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Development and application of new nucleic acid-based technologies for microbial community analyses in foods

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Abstract

Several challenges still persist in the analysis of microorganisms in foods, particularly in studies of complex communities. Nucleic acid-based methods are promising tools in addressing new questions concerning microbial communities. We have developed several new methods in the field of nucleic acid-based microbial community analyses. These methods cover both sample preparation and detection approaches. The sample preparation method involves simplified DNA purification using paramagnetic beads. As an extension of this method, the same paramagnetic beads are used for both cell separation and DNA purification. This enables full automation. The separate detection of viable and dead bacteria is a major issue in nucleic acid-based diagnostics. We have applied a living/dead dye that binds covalently to DNA and inhibits the PCR from dead cells. In addition, a DNA array-based detection assay has been developed. The assay combines the specificity obtained by enzymatic labeling of DNA probes with the possibility of detecting several targets simultaneously by DNA array hybridization. In combination with 16S rDNA amplification, this is a promising tool for community analyses. Also, we have developed a novel approach for multiplex quantitative PCR. The multiplex PCR has been combined with our DNA array-based detection method. Finally, we are now in the process of adapting a system for monitoring microbial growth and death in real-time through the tagging of bacteria with green fluorescent protein (GFP) combined with fluorescence detection using a high-resolution confocal laser scanner.

Keywords: DNA purification; Detection; Quantification; Viable/dead; Multiplex

1. Introduction

Several challenges still persist in the analyses of microbial communities in foods. However, the recent sequencing of a number of bacterial genomes and the development of new analytical tools will have a profound impact on our ability to understand, manipulate, detect, exploit, or combat bacteria (Pennisi, 1999). Techniques for quantitative PCR (Bassler et al., 1995) and exponentially increasing DNA-sequence databases, yielding better probe design, lead to improved methods for detection and quantification of bacteria (Olsen et al., 1995). Most of the techniques

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available, however, are designed for the detection of one or a few target organisms. Thus, in microbial community analyses, there is an urgent need for approaches where several different microorganisms can be detected simultaneously (Rudi, 2002a). Important fields for development are methods for simultaneous detection of a variety of bacterial species in one sample, methods to discriminate between dead and living cells, and new tagging techniques that permit analysis of the spatial distribution and colonization of microorganisms in real-time. Used in combination, techniques in these fields will permit direct analyses of increasingly complex microbial communities, such as those present in various food products.

Even though analytical limitations in many cases are due to the sample preparation step, e.g. separation of the cells or organisms from the food matrix, and subsequent DNA purification, the development of new strategies in these areas has been relatively limited (Rudi, 2002a). Generally, the approaches used are based on centrifugation, filtration, or immunocapture (Swaminathan and Feng, 1994). Approaches for adsorbing bacteria to a solid phase have also been applied (Duffy et al., 1999; Lucore et al., 2000; Payne et al., 1992). In special cases, for liquids with low conductivity, oscillating electric fields (dielectrophoresis) may also be applied for bacterial isolation (Cheng et al., 1998). Microorganisms in foods may be present in low-copy numbers and in an environment that may disrupt the integrity of the nucleic acids and/or the enzymes used for the downstream nucleic acid analyses (Al-Soud, 2000). Most sample preparation methods for DNA analyses are designed for defined materials, such as tissues and cultures (Rudi et al., 1997). The challenges presented by food samples are not only that the target nucleic acids are present in low concentrations, but also that these samples may be heterogeneous, and in many cases impossible to define. Finally, when analyzing nucleic acids from foods, it is important to determine the origin of the nucleic acids and whether the analyzed nucleic acids are obtained from living or dead organisms (Nogva et al., 2000a).

This review focuses on the method developments that we have made in the field of microbial detection and community analyses, and on the application of these methods to food-related problems. We will address the use of paramagnetic beads in DNA purification, and present a method that integrates cell concentration and DNA purification using the same beads for both steps. An application of single-cell PCR is included. A DNA-based approach for separate detection of viable and dead bacteria will be discussed. In combination with the bead-based sample preparation approach, a real-time quantitative detection method which has been adapted for the absolute quantification of *Listeria monocytogenes* in milk will be presented. A similar approach has been applied for the detection of *Campylobacter jejuni* directly from chicken fecal samples. We will also describe a novel approach for multiplex quantitative PCR. A DNA array-based detection approach combining the specificity obtained by enzymatic labeling, with the possibility of detecting several different targets simultaneously by array hybridization, is described. This method has been used to analyze the complex communities in ready-to-eat vegetable salads, and as a new bacterial fingerprinting tool. Finally, an application where green fluorescent protein (GFP) has been used to measure the real-time colonization by bacteria will be presented.

2. Sample preparation and DNA purification approaches

Representative samples are of primary importance in the analysis of foods. The fact that microorganisms often are randomly distributed in foods is illustrated by the bacteria colonizing the cut surface of crisphead lettuce (Fig. 1). There is currently no single approach for separating microorganisms from the matrices of the diverse range of existing food products. Major challenges remain both in relation to the separation of microorganisms from the food matrix, and processing sample volumes large enough to detect a small number of organisms (Olsen et al., 1995).

2.1. Single-cell analyses

A special case for limited samples is the amplification of DNA directly from single cells. This approach was tested for the amplification of toxic dinoflagellates in the presence of heterotrophic bacteria. The PCR was performed without purification to avoid target loss (Tengs et al., 2000). The advantage is that the mor-

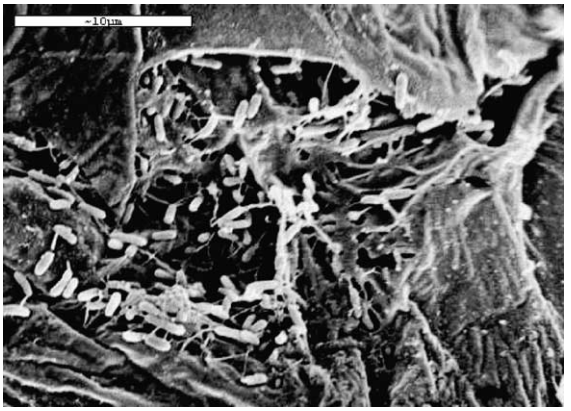

Fig. 1. Scanning electron micrographs (SEM) of bacterial colonization of plant matrices. The picture shows the colonization of a lettuce cut surface after storage in a modified atmosphere at 10 °C for 10 days. The bacteria colonize the cut surface and growth occurs in the interior of the salad matrix.

phological and cytological data can be directly linked to the genetic data. The obvious disadvantage is the complicated execution of the DNA isolation step, if this is done manually. However, combined with limited dilution, one has the possibility of detecting single cells without enrichment. Such an approach could be useful for microbial community analyses.

2.2. DNA purification with paramagnetic beads

When working with complex samples, it is important to have a rigid lysis procedure that does not introduce errors due to differential lysis of different microorganisms in the sample (Both et al., 1991; Field et al., 1997; Rudi et al., 2000). Here, mechanical, chemical, and enzymatic approaches can be applied (Sambrook and Russel, 2000). It is also of importance for large-scale screenings that the methods are simple, and can be automated. The reason for purifying DNA is to remove substances that may interfere (even marginally) with the enzymatic reactions in order to generate a DNA preparation yielding reproducible analytical results.

We have developed a simplified DNA purification protocol that involves the application of paramagnetic beads with a polymer surface. Combinations of chaotropic salts and/or detergents have been used. The system, utilizing monodispersed beads, was developed in order to obtain DNA, pure enough for PCR amplification (Rudi et al., 1997). The procedure could be automated (Deggerdal and Larsen, 1997). The system was tested on diverse organisms: bacteria, fungi, algae, vascular plants, and vertebrates. Optimization of sample amounts and lysis conditions was done using several types of tissue (fish epithelium, plant leaves, mammalian liver and muscle tissues, and fungal fruit bodies and mycelium). The standard lysis conditions used for blood could be applied successfully for most bacteria, algae, and vertebrates, while plant leaves and fungal fruit bodies had to be mechanically broken to obtain proper lysis. For vascular plants and some Cyanobacteria, lysis by heating to 65 °C gave better DNA yields than standard lysis at room temperature. Changing the buffer system to buffers containing a combination of a detergent and a chaotropic salt led to improved lysis of bacteria. The use of 70% ethanol during the washing step reduced the DNA loss when analyzing a limited amount of samples (Rudi et al., 1998).

2.3. Automated sample preparation by the application of the same paramagnetic beads for both cell concentration and DNA purification

Automation of the sample preparation process is a requirement for all large-scale screenings. Automated DNA purification approaches have already been developed for relatively defined substances, e.g. blood (Holmberg et al., 1995) and for cell cultures (Meldrum, 2000a,b). Additional problems, however, may be encountered with complex and undefined samples (Rudi, 2002a; Rudi and Jakobsen, 2001). In many applications, large sample volumes have to be processed. Another major problem might be the integration of the separation of the microorganisms from the environmental matrix with the DNA purification. Several separation methods such as filtration and centrifugation may not easily be automated (Rudi et al., 1998).

An approach that enables a fully automated sample preparation system has been developed (Fig. 2). This method allows the integration of cell capture and DNA purification by making use of the same paramagnetic beads for both steps (Rudi et al., 1998). This is a promising strategy for a fully automated sample preparation system directly from liquid samples (Rudi et al., 2000). Semisolid materials such as feces have also been tested. A fully automated quantitative

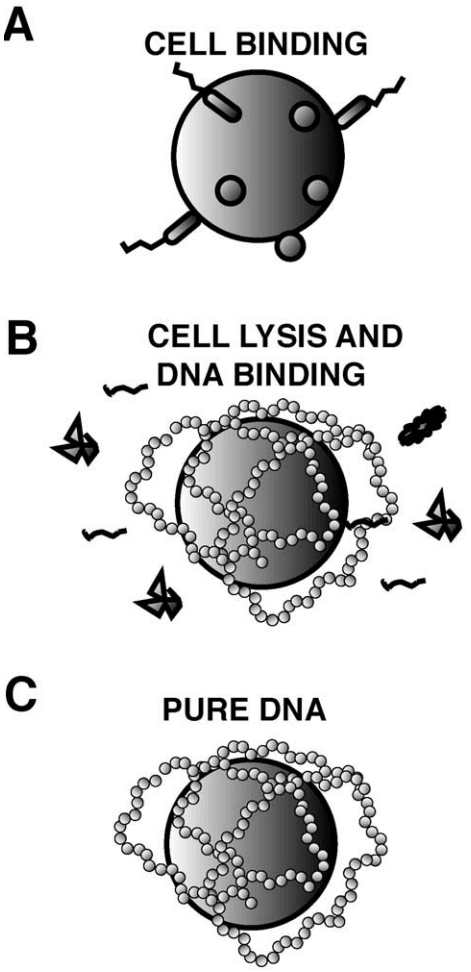

Fig. 2. Schematic representation of the integrated cell concentration and DNA purification through the application of the same paramagnetic beads for both steps. Panel (A) shows the bacteria bound to the beads. In panel (B), the cells are disrupted, and DNA binds to the beads. Panel (C) shows the situation after washing. The cell debris is removed, and pure DNA is associated with the beads. The DNA–bead complex can subsequently be used for downstream applications such as PCR amplification.

method has been developed for the detection of *C. jejuni* in chicken feces (Rudi, 2001).

2.4. Application of ethidium monoazide (EMA) for separate DNA-based detection of viable and dead bacteria

PCR techniques have significantly improved the detection and identification of bacterial pathogens (Saiki et al., 1985). Still, the lack of differentiation between DNA from living and dead cells is one of the major challenges for DNA-based methods in diagnostics.

Several nucleic acid-binding dyes are reported to selectively enter dead bacteria with compromised membranes. In addition, the dye ethidium monoazide (EMA) may be covalently bound to DNA (Bolton and Kearns, 1978; Marx et al., 1997; Caron et al., 1998) (Fig. 3). Our goal was to utilize the irreversible binding of photoactivated EMA to DNA to inhibit the PCR of DNA from dead bacteria. EMA was thus evaluated as a means of reducing the background signal from dead cells in DNA-based diagnostics, using quantitative 5′ nuclease PCR for detection (Bassler et al., 1995). The conclusions from these experiments were that EMA covalently bound to DNA was able to inhibit the 5′ nuclease PCR, whereas EMA alone was only inhibitory to 5′ nuclease PCR at high concentrations. Maximum inhibition of free DNA was 4–5 log. One hundred micrograms per milliliter EMA reduced the PCR signal by 2.5 log in boiled or ethanol-treated *E. coli* O157:H7 cells. The reduction was 1.5 and 2.5 log in boiled or benzalkonium chloride-treated *Salmonella* and *L. monocytogenes* cells, respectively. However, *L. monocytogenes* showed more variable results. Generally, Gram-positive bacteria are more permeable to ethidium dyes than Gram-negative bacteria (Caron et al., 1998). The use of lower amounts of EMA, however, gave better differentiation between living and dead *L. monocytogenes*. The potential use of EMA in future DNA diagnostics to differentiate between living and dead cells is promising (Nogva et al., 2002).

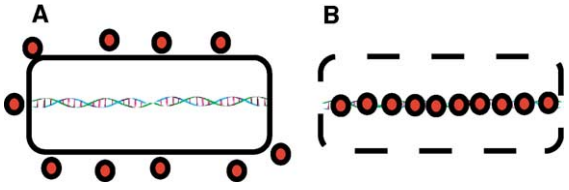

Fig. 3. Schematic representation of the PCR-based viable/dead method. The dye is prevented from entering viable cells (A) by intact membrane systems and pumps, while these barriers are compromised in dead bacteria (B), resulting in dye entry.

3. Real-time quantitative PCR detection analyses

Generally, it is not necessary with an organism-specific DNA purification, since the discrimination between different bacterial species can be carried out at the DNA detection stage (Louie et al., 2000). Different analytic applications may impose different requirements for the sample preparation step. Quantitative analyses, with low concentration of target organisms, require both quantitative sample preparation and high DNA recoveries (Nogva et al., 2000a).

3.1. Quantification of L. monocytogenes in milk

L. monocytogenes contamination can be a problem in dairy products. For instance, there have been several *L. monocytogenes* outbreaks associated with the consumption of soft cheeses made from unpasteurized milk (Pearson and Marth, 1990).

We have developed an assay for the quantitative detection of *L. monocytogenes* based on the 5′ nuclease PCR, using a113-bp amplicon from the listeriolysin O gene (*hlyA*) as a target (Nogva et al., 2000b). The assay was positive for all *L. monocytogenes* strains tested (65 isolates including the type strain), and negative for all other *Listeria* species (16 isolates from five species) and several other bacteria (18 species). The application of 5′ nuclease PCR in milk diagnostics requires a quantitative sample preparation step. Several magnetic bead-based strategies were evaluated, since these systems are simple and relatively easy to automate. The combination of non-specific binding of bacteria to paramagnetic beads, with subsequent DNA purification using the same beads, gave satisfactory results. The detection limit was approximately 6–60 CFU per PCR reaction; quantification was linear over at least 7 log units and the method could be completed within 3 h (Nogva et al., 2000b).

Listeriosis is often associated with the consumption of foods heavily contaminated with *L. monocytogenes* (Jay, 1996). The infective dose, however, depends on many different factors, among others, susceptibility of the host. In addition, it takes approximately 10–12 days for symptoms to develop by which the bacterial numbers have increased in the contaminated food. The detection limits, however, for the direct methods are within the range of what is required for the detection of *L. monocytogenes*. Furthermore, due to the uncertainties of the infectious dose, and since *L. monocytogenes* is abundant in the environment, an important aspect is the quantification of this organism (Schlech, 2000).

3.2. Quantification of C. jejuni in water, chicken fecal and caecal content samples

C. jejuni is one of the main causes of diarrhea and food-borne gastroenteritis (Solomon and Hoover, 1999). *C. jejuni* is a zoonotic microorganism and can be isolated from poultry, cattle, pigs, pets, and wild animals/birds. Diagnosis of this important pathogen is difficult due to special growth requirements and low infectious doses. Water and poultry are major sources of infections. The main reservoir of *C. jejuni* in poultry is the caecum, with an estimated content of 10^6–10^8 cells/g. If a flock is infected with *C. jejuni*, the majority of the birds in that flock will harbor the bacteria (Stern, 1992). Diagnosis at the flock level could thus be an important control point.

Our aim was to develop a complete quantitative PCR-based detection assay of *C. jejuni* directly from water, and from caecal and fecal samples. We applied the approach described above, where the same paramagnetic beads were used for both cell isolation and DNA purification (Rudi et al., 1998; Nogva et al., 2000a). This integrated approach enabled both a fully automated and quantitative sample preparation, and DNA extraction. By combining the sample preparation method with real-time quantitative PCR, we developed a complete quantitative diagnostic assay. The assay was evaluated both by spiking the samples with *C. jejuni*, and by detecting *C. jejuni* in naturally infected chickens. The conclusion from these experiments is that the detection limit and quantitative range of the assay are within what is practical for the direct detection of *C. jejuni* (Rudi, 2001).

4. DNA array-based analyses

Since the pioneering work by P. Brown in the application of high-density microarrays in biological

analyses (Schena et al., 1995), there has been a tremendous development of DNA array-based applications (Meldrum, 2000a). Generally, microarrays are either used for global gene expression analyses, or for screening of genes present or absent in a given genome. These arrays may include several thousand different probes in a single analysis. We have focused on using DNA arrays for microbial community analyses. These types of analyses require the processing of many parallel samples with relatively few probes (Rudi, 2002a). A major issue in these assays is the signal-to-noise ratios, since single nucleotide polymorphisms (SNPs) are commonly targeted (Ross et al., 1998). We have developed a novel approach that combines the specificity obtained by sequence-specific labeling of DNA probes with DNA array hybridization (Fig. 4). This approach gives a very high signal-to-noise ratio (Rudi et al., 1998, 2002).

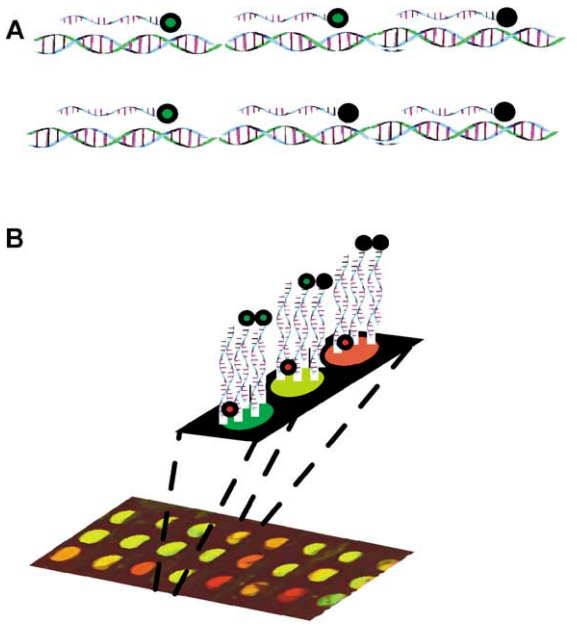

Fig. 4. Schematic representation of the DNA array-based detection approach. (A) The DNA after amplification, for instance ribosomal DNA from two different species. The probes are 3′-labeled with fluorescein ddCTP (green dots) if the target is present. If the target is not present then the probes will be unlabeled (black dots). (B) 5′-CY5 labeled internal control probes are added and the relative signal intensities are then determined by hybridization to the respective complementary sequences spotted on a solid phase.

4.1. Analysis of microbial communities on vegetable salads packed in a modified atmosphere

There is a clear need for new approaches in the field of microbial community analyses. We have developed a DNA array-based method, targeting 16S rDNA, that enables the direct detection and quantification of microorganisms from complex communities without cultivation (Rudi et al., 2002). The approach is based on construction of specific probes from the 16S rDNA sequence data retrieved directly from the communities. The specificity of the assay was obtained through a combination of DNA array hybridization and enzymatic labeling of the constructed probes (see Fig. 4).

Cultivation-dependent (enrichment and plating) and cultivation-independent (direct fluorescence microscopy, scanning electron microscopy) assays were used as reference methods in the development and evaluation of the method. The description of microbial communities in ready-to-eat vegetable salads in modified atmosphere was used as the experimental model. Comparisons were made with respect to the effect of storage conditions at different temperatures for up to 12 days, and with respect to the geographic origin of the crisp-head lettuce (Spanish or Norwegian), being the main salad component.

The conclusion drawn from the evaluation of the methods was that the DNA array method gave an accurate description of the microbial communities. *Pseudomonas* spp. dominated in salad batches of both Norwegian and Spanish lettuce before storage and after storage at 4 °C. The *Pseudomonas* population also dominated the batch containing Norwegian lettuce after storage at 10 °C. In contrast, *Enterobacteriaceae* together with lactic acid bacteria dominated the microbial community of the batch containing Spanish lettuce after storage at 10 °C, while *Enterobacteriaceae* were also abundant after storage at 4 °C. The practical implication of these results is that the microbial communities in ready-to-eat vegetable salads can be diverse, and that the microbial composition is both dependent on the origin of the raw material and on the storage conditions (Rudi et al., 2002).

4.2. Multiplex DNA array-based quantitative PCR

We have developed a method for multiplex quantitative DNA array-based PCR (Rudi, 2002b) that may

be used for the detection of any sets of genes. The method was initially used for the detection of genetically modified food. We now extend the analysis to include prokaryotes. The method is based on the inclusion of a universal region in each of the amplified fragments (HEAD) in the first few rounds of the PCR amplification. The leftovers of the primers are removed with a single-strand DNA-specific exonuclease. The next round of PCR is then competitive, conserving the ratio between the amplified fragments, by using primers complementary to universal HEAD region. Finally, in order to improve the specificity and the sensitivity, a linear amplification by sequence-specific labeling of DNA probes is included. The detection is carried out in a DNA array format by hybridizing the labeled probes to their complementary sequences spotted on a solid phase, e.g. glass or membrane (Fig. 4). The method was thoroughly evaluated by an eightplex quantitative detection assay for the maize GM constructs BT 11, BT 176, and MON 810 using construct-specific or event-specific regions as templates for the PCR reactions (Fig. 5). The system has a synthetic DNA as an internal reference standard for the absolute quantification of input DNA. The method is modular and may easily be expanded. The final version of the test is a 12-plex system for simultaneous quantitative detection of seven different GMO maize constructs (events: Bt176, Bt11, Mon810, T25, Ga21, CBH351, and DBT418) (Rud, 2002). We are currently adapting the system for gene expression studies where the transcription apparatus in *E. coli* is targeted (Moen et al., in preparation).

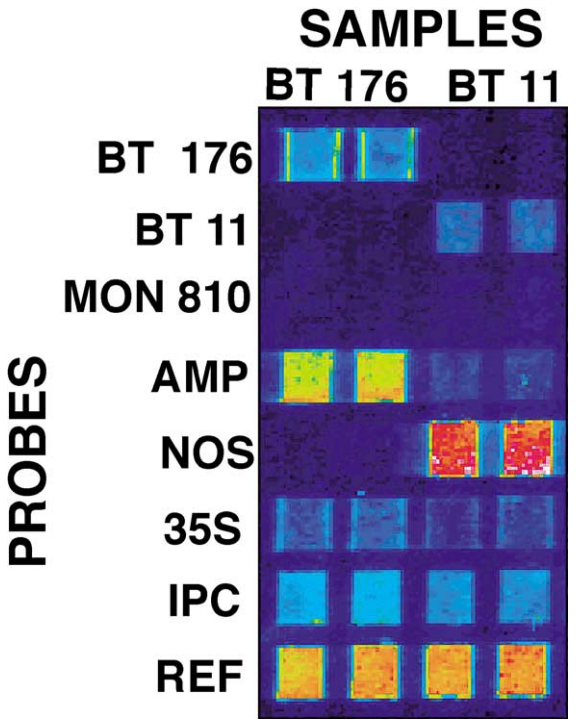

Fig. 5. Multiplex DNA array-based quantitative detection. An eightplex detection of the maize GM constructs BT 176 and BT 11 is shown. In each of the experiments, 1% GM maize was mixed wild-type maize. The signals represent the different elements in the respective GM constructs. An internal positive control (IPC) was included to determine the absolute amount of maize material in the sample. The quantifications of the GM constructs are done relative to the reference gene (REF GENE).

5. Fingerprinting and accumulation of data in databases

Multi Locus Sequence Typing (MLST) is emerging as an alternative typing technique. MLST is based on sequence determination of several different genetic loci, and the application of the variable sites in the DNA sequences to determine the relatedness between the different strains analyzed (Struelens, 1998).

We have developed an alternative MLST approach that targets the variable genetic changes directly in a DNA array format. Our approach is based on DNA array hybridization in combination with sequence-specific labeling of oligonucleotide probes (Rudi and Naterstad, 2002). *L. monocytogenes* was chosen for the development of the assay. The three-virulence genes *hlyA*, *iap* and *flaA* were targeted (Rasmussen et al., 1995). Five probes were constructed for the *hlyA* gene, eight for the *iap* gene, and four for the *flaA* gene. Reproducible signal profiles were obtained for 14 selected *L. monocytogenes* strains. The profiles were used in a maximum parsimony phylogenetic reconstruction. This analysis revealed that the strains could be divided in seven different profiles, each consisting of two statistically supported main clusters. One of these clusters could be divided into two subgroups. Furthermore, comparisons with strain serotypes and Amplified Fragment Length Polymorphism (AFLP) data gave good correlations. In conclusion, our DNA

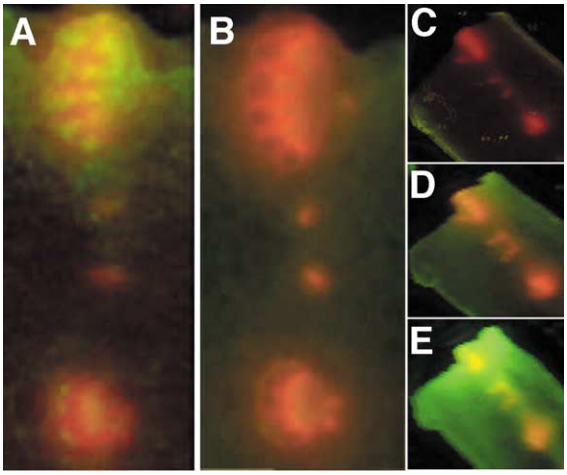

Fig. 6. Real-time measurements of the colonization of plant matrix by GFP-labeled *E. coli*. Vertical cuts of Chinese cabbage were analyzed. The red color represents the fluorescence from the plant matrix, while the green color represents the GFP fluorescence. Panel (A) shows the salad after dipping in a solution containing approximately 5×10^8 cells/ml of *E. coli* DH5α and containing the plasmid pEYFP (Clontech, Palo Alto, CA, USA) with a GFP marker and an antibiotic selection gene in the presence of 100 μg/ml ampicillin. The fluorescent bacteria are mainly trapped within the vascular tissue. Panel (B) shows that after 24 h at 37 °C, no GFP fluorescence was left. Panels (C–E) show the bacterial colonization in the absence of antibiotics. Panel (C) at the beginning of the incubation, panel (D) after 24 h, and panel (E) after 48 h. Here, GFP-labeled *E. coli* was able to colonize the plant matrix.

array-based MLST method is a promising tool for fingerprinting bacteria.

We are currently building a bacterial strain collection where the fingerprint data are linked with each strain (MATFORSK Microbial Strain Collection). In the future implementation of our strain collection, we will have searchable fingerprint patterns directly linked to other accumulated information for each strain.

6. Future on-line monitoring of microbial colonization

A major limitation in the current analyses of microbial communities is that the analyses are done for fixed-time-points. Analyzing fixed time-points will only give limited information about the population dynamics. Tools for the analysis of intact living communities will be important in the future.

6.1. Application of green fluorescent protein (GFP) for monitoring real-time colonization of a salad matrix

Labeling of *E. coli* with green fluorescent protein (GFP) (Chalfie et al., 1994) enabled real-time monitoring of the colonization of a plant matrix by this bacterium (Fig. 6). This application gives information as to how the bacteria colonize the matrix, and information on the distribution of the bacteria. This is important for subsequent diagnostics in order to obtain representative samples.

In future applications, the information obtained from the GFP-labeled bacteria will be linked to the information obtained with both real-time quantitative PCR and the DNA array method. The analyses will then include quantification, biodiversity, distribution, and real-time colonization. This set of methods gives unique possibilities for studying microbial colonization of food systems.

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