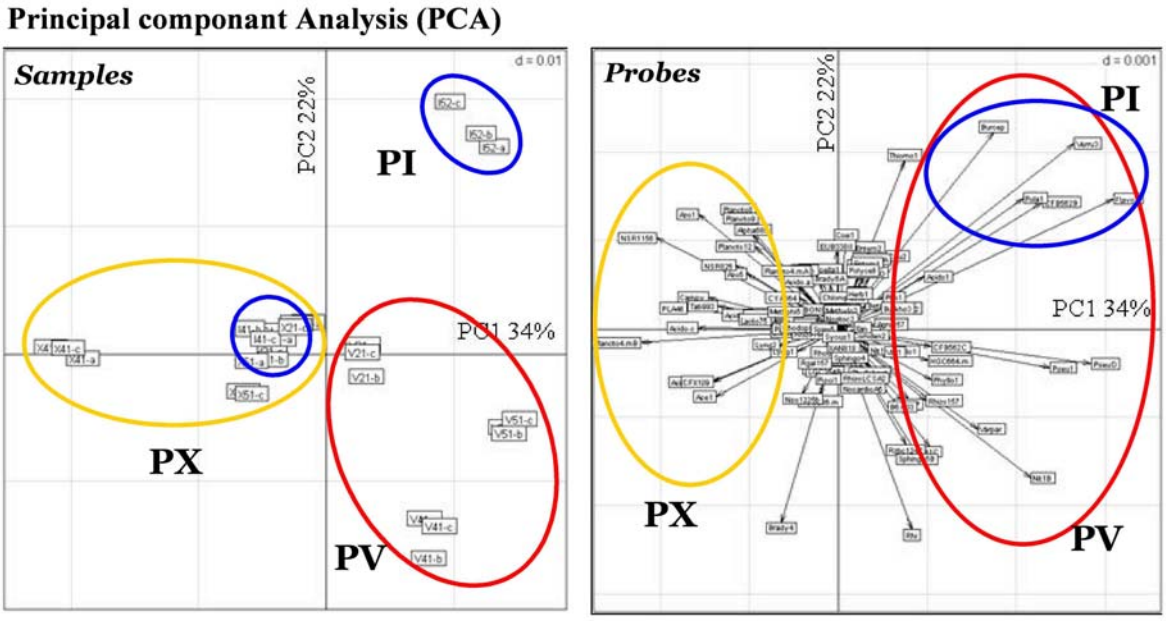

Figure 5: Cluster analysis of microarray data obtained after PCR of *Pseudomonas* populations in the wheat rhizosphere. Marked and reproducible differences were found between: PI (safe soil), PV (conducive soil) and PX (supressive soil for take-all) (adapted from Sanguin et al., 2006c). Results are in accordance with quantitative PCR data obtained for the total pseudomonads. Overall, treatment PX appears to be associated with a particular composition in biocontrol pseudomonads (which comprise biocontrol strains producing 2,4- diacetylphloroglucinol).

PCA analyses revealed a clustering of both samples and probe intensity according to treatments (Figure 5). The main differences were found between conducive (PV) and suppressive (PX) soils.
Major taxa of conducive soil were: *Gammaproteobacteria* (*Pseudomonas*), *Alphaproteobacteria* (*Sphingomonadaceae, Rhizobiaceae*, *Phyllobacteriaceae*), *Betaproteobacteria* (*Burkholderia cepacia*, *Nitrosospira*, *Variovorax*, *Acidovorax*, *Thiomonas*, *Polaromonas*), *Bacteroidetes*, *Flavobacteria* (*Flavobacterium*), *Verrucomicrobia*, *Actinobacteria*.
Major taxa of suppressive soil were: *Alphaproteobacteria* (*Azospirillum*), *Planctomycetes*, *Nitrospira*, *Acidobacteria*, *Chloroflexi, Firmicutes (Thermoanaerobacter)*.
At the whole community level, the conducive soil showed the most marked differences. However, while whole taxonomic patterns of PI and PX are similar, suggesting a certain degree of resilience after the disease peak, microarray analysis of the *Pseudomonas* sub-community revealed that pseudomonads from the suppressive (PX) soil clearly differ from those of the safe initial soil (PI). This is in agreement with previous knowledge about *Pseudomonas* taxa involved in take-all (McSpadden-Gardener and Weller, 2001). However, this work provided these information in a quite instant manner compared to former time-consuming methodologies. In addition, it also delivered a lot of yet unknown information about simultaneous and unforeseen variations of a lot of other taxa. The role or the sensitivity of those taxa in the evolution of soils during the take-all disease sequence is presently completely unknown. However, whole taxa variation certainly disserves to be studied in order to better managed take-all supressiveness.

Conclusion

The full scale application of 16S rRNA-based taxonomic microarray relies on a highly efficient probe set. The exploration of 16S rRNA databases carried out in this project led to the conclusion that the potential targets can be predicted based on a level of WMM defined by the hybridization conditions. The comparison of microarray and cloning-sequencing results demonstrated the efficacy of the microarray on complex samples. Microarray analyzes demonstrated changes in community structures due for instance to rhizosphere effect or temporal evolution in response to take-all. Probe set validation, detection limit, and the discrimination between related environmental samples confirms the potential of the 16S rRNA microarray for a systematic exploration of bacterial diversity. The 16S rRNA-based taxonomic microarray is thus a promising tool to pursue bacterial diversity studies aiming at detecting, identifying and comparing the members of bacterial communities at various taxonomic levels in complex environments.

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Analysis of data from expression microarrays for studying plant-pathogen interactions

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Abstract

In recent years microarrays have been employed to monitor gene expression in plant – pathogen interaction studies on a scale much larger than previously possible. Nevertheless, data analysis still remains a challenge. Several software packages are available for data analysis. Using gene ontologies, differentially expressed genes can be functionally classified. Recently, tools have been developed that integrate microarray data with biological processes associated with the genes, which enables visualization of plant-specific metabolic pathways. One of the biggest tasks in data analysis is the final interpretation of differentially expressed gene functions in plant metabolism. However, newly developed software and visualization tools, developed to connect genes with their roles and involvement in certain metabolic pathways, offer an insight into alterations in metabolic processes and have contributed to more ready explanations of biological processes.

Introduction

The surfaces of plant organs, both above and below ground, are continuously and permanently exposed to a diverse range of organisms, including microbial pathogens, nematodes and insects. Microorganisms are capable of causing disease in various plant hosts and are responsible for important economic damage to crops. Plants, in fact, are equipped with a variety of defence mechanisms, which include preformed defences such as waxes, cell wall components, and secondary metabolites. When detecting a pathogen, plants activate a number of defences, such as the hypersensitive response, increased expression of defence-related genes, production of antimicrobial compounds, lignin formation and oxidative burst. Thus, resistance is due to a combination of physical and chemical barriers, which are either preformed or induced after the infection. Disease results from either failure of the recognition event or the ability of the pathogen to avoid or overcome the resistance response (Gómez-Gómez, 2004). To understand the development of the disease, changes in plant metabolic pathways that include alterations in the levels and/or partitioning of different metabolites need to be investigated. Many different approaches have been used in studying metabolic pathway alterations, including studies of transcriptomes, proteomes, metabolomes and fluxomes, which provide systematic data.

Several analytical approaches are available for studying changes in gene expression, among them hybridization based methods (Northern blot hybridization, Dot blot hybridization, *in situ* hybridization), polymerase reaction based methods (primer extension, reverse transcription PCR (RT-PCR), competitive RT-PCR, RT-qPCR, serial analysis of gene expression (SAGE), AFLP (amplified fragment length polymorphism), subtractive/suppressive cDNA libraries (SSH libraries)), i*n silico* methods (expressed sequence tags (EST) analysis (digital northern)), and microarray-based approaches (cDNAs or oligonucleotides). Microarray technology is one of the most powerful methods of monitoring gene expression, as it enables simultaneous analysis of the expression of a whole population of genes, in some cases even of the whole transcriptome (Barrett and Kawasaki, 2003).

Besides its successful application to detecting and identifying plant viruses on several agricultural important crops (Boonham et al., 2003; Bystricka et al., 2005), microarray technology has received the most widespread interest of all transcript profiling approaches (reviewed in Fiehn et al., 2001).

The first microarray study of gene expression in plant-pathogen interaction was on maize infected with pathogenic fungi *Cochliobolus carbonum* (Baldwin et al., 1999). Later studies reported changes in gene expression in *Arabidopsis* following infection with different fungi and bacteria or treatment with elicitors (reviewed in Wan et al., 2002). Schenk and coworkers used cDNA microarrays for following the expression of 2375 genes in *Arabidopsis thaliana* infected with *Alternaria brassicicola* and after treatment with salicylic acid, methyl jasmonate or ethylene (Schenk et al., 2000). Genes involved in systemic acquired resistance in *A. thaliana* have also been investigated (Maleck et al., 2000). The first microarray study on plant – virus interactions was published in 2003, reporting alteration in the gene expression profile of *Arabidopsis thaliana* following infection with *Tobacco mosaic virus* (Golem et al., 2003). In more recent research, responses to other viruses in *Arabidopsis* (Huang et al., 2005; Marathe et al., 2004; Whitham et al., 2003), as well as in poplar (Smith et al., 2004), maize (Shi et al., 2005), *Nicotiana benthamiana* (Senthil et al., 2005) and potato (Pompe-Novak et al., 2006), were monitored.

Materials and Methods

In contrast to diagnostic microarrays, expression microarrays provide huge amount of data which need correct analysis to provide biologically meaningful interpretation (Fiehn et al., 2001). Microarray data analysis consists of a few steps from the analysis of the scanned microarray image and normalization, to searching for significantly differentially expressed genes and data visualization. With image analysis software, spots on the array are annotated and their signal and background intensities calculated. Intensity of the background can be determined either as the average of the spot intensities where no hybridisation is expected, or as the average of the spot's surroundings. A spot where no hybridisation is expected could arise from genes from non-related species; alternatively, 'empty spots' correspond to spots without any probes. If the spot's surroundings are used for background estimation, global (over the whole array) or local (around each spot) background can be used in calculations. Image analysis software is also used for quality control of the spots, where spots are included or excluded from further analysis on the basis of spot shape, intensity of the signal, signal to noise ratio or regression correlation (Baebler et al., 2005). Because of unequal incorporation ability and unequal brightness of dyes, and the possibility of differences in the amounts of control and treated cDNA samples, normalization of spot signals is needed (Huang et al., 2005). The signals can be normalized to a group of 'normalization genes' (spike-in controls, e.g. luciferase), internal controls (housekeeping genes or 18S rRNA) or to the overall hybridization signal. Most often, the microarray data are normalized with the loess (locally weighted linear regression), the signal-dependent cubic method or vsn (variance stabilization normalization) (Huang et al., 2005).

Results and Discussion

In the process of searching for significantly differentially expressed genes, statistical analysis is used, following the statistical model that accounts for most of the variability of the experiment. Statistical analysis is followed by exploratory analysis and data visualization. Data mining, extraction of useful and understandable patterns from potentially large volumes of heterogeneous data, can also be very informative and is often used as a complement to statistical analyses (Kralj et al, 2006). Very informative presentation of the data can be obtained by exploratory methods such as principal component analysis (PCA) or self-organizing maps (SOM; Aharoni et al., 2002). Several software packages are available for data analysis, like packages designed for microarray analyses in R (limma, affy, marray, arraymagic etc., www.bioconductor.org) and the TIGR microarray analysis suite TM4 (www.tm4.org). Differentially expressed genes can be functionally classified using several gene ontologies. The most popular Gene Ontology (www.geneontology.org) categorizes each gene with respect to the molecular function, biological process, and cellular component of the gene product (MIPS - Munich Information Center for Protein Sequences; Arabidopsis Functional genomics consortium classifications). Until recently, many microarray results of plant-pathogen interactions have been presented as tables of differentially expressed genes classified into functional categories (review in Wan et al, 2002; Dardick 2007) that lack the connection to actual metabolic pathways.

Recently, tools have been developed that integrate microarray data with biological processes associated with the genes, which enables visualization of plant-specific metabolic pathways (AraCyc, MapMan and KaPPA-view). The use of MapMan offers the possibility to paint out microarray profiling experiments onto diagrams of metabolic pathways or processes, and to visualize the responses of gene expression in a

biological context. The principle of the MapMan ontology is a hierarchical BIN-based structure. Each BIN comprises items of similar biological function, and can be further split into sub-BINs corresponding to sub-modes of the biological function (Thimm et al. 2004; Usadel et al. 2005).
In Figure 1, a metabolism overview scheme exported from MapMan is presented. Each field on the scheme presents a metabolic pathway and blocks correspond to genes involved in that metabolic pathway. The intensity of the colour is relative to the ratio of gene expression in treated and control sample. However, in the metabolic pathways both gene inhibitors and enhancers are included and in the same field the expression of genes in the different directions are expected.
The high quality statistic analysis is crucial step before data importation to the MapMan or in any other visualisation software as visualisation of raw or statistically not significant data can be misleading.
The advantages of MapMan include specificity for plant metabolic pathways and processes, and flexibility, since new metabolic schemes can be added. In addition, the system can be adapted for different microarray platforms, such as potato, tomato and others (Rotter et al., 2007).

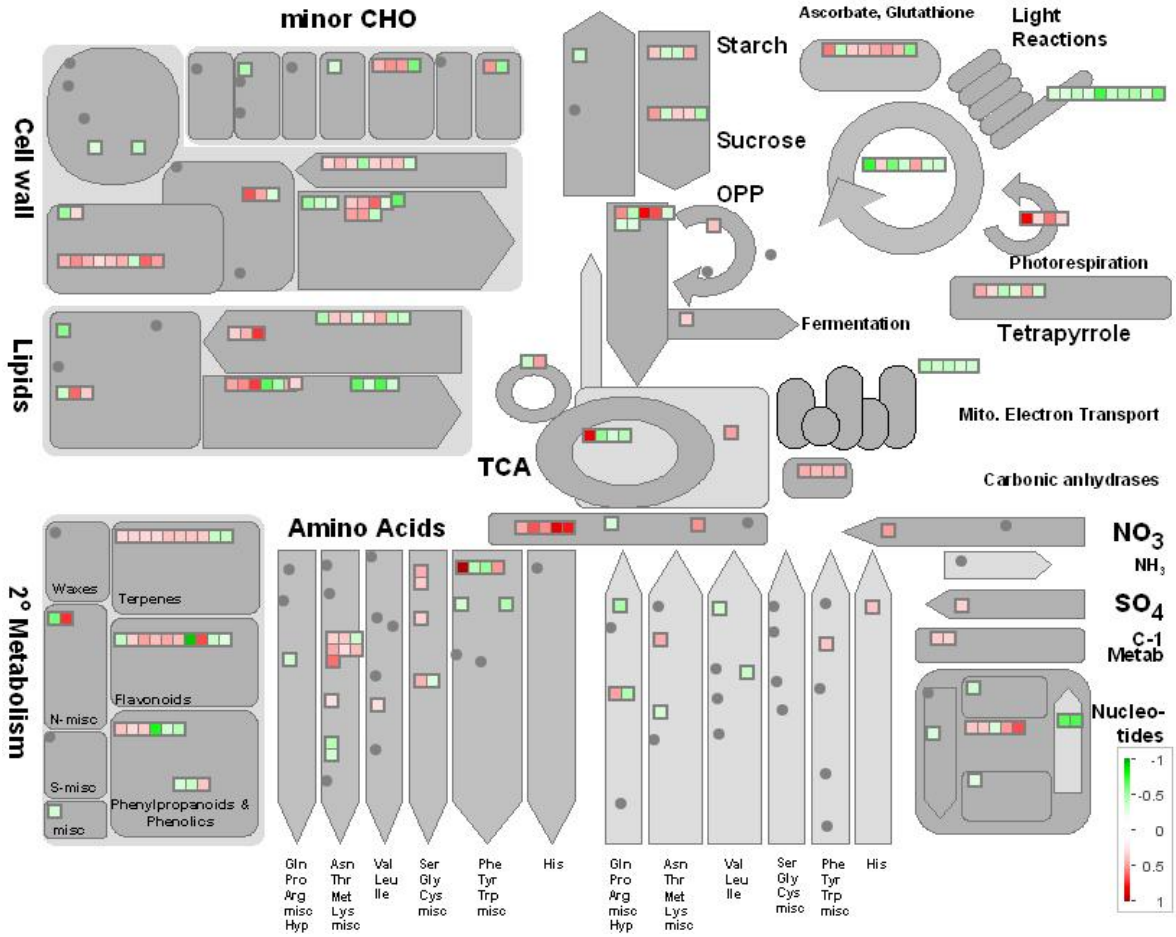

Figure 1: Metabolism overview scheme exported from MapMan. Each block presents a gene/clone within a given pathway and the intensity of the colour corresponds to the ratio between the gene expressions in treated and control sample.

Conclusions

Microarray experiments have been used in different plant-pathogen studies. Up to now it has been shown that all steps in the experiment, from the homogeneity of plant material, environmental conditions and technical performance, to the data analysis, have to be planned and done precisely in order to obtain meaningful results. Microarrays enable analysis of a huge number of genes simultaneously but the analysis and interpretation of the large data sets obtained still presents a challenge. In the data analysis step there are many possible ways to normalize the data and to determine differentially expressed genes. The final interpretation of differentially expressed gene functions in plant metabolism is one of the biggest tasks in

data analysis. However, newly developed software and visualization tools, developed to connect genes with their roles and involvement in certain metabolic pathways, offer an insight into alterations in metabolic processes and have contributed to more ready explanations of biological processes.

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