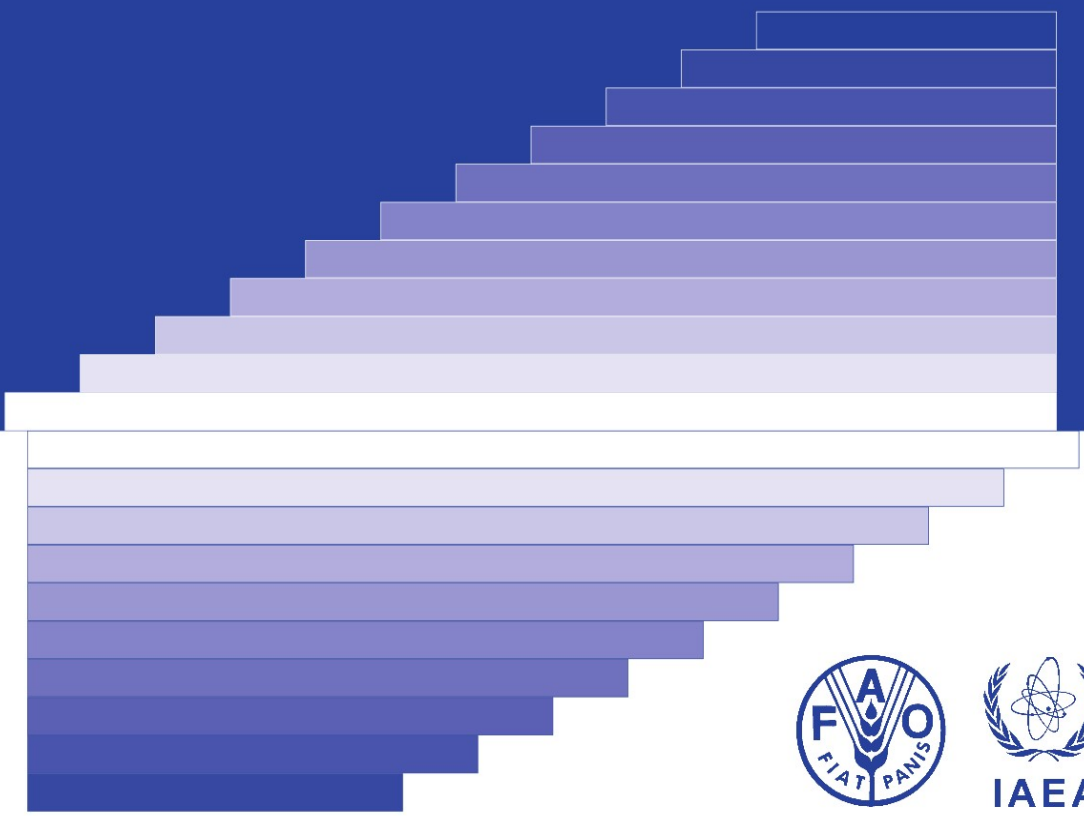
Methods in Gut Microbial Ecology for Ruminants

Edited by
Harinder P.S. Makkar and
Christopher S. McSweeney



Springer

METHODS IN GUT MICROBIAL ECOLOGY FOR RUMINANTS

Methods in Gut Microbial Ecology for Ruminants

Edited by

HARINDER P.S. MAKKAR

Animal Production and Health Section
Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture
International Atomic Energy Agency
Vienna, Austria

CHRISTOPHER S. McSWEENEY

CSIRO Livestock Industries
Queensland Bioscience Precinct
St Lucia, Queensland, Australia

A C.I.P. Catalogue record for this book is available from the Library of Congress.

ISBN-10 1-4020-3790-2 (HB)
ISBN-13 978-1-4020-3790-0 (HB)
ISBN-10 1-4020-3791-0 (e-book)
ISBN-13 978-1-4020-3791-7 (e-book)

Published by Springer,
P.O. Box 17, 3300 AA Dordrecht, The Netherlands

www.springeronline.com

Printed on acid-free paper

© 2005 International Atomic Energy Agency
No part of this book may be reproduced, stored in a retrieval system, or transmitted
in any form or by any means, electronic, mechanical, photocopying, microfilming, recording,
or otherwise, without written permission from the Publisher, with the exception
of any material supplied specifically for the purpose of being entered
and executed on a computer system, for exclusive use by the purchaser of the work.

Printed in the Netherlands.

Contents

Foreword

As a result of various human activities, such as increase in human population, decrease in arable land due to soil degradation, urbanization, industrialization and associated increase in the demand for livestock products, dramatic changes are occurring in the global ruminant livestock sector. These changes include shift in the size of regional livestock populations and in the types of management and feeding systems under which ruminant livestock are held, and increased demand of a wider range of quality attributes from animal agriculture, not just of the products themselves but also of the methods used in their production. The livestock sector will need to respond to new challenges of increasing livestock productivity while protecting environment and human health and conserving biodiversity and natural resources.

The micro-organisms in the digestive tracts of ruminant livestock have a profound influence on the conversion of feed into end products, which can impact on the animal and the environment. As the livestock sector grows particularly in developing countries, there will be an increasing need to understand these processes for better management and use of both feed and other natural resources that underpin the development of sustainable feeding systems.

Until recently, knowledge of ruminant gut microbiology was primarily obtained using classical culture-based techniques, such as isolation, enumeration and nutritional characterization, which probably only account for 10–20% of the rumen microbial population. New gene-based technologies can now be employed to examine microbial diversity through the use of small sub-unit ribosomal DNA analysis (e.g. 16S rDNA) and to understand the function of complex microbial ecosystems in the rumen through metagenomic analysis. These technologies have the potential to revolutionize the understanding of rumen function and will overcome the limitations of classical-based techniques, including isolation and taxonomic identification of strains important to efficient rumen function and better understanding of the roles of micro-organisms in relation to achieving high productivity and decreasing environmental pollutants.

This book has been produced by the Joint FAO/IAEA Division of Nuclear Technique in Food and Agriculture, IAEA Vienna, Austria in collaboration with the CSIRO Livestock Industries, Brisbane, Australia. It gives a comprehensive up-to-date account of the methodologies and the protocols for conventional and modern molecular

H.P.S. Makkar and C.S. McSweeney (eds.), Methods in Gut Microbial Ecology for Ruminants, vii.
© 2005 *IAEA. Printed in the Netherlands.*

techniques that are currently in use for studying the gut microbial ecology of ruminants. Each chapter has been contributed by experts in the field. The techniques and procedures described are also relevant and adaptable to other gastrointestinal ecosystems and the microbiology of anaerobic environments in general. The future of ruminant gut microbiology research is dependent upon the adoption of these molecular-based research technologies, and the challenge at present is the use of these technologies to improve ruminant production and decrease environment pollutants through a better understanding of microbial function and ecology. It is hoped that this book will equip the readers better in order to meet this unprecedented challenge.

James D. Dargie
Director, Joint FAO/IAEA Division
of Nuclear Techniques in Food and Agriculture
Vienna, Austria

Shaun G. Coffey
Chief, CSIRO Livestock Industries
Brisbane, Australia

Introduction

Current approaches to the evaluation of digestibility and the nutritive value of feed resources using conventional *in vitro* feed evaluation and animal studies have resulted in a large body of information about nutrient composition, digestion kinetics and digestibility. However, these techniques are unable to describe the microbial mechanisms involved in digestion by ruminants and other herbivores, and are unlikely to result in the development of new feeding strategies. Conventional culture-based methods of enumerating and identifying rumen bacteria are being rapidly replaced by the development of nucleic acid-based techniques that can be used to characterise complex microbial communities. Ruminant nutritionists and microbiologists have recognized the importance of molecular microbial ecology, but many have found it difficult to employ the most appropriate techniques because they are not familiar with the methods. In addition, this field is developing very rapidly and even researchers with experience in molecular microbial ecology find it difficult to keep abreast with the increasing number of techniques and alternatives.

This manual is written by an expert group of scientists interested in ruminant digestion and gut microbiology. The most recent and up-to-date methods in molecular microbial ecology with special emphasis on ruminants are collated and interpreted in this book. The methods will provide the readers an easy access to molecular techniques that are most relevant and useful to their area of interest. The authors have attempted to write in a recipe-like format designed for direct practical use in the laboratory and also to provide insight into the most appropriate techniques, their applications and the type of information that could be expected. These aspects have been supported by inclusion of the relevant literature.

The contents of the manual are presented in a sequence that recognizes the key elements in studying gut microbial ecology. The first chapter provides a perspective on how to design animal trials in which microbial ecology is studied. Often the power of the new molecular techniques is diminished by an inappropriate design in terms of animal number, sampling frequency, location and replication. The second chapter describes the classical culture-based methods for studying rumen microbes, as these methods are often a pre-requisite to employing molecular techniques. Chapters 3–6 provide information on the basic underpinning techniques and the protocols in

H.P.S. Makkar and C.S. McSweeney (eds.), Methods in Gut Microbial Ecology for Ruminants, ix–x.
© 2005 *IAEA. Printed in the Netherlands.*

molecular ecology, such as DNA extraction from environmental samples, the polymerase chain reaction (PCR), oligonucleotide probe and primer design and DNA fingerprinting amongst others. The application of these techniques to microbial detection and identification are discussed. Specialized techniques such as denaturing gradient gel electrophoresis (DGGE) and 16S/18S ribosomal DNA libraries for studying complex communities that contain unculturable organisms are also described. Many of these techniques are used to identify and enumerate the population of organisms that are present in a sample. However, the field is rapidly moving to a functional analysis of the microbes in an ecosystem, and some of the methods being employed to measure genes expression are described in Chapter 3. In Chapter 6, knowledge about location and spatial relationships of micro-organisms in their natural environment that are often essential for understanding the function of these organisms are discussed. The final chapter deals with metagenomic technologies, which provide the potential to capture and study the entire microbiome (the predominant genomes) from a complex microbial community, such as the rumen. The rapid high-throughput technologies developed in mapping the human genome are now being deployed to study microbial ecosystems. An explosion of knowledge in the field of microbial ecology is now expected.

The editors wish to acknowledge the contributions made by all the authors who participated in the publication of this manual. They have spent considerable time gathering information from many sources into a focussed document that enables the reader to understand how techniques have evolved and the context in which the methods should be applied to address specific issues relating to gut microbial ecology. We believe that this manual will 'demystify' the methods in molecular microbial ecology for readers, who are novice in the field but are excited by the prospects of the technology. It would also be invaluable for the experienced workers striving for giving new dimension to their research – expanding the work in other fields and initiating cross-cutting activities. This manual is seen as the first step towards understanding and manipulating gut micro-organisms as it is expected that the techniques and the methodologies associated with the study of molecular microbial ecology will continue to grow and evolve. A key challenge for the future will be the simplification of these techniques, so that these become tools of routine use in nutritional, environmental and ecological laboratories.

Harinder P.S. Makkar
Animal Production and Health Section
Joint FAO/IAEA Division
International Atomic Energy Agency
Vienna, Austria

Christopher S. McSweeney
CSIRO Livestock Industries
Queensland Bioscience Precinct
St Lucia, Queensland, Australia

Editorial Note

The use of particular designations of countries or territories does not imply any judgement by the publisher, and the IAEA, as to the legal status of such countries or territories, of their authorities and institutions or of the delimitation of their boundaries.

The mention of names of specific companies or products (whether or not indicated as registered) does not imply any intention to infringe proprietary rights, nor should it be construed as an endorsement or recommendation on the part of the IAEA.

All patents, registered trademarks and ownership for products, reagents and processes mentioned in this publication should be respected.

H.P.S. Makkar and C.S. McSweeney (eds.), Methods in Gut Microbial Ecology for Ruminants, xi.
© 2005 *IAEA. Printed in the Netherlands.*

PART ONE

Designing **in vivo microbial ecology studies

1.1. Experimental designs for rumen microbiology

ADRIAN R. EGAN
Institute of Land and Food Resources, The University of Melbourne, Parkville, Victoria 3010, Australia

Introduction

Research and innovation in relation to microbiology of the rumen is based principally around functional attributes of the populations as they affect digestion and performance of the host animal. What is sought is a better understanding of the complex microbiological communities [4, 24] and identification of ways to manipulate these populations for specified objectives in ruminant production and environmental impact [27, 34]. Further objectives are to develop from that knowledge base, novel anaerobic systems for a range of purposes, such as generation of fuels, detoxification and degradation of waste materials [46].

This chapter is primarily aimed at design of experiments to describe the diversity of rumen microbial populations, identify the factors that influence the composition and nature of associations and quantify relative and absolute growth rates and functional performance of those populations. Many of the principles outlined are applicable to other types of anaerobic microbial systems.

The nature of rumen microbial populations

In research into complex microbial populations, it is well to remember that the population present at any given time is the outcome of prior successions [5]. The population is dynamic in relation to relative growth rates [15], determined by competitive advantage along with interdependencies in relation to the supply of preferred substrates and the prevailing environmental conditions [38, 40, 44, 47]. Microbes occupy microenvironments and in a system such as a compartment of the digestive tract there is always a degree of heterogeneity [1, 3, 7, 9]. Thus, microbes are distributed in broad terms between fluid phase, suspended particulate phase and the wall of compartment; in the latter two phases, they may be adherent or associated but unattached [13, 36]. The degree of heterogeneity in the environment determines which organisms are successful and what symbiotic or interdependent relationships are critical to that success. It also dictates the ease or otherwise of drawing a representative sample. These issues are raised at the outset not to deter investigation but to provide a

H.P.S. Makkar and C.S. McSweeney (eds.), Methods in Gut Microbial Ecology for Ruminants, 3–19.
© 2005 *IAEA. Printed in the Netherlands.*

conceptual framework in which hypotheses are set and experimental protocols are established.

The definition of objective or statement of the hypothesis

What is it that you wish to know? Is it how much microbial protein is generated on a daily basis? Is it whether a specified species or group of species are present, something about their relative numbers or biomass, and perhaps the relationship to processes of degradation of substrates? Or is it the broad profile of all major species or functionally identifiable groups present and the changes due to a set of dietary or other treatments imposed? Is it a qualitative or quantitative question? Are the questions about general trends that can be expected in response to a given set of variable conditions, so that the experiment is conducted to derive empirical equations for incorporation into a mechanistic model? Or are they about a specific result to explain performance of animals under specific sets of dietary conditions? Or combinations of the above?

Investigations of microbial populations therefore require very clearly defined objectives or specific hypotheses in order to specify the necessary and sufficient conditions, the experimental design and the protocol for all measurements made. Thus, for example, some questions can be answered under conditions where substrate supply is continuous and the system tightly controlled to minimize variability in conditions through time – so-called *steady state*. Many experiments *in vivo* or in continuous fermenters in which attempts have been made to quantify the rate of microbial growth or flow of microbial cells from the rumen have been based on such protocols [25].

Other important questions, however, relate to the transitional and cumulative effects of changing conditions on the growth rates or population density of specific organisms or groups. Under *non-steady* state dynamics, there is potential for changes in pool size, dilution rates and relative efficiencies of growth that can dramatically affect the nature of the population present at any given sampling time [15, 25]. Most questions relating to microbial activity and the species composition of the microbial population under normal animal behavioural patterns of intake of feed and water call for protocols that allow for this, particularly those involving grazing and/or the feeding of supplements. Here, the patterns of intake may be relatively repeatable in cycles on a 24 h basis [43], but any regularity will depend on frequency of feeding and even prevailing weather conditions.

Variables

The potential sources of variability in experiments to explore the microbial population of the rumen (or any other gut compartment), its diversity and the factors affecting the structure of that population include combinations of the following:

The animals

- between species of animals
- between animals of the same species at different ages/stages of development
- between animals of the same species and age but reared under different conditions
- between like animals in a cohort from the same rearing conditions (the most common approach in selecting animals for nutritional experiments)
- between fermenters started with the same innoculum (replicate systems)
- within individual animals (replicated in time)

The diets

- between previous diets (carry-over effects)
- between current diets
- between levels of intake (*ad libitum* or controlled)
- between meal eating patterns or periodicity of feeding of components of the diet

Time of sampling

- between samples taken at a specified time relative to the feeding regime
- between bulked samples taken at several specified times in the feeding cycle
- between individual samples taken at specified times in a feeding regime

Site of sampling

- between samples drawn at a set position of the sampling device within the digesta
- between samples taken from several set positions but with samples bulked
- between several set positions of sampling with samples analysed separately

Fraction sampled

- between samples of mixed digesta
- between samples of strained fluid phase
- between samples of strained particulate phase
- between samples extracted, for example, by centrifugation methods

Experimental conditions, treatments and sampling protocols are designed to remove the influence of selected sources of potential variability in accordance with the demands of the specific objective or hypothesis. Interactions can occur between the various sources of variability, so that, for example, there may be animal by diet interactions revealed only at specific times of sampling. That may or may not be of immediate interest, depending on the objectives of the experiment, but may be of importance when separate experiments are compared and we seek to explain differences in results or interpretation. Replication at the sampling level is necessary if one is to evaluate the influence of any one of the above potential variables to the total variability.

Variability associated with unresolvable interactions plus the variance due to replicates is treated as residual 'error'.

The experimental unit

A critical element in design of experiments involves the establishment of the variables that must be isolatable in subsequent statistical analysis of the data. The experimental unit is the finest subdivision of data that can legitimately be treated as truly independent. Clearly the objective or hypothesis will determine one layer or set of such isolatable variables. However, additional variables become important if, for example, comparisons to related work of others are important and the experiment can provide some support towards being 'right for the right reason' or contribute to explanations of differences in results.

The degree to which a researcher can add complications either of additional experimental treatments or sampling schedules to cover suspected sources of variability obviously depends on cost and time constraints. The question resolves to the importance placed on getting a result that represents a good 'general case' or getting a result that defines the magnitude and impact of the various sources of variability. For example, samples taken at different times of the day and from different sites in the rumen can be bulked to 'average out' the broad picture of differences due to diet. The experimental unit is clearly the bulked sample. However, if it is desirable to get a more intimate picture of the changes going on or to ensure that the chosen sampling and bulking schedule (e.g. equal volumes only before and 6 h after feeding) does not grossly bias the results derived through the bulking process, the individual samples should be analysed separately and become the experimental unit.

Individual animals differ in the microbial populations established, which may reflect the source of the inoculum, but importantly also anatomical and physiological variables [18]. These include factors, such as digesta pool size, effectiveness of rumination, the kinetics of fluid and solid particle entry and exit rates [20], overlain by the individual animal response to diet composition expressed in selection and/or meal patterns where these are not constrained in the management system applied. While the broad outcomes in terms of digestion rates for dietary constituents may be similar, the organisms occupying the various microenvironmental and particularly substrate niches can differ. Likewise, the patterns of production of fermentation products, rates and energetic efficiencies of microbial growth and the net microbial cell yields presented for subsequent digestion vary. Such diversity in the solutions of microbial success under the prevailing conditions in each animal constitutes the so-called biological variability and will have an influence on the numbers of animals required for robust statistical analysis and interpretation, and the appropriate source, selection and preparatory treatment of those animals. From this, it is also clear that in any experimental program that is undertaken *in vitro* (e.g. continuous flow fermenters) the source and constitution of rumen digesta inoculum should be well described. Guidelines can be established in order that more secure comparisons between experiments can be made. However, guidelines are often aimed at reducing variability and so may

constrain the circumstances to which the results can be extrapolated. While we may justify the simplifications inherent in over-riding the complicated realities of microbial dynamics *in vivo*, we need to be alert to those matters where such simplification could lead to incorrect interpretations.

All that said, the following discussion has the aim of assisting in establishing robust, purpose-specific experimental designs and protocols for investigations of microbial populations in the rumen and their contribution to processes of digestion and the supply of nutrients to the host animal. Because of the diverse objectives of individual experiments in such research what is presented is in the form of principles and processes in arriving at best solutions for specific cases.

Design, conditions, sampling and measurements

In many studies of the rumen microbes, the studies have drawn on samples obtained from digesta of free-ranging ruminants or from animals in experiments designed to investigate wider aspects of animal performance. Samples taken have been used to establish in the laboratory libraries of readily culturable anaerobic genotypes. Once isolated, the organism can be characterized on the basis of substrate range and specificities and the nature of the end products of fermentation. The challenge has been to increase the array of culturable organisms by finding the conditions under which each can be maintained. This has allowed development since the early 1940s of knowledge of substrate range, cofactor requirements and end products for many rumen anaerobes.

While these objectives remain, new opportunities have arisen through advances in molecular genetics permitting, for example, description of hereto uncultured organisms using metagenomic approaches and the application of biotechnological approaches to manipulation of organisms.

For all experiments, there are several guiding principles.

1. A *full description of the experimental conditions* is mandatory, to provide key information in terms of the type and sources of animals, where and under what environmental conditions they are held, the diet composition and feeding regime. This is necessary but rarely sufficient.
2. The *specific objective and hypothesis* to be tested must be explicit, because it determines the constraints to be set on the design and protocol to be followed.
   Many experiments are designed on the basis of constraining sources of variability other than the primary (treatment) variables or to obviate spatial and time-sensitive differences. Thus, many experiments and much of the data used in construction of mechanistic models are based on experiments using total mixed rations (TMR) (dietary mixtures aimed at delivery of all feed components synchronously) and short-interval feeding regimes (e.g. 2 h feeding in equal-sized meals). While such conditions produce relatively stable and therefore more easily measured digestion parameters, they do not provide an understanding of the effects of the fluctuating conditions established during many natural feeding and particularly grazing cycles.
3. When setting the *experimental design*, decisions are required not only on what *treatments* are to be imposed, but also on the nature of the baseline conditions.

Often there needs to be a control treatment that will allow inter-experiment comparisons through some consistent baseline condition and perhaps provide data on between-animal variability. There are some traditions about the length of any preliminary treatment or feeding period, the numbers of animals required for robust statistical analysis, the use of Factorial, Latin Square or Cross-over designs and the benefits of a covariate period. However, as we move into an exploration of the functional diversity of rumen organisms and the potential for reliable manipulation for production purposes, longitudinal studies involving dietary changes in individual animals in the treatment cohort may prove more illuminating.

4. *Individual animals differ*, for example, protozoa or anaerobic fungi may be abundant in some but not all animals particularly on some but not all diets studied [14, 15]; the reasons for this again call for further experimental work. This imposes a degree of statistical heterogeneity in data obtained with any type of design, and designs are selected either to explore the differences by keeping individual animal as the experimental unit or to gain a 'coarser' view by bulking samples or combining data obtained over groups of animals as the experimental unit.

   In all cases, the animals are randomly assigned to groups to receive the respective experimental treatments *except* where the class of animal is to be an experimental variable. If the animals are deemed to be of a single class, unbiased allocation to treatments is by simple random number drafting. Where the animals are clearly differing in some respect and there is no immediate interest in the variance due to such differences, randomization should be on a stratified basis. *Stratified randomization* requires animals to first be assigned to a defined class such as breed, sex, age and/or weight and members of each class are assigned in rotation to the respective treatments randomly.

   Animals for which results appear to be 'outliers' in relation to any measurement made contribute to the overall variability and create a greater level of heterogeneity in the cell into which their data are assigned. Their unusual status may make them a target for closer examination. In terms of data relating to microbial populations, such animals may have special significance.
5. In any given design, *the measurements* to be made are selected on two bases. They are the measurements that are essential in testing the primary hypothesis. Additional measurements to be considered are those that characterize more thoroughly the conditions of the experiment, inform the interpretation and support efforts to compare and contrast results with those of other apparently similar experiments.
6. The numbers of *samples* and the times and the sites of sampling need close attention. The decisions revolve around the nature and magnitude of differences due to time, to any stratification or imperfection in digesta mixing and to interactions between these factors.

   Simply adopting the protocols of others in the field is not always best practice. Always the capacity for analysis of samples depends on time and funds available, but the compromise arrived at needs to acknowledge that the reason for spending any time or money is to take a robust step towards reliable additional knowledge.

Pragmatic solutions such as sampling cows only at milking times or choosing a single 'best time' of the day for sampling need to be challenged and strong biological reasons advanced that this is sufficient to the objective. In terms of sample size and sampling site, in some cases extreme efforts to take a 'representative sample' may be unwarranted; in other cases samples taken at the same time from different sites may need to be viewed as describing the basic heterogeneity rather than to be pooled to provide an aggregate result.

Key questions

The following considerations, expressed as questions to be addressed, form an important step in planning for most experiments and have general application here. They cannot all be answered once and for all in a stepwise fashion but have to be revisited as provisional decisions are reached.

- To what degree do I have control over each of the variables?
- Which of the potential variables am I interested in, in terms of main effects and possible interactions?
- Which of the potential variables must be 'removed' to address the objective or test the hypothesis?
- Which of the potential variables cannot be removed given the constraints on the experiment and the conditions under which it will be conducted *and* how then do I provide sufficient information to ensure that others can see the results in that context?
- How many treatments are necessary and sufficient to the objective?
- Over what ranges do I seek to set the levels for treatment variables?
- What samples are to be taken from all animals, in relation to time, site and fractionation of the sample?
- What replication is required in order to establish a sufficient basis for robust statistical analysis at the level of the experimental unit?
- What are the samples to be analysed for in terms both of data essential to the objective and data desirable for more effective description of the conditions achieved in the experiment?
- How many samples can be analysed (level of precision, time, cost) and what is the compromise on issues such as bulking of samples?

Strengths and weaknesses of experimental designs and protocols for evaluation of microbial populations

In the following section, several common designs are reviewed and comments made on the issues that arise in their application. All readers are advised to discuss fully with their statistical adviser the design that they consider most appropriate to their objectives and ensure at the outset that they have a clear view of the way the data will be treated in subsequent statistical analysis.

Samples take at one time from individual animals

The results are a snapshot of the microbial population present. Samples may be taken from one or more animals, from different sites, fractionated and replicated to allow analysis for variance due to animals, sites and fractions [8, 26, 33, 41]. Such studies may provide the initial basis for a hypothesis or yield unusual data of microbiological importance setting the scene for further experimental work. Results cannot reveal what factors influenced the arrival at that population; any relationships to diet, season and digestive physiology of the animal are by inference.

Longitudinal studies on individual animals

Each animal is its own control and data obtained through time relate to the sequence of changes in conditions over that time course and the consequent patterns of microbial successions [10, 12, 18, 21]. Samples may be taken at successive intervals at times within a day or over an extended period, relating to events or time elapsing since imposition of a treatment. Samples may be taken from different sites, fractionated and replicated, to allow analysis for variance due to animals, times, sites and fractions. Relationships to season, diets and physiological changes over the period can be inferred, but because of confounding of these influences, direct evidence of the influence of any critical variable can only be derived by further testing of hypotheses under more controlled experimental conditions. However, longitudinal studies can be established within more complex designs described below.

Studies on animals subjected to different treatments within the same time period

These types of experiments provide opportunity to investigate the influence of a limited array of selected variables such as species, age, diet, environmental conditions, physiological state or physiological intervention where these are imposed as 'treatments' [5, 30]. Animals are usually, but not always, drawn from groups with a known common history and are assigned to treatments by randomization or by stratified randomization. Any differences in recent dietary or drug treatment or in familiarity with the conditions for the experiment are to be reported and are usually dealt with by including a preparatory or preliminary period under a common management system. Replication is needed and individual animals can be treated as replicates if they correctly define the *experimental unit*.

Block design
Individual animals or groups of animals (in each case replicated) are subjected to several treatments to compare effects of, say, Treatment A vs. Treatment B etc. in a single experimental period [11, 23, 28]. The variance due to animals within a treatment may be significant but such interactions can result in high residual variances (error term). Samples may be taken at successive intervals at times within a day or over an extended period, relating to events or time elapsing since imposition of a treatment [32]. Samples may be taken from different sites, fractionated and

replicated, to allow analysis for variance due to treatments, animals, times, sites and fractions.

Factorial design

Replicate animals or groups of animals are necessary. The way that animals are managed (e.g. individually fed vs. group fed) and the way the samples taken are treated for analysis determine the experimental unit.

Under these types of design, it is possible to investigate interactions between treatments by imposing several treatments separately and in selected combinations on randomized groups of animals [29, 35, 37]. For example, a basic treatment might be pasture or roughage diet (R), and the further treatments imposed may be added, for example, type of supplement (R + A, R + B), level of supplementation (R + A, R + 2A) or various combinations of supplements (R + A + B). Samples may be taken at successive intervals at times within the experimental period relating to events or time elapsing since imposition of the respective treatment. Samples may be taken from different sites, fractionated and replicated, to allow analysis for variance due to treatment, times, sites and fractions. However, even when the experimental unit is set correctly, differences due specifically to individual animals within groups cannot be separated from other residual variability (error term).

Studies on animals subjected to different treatments in a sequence over time

These designs are aimed at increasing the database and ensuring that all animals receive all treatments, but they increase the length of time and hence the opportunity for time-related factors to influence the results. There are advantages particularly where infrastructure and equipment are limiting.

Cross-over design experiments allow for each animal or group of animals as a set to receive one of a number of treatments in one period of time and other treatments in following periods in a balanced design [19]. Often this design is used to make simple comparisons between two treatments; Group 1 receives Treatment A in period 1 and Treatment B in period 2, while a second group receives the same two treatments but in the reverse order. Usually the analysis is most robust when the experimental unit is an individual animal (i.e. each animal is managed on a truly independent basis). Replication is needed. Samples may be taken at successive intervals at times within the experimental period relating to events or time elapsing since imposition of the respective treatment. Samples may be taken from different sites, fractionated and replicated, to allow analysis for variance due to treatment, times, sites and fractions.

Latin Square design experiments provide a basis for investigation of variance due to individual animals. It can help uncover a consistent bias in data due to some peculiarity of the individual. In its basic form, there are as many animals as there are treatments, and each animal receives each treatment in a randomized sequence over successive periods of time (Table 1). In any period, no two animals receive the same treatment [42]. The data can be analysed for variance due to treatment, period and animal; any interactions are treated as residual variability (error). In this case, the animal is managed as an individual and is the experimental unit. Interactions

Table 1. A Latin Square design

	Period 1	Period 2	Period 3	Period 4
Treatment 1	Animal (Group) 2	Animal (Group) 4	Animal (Group) 1	Animal (Group) 3
Treatment 2	Animal (Group) 4	Animal (Group) 1	Animal (Group) 3	Animal (Group) 2
Treatment 3	Animal (Group) 3	Animal (Group) 2	Animal (Group) 4	Animal (Group) 1
Treatment 4	Animal (Group) 1	Animal (Group) 3	Animal (Group) 2	Animal (Group) 4

between animal, treatment and time period are embedded in the residual variability (error term).

A Latin Square design can also be based on a group of animals managed together as the experimental unit, so that a more aggregated view of effects of treatment and period is achieved. Samples may be taken at successive intervals at times within the experimental period relating to events or time elapsing since imposition of the respective treatment. Samples may be taken from different sites, fractionated and replicated, to allow analysis for variance due to treatment, animals, periods, times within periods, sites and fractions. The samples can be physically bulked across animals within a given treatment group for each time, site and fraction of sample. However, this means that individual animal variability cannot be isolated. If the samples are analysed separately, individual data can be viewed (any outliers?), but the data will still be analysed on the basis of the treatment group; the individual variability within groups becomes part of the residual variability (error term).

The issue of treatment sequence and its effects on the microbial population also becomes important in the Cross-over and Latin Square designs, because no two animals receive the same sequence of test treatments. In analysis of the data, this has the effect of lumping together the different carry-over effects. If there are any carry-over effects of a preceding treatment on the microbial succession under the new treatment, this will increase the heterogeneity of the data attributed to the current treatment, and in analysis this will appear in the error term. Therefore, there is a need to reduce any influence of carry-over effects. This can be achieved by including longer periods for adaptation to the new set of treatments. Another approach is to return all animals to a common set of conditions during an interval before imposing the new treatment. All these strategies are expensive in use of resources including time and in some cases are not warranted.

Time and site of sampling

The following section relates particularly to the study of the microbiology of the rumen, though some considerations may help in choice of sampling procedures in other compartments of the digestive tract.

Sampling time can be a most critical decision depending on the objectives of the experiment. Since the current potential to track the dynamics of microbial population change by repeated sampling is strongly constrained by cost and time, most researchers

will have to arrive at a restricted sampling schedule based on their knowledge of time patterns in the changing environment in the compartment of the digestive tract under investigation. The major environmental factors implicated are rates of entry of new substrates, their individual rates of fermentation and the concentrations or rates of accumulation of end products (rate of production minus rate of removal). A review of existing mechanistic models that predict rumen function can help gain some overview of the important factors involved, but most of these aggregate to a daily average level for predicted variables [6, 16, 22, 39]. For shorter-term fluctuations during a day, the reader should refer to individual published papers such as Dixon et al. [17] and Williams et al. [45]. In broadest terms, the chemical composition and physical form of the dietary ingredients and time patterns of ingestion set the substrate entry rates and changes in rates of their fermentation. For dietary carbohydrates, the rate of accumulation of fermentation end products is very broadly associated with pH of the digesta and for dietary N compounds, with digesta ammonia concentration. Both of these variables can reflect important changes in the conditions affecting the relative competitive success or fitness of various functional classes of micro-organisms, though evidence has mostly been indirect through measured changes in rates of digestion of, for example, dietary fibre. In Figs. 1 and 2, taken from Williams et al. [45] a few times for sampling are proposed in order to detect the most likely times at which important changes in numbers, growth rates or species composition of the microbial population will be apparent.

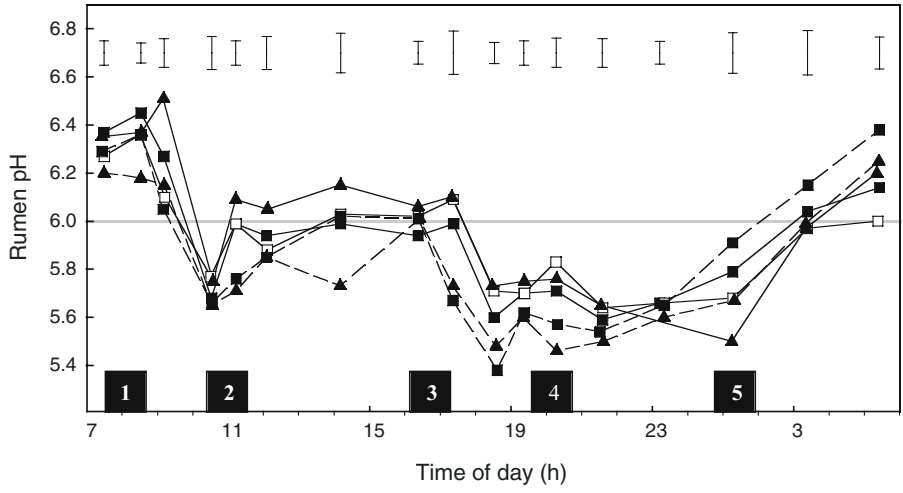


Figure 1. Diurnal pattern of rumen pH in cows grazing perennial ryegrass – based pastures alone at low (■—) or high (—) allowances or at low allowance and receiving a grain pellet (■- - -), hay cube (▲—) or grain/hay cube (▲- - -). Bar blocks along the 'time' axis indicate priority sampling times; open blocks indicate transition sampling times for comparison of microbial population as they change with time and different dietary conditions. The error bars indicate the s.e.d. for comparing between dietary treatments at each time. Based on Williams et al. [45].

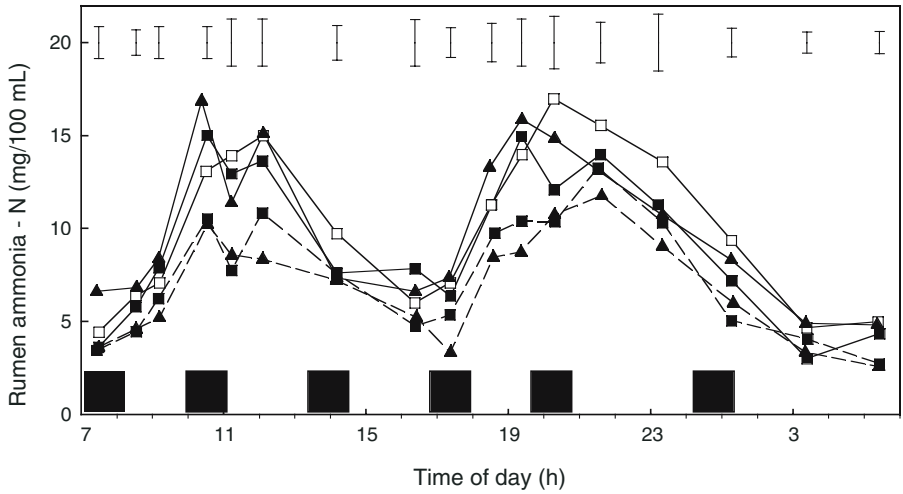


Figure 2. Diurnal pattern of ruminal fluid ammonia-N concentration in cows grazing perennial ryegrass—based pastures alone at low (■—) or high (—) allowances or at low allowance and receiving a grain pellet (■- - -), hay cube (▲—) or grain/hay cube (▲- - -). Bar blocks along the 'time' axis indicate priority sampling times; open blocks indicate transition sampling times for comparison of microbial population as they change with time and different dietary conditions. The error bars indicate the s.e.d. for comparing between dietary treatments at each time. Based on Williams et al. [45].

These sampling times are based on the logic that they represent 'peak and trough' situations that appear to differ between treatments and where substrate availability and rumen condition may have swung to favour an entirely different array and functional predominance of organisms. The suggested choices of sampling time related to pH and to ammonia concentration differ because they indicate independent sets of factors contributing to fermentation rates, substrate utilization rates and other conditions that alter the competitive advantages of organisms. All the factors that contribute to the changes and the possible lag times in measurable responses of the microbial species are of course not fully accounted for. Hence, it is doubly important that the description of sampling procedures and relationships to times of feeding and other data describing the prevailing conditions are provided.

Sampling procedures

In order that samples taken from the rumen (or other gut compartment) are representative of the digesta content as a whole, or of defined strata or positions in the digesta body, various approaches can be taken. The question is, do we know how the various constituent microbial groups in which we have immediate interest are distributed?

Because there are many objectives and many approaches have been developed (see previous section) advice at this point must be generalized. Study carefully the sampling methods reported in that body of literature most relevant to the

objective of your experiment, consider carefully the adoption or adaptation of the respective options, explain very clearly how sampling has been undertaken and justify the choice. Several approaches and some issues to consider are presented below.

- With slaughtered animals or fistulated animals, most of the digesta can be removed, mixed and sampled in replicate but anaerobic conditions will not be easily maintained. For some objectives, this will bring into doubt the degree to which the result can be taken as definitive.
- In live unfistulated animals, rumen digesta can be obtained by passing a tube down the oesophagus into the rumen digesta. Samples will inevitably be biased to the fluid fraction, and depending on whether the tube passes to the dorsal rumen or the reticulum, may be diluted with saliva and poorly representative of even the same fraction in the deep rumen digesta.
- If a fistulated animal is used, 'whole digesta' samples can be taken using a 'core-sampling' device made up as described by Walker et al. [42]. The representativeness of the combined fluid and the solid samples depends on the effectiveness of closure of the device.
- Samples of liquid fraction (by open tube or probe with a filter attached) and solid fraction (by grab sample with forceps) can be removed separately from several sites around the rumen, and can be investigated as independent samples or be bulked to provide a 'mixed digesta' sample. If the questions are quantitative about the make up of the whole rumen population, it is possible that bulking can give a biased result. However, putting together the correct proportions of solids and liquids fractions requires much more work (e.g. Faichney [20]). Generating data for 'validly recombined whole digesta' may be by physical recombination or by mathematical treatment of the data from each fraction.
- Sampling tubes can be inserted and pumps used to draw samples continuously throughout any given period into fraction collectors or into a bulk container. Collections by this means require rapid transfer into sealed frozen collectors. This has been used principally to obtain samples of fluid fractions, which may not be what we want.

Fractionation of samples

The microbial population was described earlier as being distributed between liquid and solid digesta phases. Planktonic organisms exist in the aqueous phase of the rumen liquor utilizing soluble substrates, while other organisms are associated with the particulate solid phase. Among the latter group are micro-organisms that form dynamic biofilms on digesta particles [2, 4], the species representation in those consortia being related to the substrate material. However, also present in the solids fraction are microbes that are only loosely associated that can be removed for study by simple buffer extraction procedures. In this group are organisms also found in the liquid phase and some that are present in greater numbers tightly adherent to solid particles.

Why would it be important to know more about the distribution of microbes in these microenvironmental terms? Objectives of research may be to get an accurate quantitative measure of the total microbial biomass, its distribution between phases or the vulnerability of different species to altered rumen conditions. While extensive environmental fluctuations may stop growth or kill large proportions of any given microbial community, small and functionally important groups may be able to survive intermittent adverse conditions because of biofilm protection or perhaps because of the ability to move to a different phase, 'migrate' and enter a different microenvironment.

Consequently, with the questions arising at this level of microbial ecology, the fractionation of digesta samples becomes important.

Following is a fractionation method used by Larue et al. [31]

A crude rumen digesta sample (approximately 200 *g*) was taken by tube inserted through the rumen fistula into the mid-rumen digesta and immediately strained through 4 layers of cheesecloth.

Liquid fraction

The liquid fraction (approximately 150 ml) was centrifuged at 10 000 *g* for 10 min, and the supernatant of clarified rumen fluid was discarded.

The pellet was re-suspended in 50 ml TE (10 mM Tris–HCl and 5 mM EDTA, pH 8.0) and taken forward for metagenomic analysis as the *Liquid Fraction.*

Solid fraction

The solids (approximately 50 g) were centrifuged at 350 *g* for 15 min.

The fluid supernatant was then taken and centrifuged at 10 000 *g* for 20 min, and the supernatant from that was discarded.

The pellet was re-suspended in 50 ml TE and taken forward for analysis as the *Associated Fraction.*

The solid fraction from low speed centrifugation was washed, 25 ml of Anaerobic dilution solution [8] plus 15% Tween 80 was added and placed on ice for 2.5 h. This was then centrifuged at 350 *g* for 15 min and the supernatant only retained.

The supernatant was centrifuged at 10 000 *g* for 20 min and the Tween 80 supernatant liquid was discarded.

The pellet was re-suspended in 50 ml TE and used for analysis as the *Adherent Fraction*.

Conclusions

Design of the experiment and the protocols in management of the animal or the microbial system ultimately come down to setting clear objectives, understanding the variables in the system and establishing the sampling schedule and data analysis on the basis of a defined experimental unit. The decisions on what to

describe, what to control, what to measure and how much data are necessary have to be made in the light of the value to those who commission the research and to the informed, critical reader of the final published scientific paper. The advice provided has been aimed at improving the planning process that precedes each experiment. Not all of the advice applies all the time, but it is valuable to consider the list each time in the interests of designing the best experiment within the resources available.

References

1. Abe, M., T. Iriki, N. Tobe, and H. Shibu. 1981. Sequestration of holotrich protozoa in the reticulo-rumen of cattle. *Appl. Environ. Microbiol*. **41:**758–765.
2. Akin, D.E. 1976. Ultrastructure of rumen bacterial attachment to forage cell walls. *Appl. Environ. Microbiol.* **31:**562–568.
3. Amman, R.I., W. Ludwig, and K.H. Schleifer. 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59:**143–169.
4. Anderson, K.L. 2003. The complex world of gastrointestinal bacteria. *Can. J. Anim. Sci.* **83:**409–427.
5. Anderson, K.L., T.G. Nagaraja, J.L. Morrill, T.B. Avery, S.J. Galitzer, and J.E. Boyer. 1987. Ruminal microbial development in conventionally or early-weaned calves. *J. Anim. Sci.* **64:**1215–1226.
6. Baldwin, R.L. 1995. *Modelling Ruminant Digestion and Metabolism*. pp. 469–518. Chapman and Hall, NY.
7. Bauchop, T. 1989. Colonization of plant fragments by protozoa and fungi, pp. 83–96. *In* J.V. Nolan, R.A. Leng, and D.I. Demeyer (eds.), *The Roles of Protozoa and Fungi in Rumen Digestion*. Penambul Books, Armidale, NSW.
8. Bryant, M.P., and L.A. Burkey. 1953. Numbers and some predominant groups of bacteria in the rumen of cows fed different rations. *J. Dairy Sci.* **36:**218.
9. Bryant, M.P., and I.M. Robinson. 1968. Effects of diet, time after feeding and position of sampling on the numbers of viable bacteria in the bovine rumen. *J. Dairy Sci.* **51:**1950–1955.
10. Bryant, M.P., N. Small, C. Bouma, and I. Robinson. 1958. Studies on the composition of the rumen flora and fauna of young calves. *J. Dairy Sci.* **41:**1747–1767.
11. Chen, M., and M.J. Wolin. 1979. Effect of monensin and lasalocid-sodium on the growth of methanogenic and rumen saccarolytic bacteria. *Appl. Environ. Microbiol.* **38:**72–78.
12. Clarke, R.T.J., M.J. Ulyatt, and A. John. 1982. Variation in numbers and mass of ciliate protozoa in the rumens of sheep fed chaffed alfalfa (*Medicago sativa*). *Appl. Environ. Microbiol.* **43:** 1202–1204.
13. Craig, W.M., G.A. Broderick, and D.B. Ricker. 1987. Quantitation of microorganisms associated with the particulate phase of ruminal digesta. *J. Nutr.* **117:**56–64.
14. Davies, D.R., M.K. Theodorou, M.I.G. Lawrence, and A.P.J. Trinci. 1993. Distribution of anaerobic fungi in the digestive tract of cattle and their survival in faeces. *J. Gen. Microbiol.* **139:**1395–1400.
15. Dehority, B.B., and C.G. Orpin. 1997. Development of and natural fluctuations in rumen microbial populations, pp. 196–245. *In* P.N. Hobson and C.S. Stewart (eds.), *The Rumen Microbial Ecosystem*. Chapman and Hall, London.
16. Dijkstra, J. 1994. Simulation of the dynamics of protozoa in the rumen. *Br. J. Nutr.* **72:**679–699.
17. Dixon, R.M., W. Karda, B.J. Hosking, and A.R. Egan. 2001. Oilseed meals and fortified grain as supplements fed infrequently to sheep consuming low quality roughage. *Anim. Feed Sci. Technol.* **50:**248–257.
18. Eadie, J.M. 1962. The development of rumen microbial populations in lambs and calves under various conditions of management. *J. Gen. Microbiol.* **29:**563–578.
19. Egan, A.R., and M.J. Ulyatt. 1980. Quantitative digestion of fresh herbages by sheep: VI. Utilization of N of five herbages. *J. Agric. Sci. Camb.* **94:**47–56.

20. Faichney, G.J. 1993. Digesta flow, pp. 53–86. *In* J.M Forbes and J. France (eds.), *Quantitative Aspects of Ruminant Digestion and Metabolism*. CAB International, Wallingford, UK.
21. Fonty, G., J.P. Jounay, J. Senaud, P. Gouet, and J. Grain. 1984. The evolution of microflora, microfauna and digestion in the rumen of lambs from birth to 4 months. *Can. J. Anim. Sci.* **64**(Suppl.)**:** 165–166.
22. Fox, D.G., L.O. Tedeschi, T.P. Tylutki, J.B. Russell, M.E. van Amburgh, L.E. Chase, A.N. Pell, and T.R. Overton. 2004. The Cornell Net Carbohydrate and Protein System model for evaluating herd nutrition and nutrient excretion. *Anim. Feed Sci. Technol.* **112:**29–78.
23. Gordon, G.L.R., and M.W. Phillips. 1993. Removal of anaerobic fungi from the rumen of sheep by chemical treatment and the effect on feed consumption and *in vivo* fibre digestion. *Lett. Appl. Microbiol.* **17:**220–223.
24. Hespell, R.B., D.E. Akin, and B.A. Dehority. 1996. Bacteria, fungi and protozoa of the rumen, Vol. II, pp. 59–141. *In* R.I. Mackie, B.A. White, and R.E. Isaacson (eds.), *Gastrointestinal Microbiology*. Chapman and Hall, NY.
25. Hespell, R.B., and M.P. Bryant. 1979. Efficiency of microbial growth: influence of some theoretical and experimental factors on Y_{ATP}. *J. Anim. Sci.* **49:**1640–1659.
26. Jarvis, G.N., C. Strompl, D.M. Burgess, L.C. Skillman, E.R. Moore, and K.N. Joblin. 2000. Isolation and identification of ruminal methanogens from grazing cattle. *Curr. Microbiol.***40:** 327–332.
27. Jounay, J.P., B. Michalet-Doreau, and M. Doreau. 2000. Manipulation of the rumen ecosystem to support high-performance beef cattle – review. *Asian Australas. J. Anim. Sci.* **13:**96–114.
28. Kocherginskaya, S.A., R.L. Aminov, and B.A. White. 2001. Analysis of the rumen bacterial diversity under two different diet conditions using denaturing gradient gel electrophoresis, random sequencing and statistical ecological approaches. *Anaerobe* **7:**119–134.
29. Krause, D.O., W.J.M. Smith, F.M.E. Ryan, R.I. Mackie, and C.S. McSweeney. 2000. Use of 16S-rDNA based techniques to investigate the ecological succession of microbial populations in the immature lamb rumen: Tracking of a specific strain of inoculated *Ruminococcus* and interactions with other microbial populations *in vivo*. *Microbiol. Ecol.* **38:**365–376.
30. Krysl, L.J., M.E. Branine, A.U. Cheema, A.U. Funk, and A.L. Galyean. 1989. Influence of soybean meal and sorghum grin supplements on intake, digesta kinetics, ruminal fermentation, site and extent of digestion and microbial protein synthesis in beef steers grazing blue grama rangeland. *J. Anim. Sci.* **61:**3040–3051.
31. Larue, R., Z. Yu, V.A. Parisi, A.R. Egan, and M. Morrison. 2005. Novel microbial diversity adherent to plant biomass in the herbivore gastrointestinal tract, as revealed by ribosomal intergenic spacer analysis and rrs gene sequencing. *Environ. Microbiol.* **7:**530–543.
32. Mackie, R.I., F.M.C. Gilchrist, A.M. Roberts, P.E. Hannah, and H.M. Schwartz. 1978. Microbiological and chemical changes in the rumen during stepwise adaptation of sheep to high concentrate diets. *J. Agric. Sci. Camb.* **90:**241–254.
33. Mackie, R.I., A. Sghir, and H.R. Gaskins. 1999. Developmental microbial ecology of the neonatal gastrointestinal tract. *Am. J. Clin. Nutr.* **69:**1035S–1045S.
34. Mackie, R.I., and B.A. White. 1990. Recent advances in rumen microbial ecology and metabolism: potential impact on nutrient output. *J. Dairy Sci.* **73:**2971–2995.
35. Martin, C., and B. Michalet-Doreau. 1995. Variations in mass and enzyme activity of rumen microorganisms: effect of barley and buffer supplements. *J. Sci. Food Agric.* **67:**407–413.
36. Rasmussen, M.A., B.A. White, and R.B. Hespell. 1989. Improved assay fro quantitating adherence of ruminal bacteria to cellulose. *Appl. Environ. Microbiol.* **55:**2089–2091.
37. Romulo, B., S.H. Bird, and R.A. Leng. 1989. Effects of defaunation and protein supplementation on intake, digestibility, N retention and fungal numbers in sheep fed straw-based diets, pp. 285–288. *In* J.V. Nolan, R.A. Leng, and D.I. Demeyer (eds.), *The Role of Prototzoa and Fungi in Ruminant Digestion*. Penambul Books, Armidale, NSW, Australia.
38. Russell, J.B., W.M. Sharp, and R.L. Baldwin. 1979. The effect of pH on maximum bacterial growth rate and its possible role as a determinant of bacterial competition in the rumen. *J. Anim. Sci.* **48:** 251–255.

39. Russell, J.B., and H.J. Strobel. 1993. Microbial energetics, pp. 165–186. *In* J.M. Forbes and J. France (eds.), *Quantitative Aspects of Ruminant Digestion and Metabolism*. CAB International, Wallingford, UK.
40. Stewart, C.S., H.J. Flint, and M.P. Bryant. 1997. The rumen bacteria, pp. 10–72. *In* P.N. Hobson and C.S. Stewart (eds.), *The Rumen Microbial Ecosystem*. Chapman and Hall, London.
41. Tajima, K., R.I. Aminov, T. Nagamine, K. Ogata, M. Nakamura, H. Matsui, and Y. Benno. 1999. Rumen bacterial diversity as determined by sequence analysis of 16S rDNA libraries. *FEMS Microbiol. Ecol.* **29:**159–169.
42. Walker, D.J., A.R. Egan, C.J. Nader, M.J. Ulyatt, and G.B. Storer. 1975. Rumen microbial protein synthesis and the proportions of microbial and non-microbial N flowing to the intestines of sheep. *Aust. J. Agric. Res.* **26:**699–708.
43. Warner, A.C.I. 1966. Diurnal changes in the concentrations of microorganisms in the rumens of sheep fed to appetite in pens or pasture. *J. Gen. Microbiol.* **45:**243–251.
44. Williams, A.G., K.N. Joblin, and G. Fonty. 1994. Interactions between the rumen chytrid fungi and other microorganisms, pp.191–228. *In* D.O. Mountford and C.G. Orpin (eds.), *Anaerobic Fungi*. Marcel Dekker, NY.
45. Williams, Y.J., G.P. Walker, P.T. Doyle, A.R. Egan, and C.R. Stockdale. 2005. Rumen fermentation characteristics of dairy cows grazing different allowances of Persian clover- or perennial ryegrass-dominant swards in spring. *Aust. J. Exp. Agric.* **45:**665–675.
46. Wolin, M.J. 1979. The rumen fermentation: a model for microbial interactions in anaerobic systems. *Adv. Micro. Ecol.* **3:**49–77.
47. Wolin, M.J., T.L. Miller, and C.S. Stewart. 1997. Microbe–microbe interactions, p. 467–491. *In* P.N. Hobson and C.S. Stewart (eds.), *The Rumen Microbial Ecosystem*. Chapman and Hall, London.

PART TWO

Classical methods for isolation, enumeration, cultivation and functional assays of rumen microbes

2.1. Rumen bacteria

CHRISTOPHER S. MCSWEENEY[1], STUART E. DENMAN[1] and RODERICK I. MACKIE[2]
[1]*CSIRO Livestock Industries, Queensland Bioscience Precinct, 306 Carmody Rd, St Lucia, Queensland, 4067, Australia*
[2]*Department of Animal Sciences, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA*

Introduction

The rumen is the most extensively studied gut community and is characterized by its high population density, wide diversity and complexity of interactions. This complex, mixed microbial culture is comprised of prokaryote organisms including methane-producing archaebacteria, eukaryote organisms, such as ciliate and flagellate protozoa, anaerobic phycomycete fungi and bacteriophage. Bacteria are predominant (up to 10^{11} viable cells per g comprising 200 species) but a variety of ciliate protozoa occur widely (10^4–10^6/g distributed over 25 genera). The anaerobic fungi are also widely distributed (zoospore population densities of 10^2–10^4/g distributed over 5 genera). The occurrence of bacteriophage is well documented (10^7–10^9 particles/g). This section focuses primarily on the widely used methods for the cultivation and the enumeration of rumen microbes, especially bacteria, which grow under anaerobic conditions. Methods that can be used to measure hydrolytic enzymes (cellulases, xylanases, amylases and proteinases) are also described, along with cell harvesting and fractionation procedures. Brief reference is also made to fungi and protozoa, but detailed explanations for culturing and enumerating these microbes is presented in Chapters 2.4 and 2.5.

Anaerobic culturing techniques

The majority of bacteria, which colonize the gastrointestinal tract of ruminants are strict anaerobes, which require an environment with a low redox potential for growth. The anaerobic techniques of Hungate [12] as modified by Bryant [3] are used by all the major rumen microbiology laboratories and have been described by Stewart et al. [28]. A brief description will be given here, but it is recommended that experience be obtained in an anaerobic microbiology laboratory before attempting these procedures.

H.P.S. Makkar and C.S. McSweeney (eds.), Methods in Gut Microbial Ecology for Ruminants, 23–37.
© 2005 *IAEA. Printed in the Netherlands.*

Preparation of anaerobic media

Combine the mineral solutions, heat stable media ingredients and water (Tables 1, 2, 3 and 4) in a large vessel, which can be autoclaved. Heating the solution to boiling point in a microwave is recommended to help eliminate dissolved oxygen. After boiling, use a gassing probe/needle to purge the solution with food grade CO_2 while the medium cools. A reducing agent (e.g. cysteine HCl) can be added after gassing for at least 0.5 h (or when the redox dye such as resazurin has become colourless) and seal the vessel with a stopper that will prevent the entry or escape of gas from the bottle if the medium is to be autoclaved (15 min at 100 kPa, 121°C) in the preparation vessel. Other reducing agents are shown in Table 2. Reducing agents such as Na_2S should be added to culture tubes immediately before inoculation rather than during medium preparation. The most commonly used dyes to indicate oxidation–reduction state in media are resazurin and indigo carmine, which become colourless in the reduced state. The stoppered bottle can also be immediately transferred to a anaerobic hood (chamber) where it is aliquoted into smaller bottles or culture tubes equipped with gas-impermeable butyl rubber stoppers and then seal with a special crimper tool and aluminium seal (Bellco Glass Inc., Vineland, New Jersey, USA) before autoclaving. It is preferable to autoclave large volumes of medium in vessels, which are coated with a membrane (Schott Glas, Germany) that prevents shattering due to explosion or implosion during autoclaving. Filter-sterilized B-vitamins [18] can be added to melted agar medium and broth just prior to use for inoculation.

A more recent development in anaerobic microbiology has been the use of the flexible plastic anaerobic glove box chamber (Coy Laboratory products, Inc., Ann Arbor, Michigan), which is more convenient than the roll tube method of isolating anaerobic bacteria. The advantage of using an anaerobic chamber is that standard microbiological techniques can be used including agar spread plates, replica plating and dispensing of media in an anaerobic environment. However, media should be prepared using anaerobic techniques prior to transferring to an anaerobic hood for dispensing into plates or culture tubes. Media should not be made under aerobic conditions and placed in an anaerobic glove box to equilibrate.

Conventional techniques for enumeration of rumen bacteria, protozoa and fungi

The design of animal trials and rumen sampling methodology for quantitative analysis of microbial populations is discussed in Chapter 1.1 by A.R. Egan. For the purposes of this chapter, a representative rumen digesta sample (50 g) for analysis of microbial populations is collected from the rumen.

Bacteria

The rumen sample taken for bacterial counts is immediately transferred to an anaerobic hood containing a gas phase of CO_2/H_2. A subsample (5 g) is diluted 1 : 10 with cold anaerobic diluent (Table 1) and processed for 1 min with a homogenizer (Bamix,

Table 1. Media recipes widely used for the non-selective isolation and selective isolation and enumeration of culturable ruminal bacteria as well as recipes for the selective isolation of rumen fungi and bacteria based on hydrolytic properties (adapted from Stewart et al. [28] and McSweeney et al. [23])

	Non-selective medium					Selective medium						
	RGCA[a] [11]	Med10[b] [4]	Med2[c] [10]	HS[d] [13]	Anaerobic diluent	Total cultur-able	Xylanolytic	Cellulolytic	Proteolytic	Pectinolytic	Amylolytic	Fungi
Clarified rumen fluid (ml)	30	–	20	–	–	30	–	–	–	–	–	20
Depleted clarified rumen fluid (ml)						–	30	30	30	30	30	–
Peptone	–	–	–	–	–	–	–	–	–	–	–	–
Casitone	–	–	1.0	–	–	–	–				–	
Trypticase	–	0.2	–	–	–	0.05	0.05	0.05	–	0.05	–	0.05
Yeast extract	–	0.05	0.25	–	–	0.05	0.05	0.05	0.05	0.05	–	0.05
Soluble starch	0.05	0.05	–	–	–	0.05	–	–	0.05	–	0.25	0.07
Sodium lactate (70% w/v)	–	–	1.0	–		0.2	–	–	–	–	–	–
Glucose	0.025	0.05	0.20	–	–	0.05	–	–	0.05	–	–	0.03
Maltose	–	–	0.20	–	–	0.05	–	–	0.05	–	–	–
Cellobiose	0.025	0.05	0.20	–	–	0.05	–	–	–	–	–	0.03
Cellulose (ball-milled)	–	–	–	0.3	–	–	–	0.2	–	–	–	–
Xylan (birchwood/oat spelt)	–	–	–	–	–	0.05	0.2	–	–	–	–	0.07
Casein (soluble)	–	–	–	–	–	1.0	—-	–	1.0	–	–	–
Pectin	–	–	–	–	–	0.05	–	–	–	0.2	–	–
Xylose	–	–	–	–	–	0.05	– –	–	0.05	–	–	–
$(NH_4)_2SO_4$	0.1	–	–	–	–	–	–	–	–	–	–	–

Table 1. Contd...

	Non-selective medium					Selective medium						
	RGCA[a] [11]	Med10[b] [4]	Med2[c] [10]	HS[d] [13]	Anaerobic diluent	Total cultur-able	Xylanolytic	Cellulolytic	Proteolytic	Pectinolytic	Amylolytic	Fungi
$NaHCO_3$	–	–	0.4	0.5	–	0.8	0.6	0.8	0.6	0.6	0.6	0.8
Na_2CO_3 solution (8%) (ml)	–	5.0	–	–	5.0	–	–	–	–	–	–	–
Solution no. (Table 3) and volume (ml)	(1) 50	(2) 3.8	(4) 15	(4) 16	(2) 3.8	(4) 15	(4) 15	(4) 15	(4) 15	(4) 15	(4) 15	(4) 15
Solution no. (Table 3) and volume (ml)		(3) 3.8	(5) 15	(5) 16	(3) 3.8	(5) 15	(5) 15	(5) 15	(5) 15	(5) 15	(5) 15	(5) 15
Trace elements (ml, Table 4)				0.1								
Pfenning trace elements (ml, Table 4)						0.1	0.1	0.1	0.1	0.1	0.1	0.1
VFA mix 1 (ml)[e]	–	0.31	–	–		0.31	–	0.31	–	–	–	0.31
VFA mix 2 (ml)[f]	–	–	–	2.0	–	–	–	–	–	–	–	
Haemin (mg)[g]	0.5	1.0	–	100 μl	–	1.0	–	1.0	–	–	–	1.0
Vitamin solution[h]	–	–	–	100 μl		–	–	–	–	–	–	–
Vitamin K_1 (mg)	0.1	–	–	–		–	–	–	–	–	–	–
L-Cysteine HCl-H_2O[i]	0.05	0.025	0.05	0.02		0.05	0.05	0.05	0.05		0.05	0.05

$Na_2S{\cdot}9H_2O$[i]	–	0.025	–	0.1	–	–	–	–	–		–	0.025
Resazurin (mg)	0.1	0.1	0.1	1.2	–	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Indigo carmine solution (0.05%) (ml)					1.0	–	–	–	–	–	–	–
Agar	2.0	2.0	2.0	–		2.0	2.0	2.0	2.0	2.0	2.0	2.0
Antibiotics (ml)[j]												0.5

Ingredients in g unless otherwise indicated. Distilled H_2O added to final volume of 100 ml.

[a] Holdeman et al. [11].

[b] Caldwell and Bryant [4], cited in Holdeman et al. [11]

[c] Hobson [10]

[d] Hungate and Stack [13]

[e] VFA mix 1 : 17 ml acetic acid; 6 ml propionic; 4 ml *n*-butyric; and 1 ml each of *n*-valeric, *iso*valeric, *iso*butyric and 2-methylbutyric acid.

[f] VFA mix 2 : 9.845 g sodium acetate; 139 μl *iso*butyric acid; 163 μl 2-methylbutyrate; and 160 μl each of *n*-valerate and *iso*valerate made up to 100 ml with distilled H_2O.

[g] Haemin: Dissolve 50 mg haemin in 1 N NaOH; make up to 100 ml with distilled H_2O. Autoclave at 121°C for 15 min.

[h] Vitamin solution: 1 mg each biotin and cobalamin; 3 mg PABA; 5 mg folic acid; 15 mg pyridoxamine made up to 100 ml with distilled H_2O. After autoclaving the medium, add 100 μl (per 100 ml medium) of a solution containing 5 mg each thiamine and riboflavin per 100 ml distilled H_2O.

[i] When added together, prepared as a single concentrated solution in dilute alkali, pH around 10 [9].

[j] Benzyl penicillin (12 mg/ml) and streptomycin sulphate (2 mg/ml) dissolved in distilled water (previously boiled and bubbled with nitrogen until cooled) and filter-sterilize whilst being gassed with nitrogen.

Table 2. Reducing agents for anaerobic media

Compounds	E_0' (mV)	Concentration in media
Cysteine HCl[a]	−210	0.025%
Dithiothreitol[a]	−330	0.05%
H_2 + palladium chloride [1]	−420	
Titanium III citrate[b] [34]	−480	0.5–2 mM
Titanium III nitrilotriacetate[c] [25]		>30 μM
$Na_2S{\cdot}9H_2O$[a] (or H_2S)	−571	0.025%

[a]Stock solutions may be autoclaved and stored under anaerobic gas.
[b]Add 5 ml of 15% titanium trichloride solution to 50 ml of 0.2 M sodium citrate and neutralize with a saturated sodium bicarbonate solution. Filter, sterilize and add 30 ml/l of sterile oxygen-free medium in the appropriate concentration. The titanium III citrate complex is blue-violet when reduced and colourless when oxidized.
[c]Nitrilotriacetic acid (NTA, free acid 9.6 g) is added to 300 ml of anaerobic water (gassed with N_2). The pH is adjusted to 9.0 with concentrated NaOH. A total of 9.6 ml of 20% $TiCl_3$ is added slowly to the chelator solution (NTA). To prevent precipitation, the pH is kept above 2.0 by the addition of an anaerobic, saturated solution of $NaCO_3$. (Use of NaOH at this stage will precipitate the titanium.) The final pH is adjusted to 7.0 with $NaCO_3$, and the final volume was adjusted to 500 ml with anaerobic water. The Ti(III) NTA reductant is filter-sterilized into a sealed serum vial containing N_2 gas. The final solution is a slight bluish-green in colour, in contrast to the blue-violet colour of a Ti(III) citrate solution. The final component concentrations are 25 mM Ti(III) and 100 mM NTA.

Mettlen, Switzerland; Ultra-turrax Homogenizer, Janke and Kunkel, Germany), serially diluted in anaerobic diluent [19] and aliquots (20 μl) of 10^{-5}–10^{-8} dilutions are inoculated in triplicate onto agar selective media [20]. Media used in agar plates to enumerate total culturable, cellulolytic, xylanolytic, pectinolytic and proteolytic bacteria are described in Table 1. Rumen fluid used in agar media is pre-incubated to deplete fermentable substrates [16]. Control plates, which contain the same media constituents minus the selective nutrient, are also inoculated for each functional group of bacteria. All colonies growing on the selective media plates are counted after 5–10 d when they were larger than or at higher concentration than those on

Table 3. Mineral solution ingredients

	Solution number				
Mineral (g/1 distilled H_2O)	1	2	3	4	5
$CaCl_2$	0.2	–	–	–	0.6
$CaCl_2{\cdot}2H_2O$	–	–	1.6	–	–
$MgSO_4$	0.2	–	–	–	–
KH_2PO_4	1.0	6.0	–	3.0	–
KH_2PO_4	–	–	6.0	–	3.0
$NaHCO_3$	10.0	–	–	–	–
NaCl	2.0	–	12.0	–	6.0
$(NH_4)_2SO_4$	–	–	6.0	–	6.0
$MgSO_4{\cdot}7H_2O$	–	–	2.5	–	0.6

Table 4. Trace mineral solution ingredients

Mineral (mg/l distilled H_2O)	Trace mineral solution	Pfenning trace mineral solution
H_3BO_3	61.8	300
$ZnCl_2$	68.1	–
$ZnSO_4 \cdot 7H_2O$	–	100
$MnCl_2$	98.9	–
$MnCl_2 \cdot 4H_2O$	–	30
$CoCl_2$	95.2	–
$CoCl_2 \cdot 6H_2O$	–	20
Na_2MoO_4	36.3	–
$Na_2MoO_4 \cdot 2H_2O$	–	30
$Na_2S_iO_3$	21.2	–
$Na_2S_eO_3$	17.3	10
$NiCl_2$	12.9	20
$CuCl_2 \cdot 2H_2O$	–	10
Na_2WO_4	3.3	–
$FeCl_2 \cdot 4H_2O$	–	150
$Al_2(SO_4)_3$	–*	–

Note: Dissolve ingredients in 100 ml (0.25 M) HCl and then make up to 1 l distilled H_2O.
*One microlitre solution of 6.6 mg/1 distilled H_2O.

the control plates. Cellulolytic colonies are identified by zones of cellulose-clearing in the opaque agar media. The agar can be stained with Congo red to aid in the visualization of clearing zones (see below). Proteolytic colonies are counted as those with clearing zones after the casein agar plate is flooded with 1 M HCl. Cellulolytic bacteria can also be enumerated in broth medium using the most probable number (MPN) procedure described by Dehority et al. [7] and summarized below. The cellulose broth medium is the same as that used in the cellulose agar medium (Table 1) except trypticase and yeast extract are omitted and the ball-milled filter paper cellulose (Whatman No. 1, Kent, England; Sigmacell-20; Sigma Chemical Co., St. Louis, MO, USA) is replaced with either four pieces of cotton thread (1 mm diameter and 50 mm long; GIMP soft cotton; Coats, Brisbane, Australia) or four discs (5 mm diameter) of acid swollen filter paper (Whatman No. 1) [26] or both (see method below). After incubation for 5–10 d, tubes are scored positive for cellulolytic activity based on increased turbidity of the culture fluid from growth of cells and visual loss (>30%) of cellulose.

The techniques used to quantify cellulolytic populations often yield variable results. Cellulolytic bacteria have been enumerated on selective media plates with ball-milled cellulose as the substrate [15, 24]. However, we have found that this method also grows bacteria, which were not cellulolytic and other experiments (McSweeney, unpublished data) have shown that many strains of cellulolytic bacteria, such as *Fibrobacter succinogenes*, growing in broth culture will not grow on or within this type of selective agar medium. In our laboratory, the MPN procedure [7] based on cellulose degradation in broth culture was a more reliable method for enumerating cellulolytic bacteria, and acid swollen cellulosic filter paper supported the growth of

a larger population of cellulolytic bacteria than cotton thread. Cotton thread tends to select for *Fibrobacter* bacteria, whereas cellulosic filter paper is readily digested by *Ruminococcus* populations as well [29]. Therefore, enrichments with cotton thread may underestimate the total cellulolytic population.

Protozoa and fungi

Rumen samples collected for enumeration of protozoa and fungi are not homogenized prior to dilution. Direct counts of protozoa can be made from rumen digesta samples that are initially diluted 1 : 1 with 10% formal saline [22]. The preserved sample is diluted 1 : 10 with anaerobic diluent solution containing 30% glycerol (vol/vol) and a dye (e.g. methylene blue) counted using a glass counting chamber (Fuchs-Rosenthal, Weber, England; Sedgwick-Rafter). Counts of each sample are made from at least 32 fields of view in two chambers that were filled separately. A detailed procedure for counting total protozoal numbers and species identification of rumen ciliate protozoa is available in the Laboratory Manual written and illustrated by Dehority [6].

The total number of rumen anaerobic fungi are determined by counting colonies (thallus forming units, TFU) that formed from zoospores and thalli in roll tubes (3 ml agar medium in 25 ml Balch tube) using the technique of Joblin [14] as follows. A rumen digesta sample is taken and serial, 10-fold dilutions made in anaerobic diluent. For each rumen sample, a 0.2 ml aliquot of 10^{-1}–10^{-3} dilutions are inoculated into triplicate roll tubes of fungal medium. The fungal medium (Table 1) contains penicillin G (1.2 mg/ml), streptomycin sulphate (0.2 mg/ml) and chloramphenicol (25 μg/ml) to inhibit bacterial growth. Colonies are counted after incubation at 39°C for 5 d. A modification of this procedure is used to enumerate those fungi which are fibre degrading. The fibrolytic fungal population is quantified as TFU using a procedure developed by Theodorou et al. [32], which is based on an end point dilution procedure and the MPN technique. Rumen samples are diluted into culture medium containing antibiotics as described previously and inoculated into triplicate tubes of cellulose broth medium which contains ground plant material with a high fibre content (e.g. crop residue or grass hay; 50 mg/10 ml) rather than pure cellulose as substrate. Tubes are scored positive when there is visual evidence of colonization and degradation of the plant material by the fungi.

Most probable number enumeration procedure

The MPN method is laborious and statistically inefficient, but it is an advantage if the bacteria cannot be cultivated or grow poorly on solidified media. The number of viable microbial cells can be estimated by the MPN procedure, which involves the mathematical calculation of the viable cells in a sample based on the number of cultures that fail to show growth in a dilution series of tubes containing a specific broth medium for the growth of a particular organism. The method involves taking several replicate (at least 3–5 replicates per dilution and termed 3–5 tube MPN) dilutions and recording the number of tubes at each dilution which support growth until a dilution range is reached where there is no growth in any of the replicates. The number of viable

cells is calculated from MPN tables [31] that use statistical methods to combine data from different dilution levels when a specified number of tubes is run at each level. MPN table calculators can be downloaded from the Internet as free software packages.

Storage of cultures

The majority of rumen bacteria can be stored in anaerobic diluent at −70°C for at least several years. One reliable method is to prepare an anaerobic storage medium, which is comprised of (by volume) 20% each of solution 2 and 3 (Table 1), 30% glycerol and 30% water. This medium (3 ml) is dispensed into 10 ml glass serum bottles under anaerobic conditions, stoppered and autoclaved. Cultures to be stored are grown to mid log phase growth and then 4 ml of culture is added to the serum bottles containing the storage medium and immediately placed in a −70°C freezer. Deep-frozen cultures are recovered by rapid thawing in water at 32–35°C [9].

Assays for major functional microbial groups in rumen

The major nutritional components of conventional ruminant diets outside of North America are fibre (pectin, cellulose and xylan) and protein, which are degraded by enzymes from the predominant micro-organisms in the rumen. Enzyme assays are routinely used to quantify the cellulase, xylanase and proteolytic activity of pure and mixed cultures of organisms as well as total activity in crude digesta samples taken from the rumen.

Preparation of microbial cells for assays

Cell pellets are collected from cultures or a rumen fluid sample by centrifugation at 10 000 *g* for 5 min. The supernatant fluid is harvested and 1 M sodium phosphate buffer (pH 6.5) is added to give a final concentration of 50 mM (extracellular fraction). Residual culture supernatant fluid is removed from the cell pellet with a pipette. The pelleted cells are resuspended in 0.5 ml 50 mM sodium phosphate buffer (pH 6.5) by vortexing and can be lysed by either sonication or passage through a French pressure cell. Cell and extracellular fractions can be stored at −20°C until use, but should be assayed as soon as possible after preparation to avoid loss of activity.

Cellulase and xylanase assays

Cellulase assays can also be performed using the following substrates: 0.4% (w/v) carboxymethyl cellulose (CMC) (low viscosity), 0.05% (w/v) *p*-nitrophenyl cellobioside and 1% (w/v) amorphous cellulose which are supplied by Sigma (Sigma). Acid swollen cellulose and ^{14}C labelled cellulose can also be used as substrates (see procedure for preparation of these substrates below). The procedure used with Avicel as the substrate is as follows: an Avicel solution is initially washed by the addition of a

1/2 vol of H_2O to a 1% Avicel suspension and then centrifuged to remove any soluble cellulose. A typical reaction which is performed against the 1% Avicel contains 100 μl of ice-cold 200 mM Na-citrate pH 6.0, 250 μl of H_2O, X μl of enzyme and made up to a total volume of 400 μl with BSA (1 mg/ml). Samples are taken at appropriate time points for at least 2 h and are always removed by first centrifuging the Avicel solution and then removing 30 μl of reaction mixture. The digestion reaction is then resuspended and returned to the 39°C shaking incubator at 225 rpm for further hydrolysis.

Cellulase assays are also performed over an extensive pH and temperature range. Assays for evaluating the pH profile of enzymes can be performed using 50 mM Na-citrate buffer for a pH range of 4–7 and 50 mM Tris NaCl for a pH range of 7.5–10. Assays for a temperature profile are performed in 50 mM Na-citrate (pH 6.0) at a temperature range of 25–70°C. Rate of hyrolysis of cellulose is measured using a reducing sugar assay (see below).

Xylanase activity against 0.5% oat spelt xylan or birchwood xylan (Sigma) is carried out essentially as mentioned above for the cellulase assay. Xylanase activity is determined by measuring reducing sugar release from xylan substrate.

Reducing sugar assay

Reducing sugar assays are often performed according to the method described by Lever [17]. A typical assay requires a set of sugar standards based on the reducing sugar which is to be detected, i.e. glucose for Avicel, CMC and MUC; and xylose when xylan is used as a substrate. Thirty microlitre of digest is mixed with 20 μl of distilled H_2O and 150 μl of *p*-hydroxybenzoic acid hydrazide (PAHBAH) working solution, which is prepared from a 5× stock solution (2 g PAHBAH, 0.39 g $CaCl_2{\cdot}2H_2O$ in 40 ml 0.5 M HCl) by addition of 4 volumes of 0.5 M NaOH. The mixture is heated in a boiling water bath for 8 min. After cooling, the tubes are centrifuged and optical density measured in a spectrophotometer at 410 nm.

All enzyme assays are performed at least in triplicate. One unit of cellulase or xylanase activity is defined as the amount that released 1 μmol reducing sugar equivalents/min) from the cellulose or xylan substrate. Enzyme activity can also be expressed per unit of cell protein in the assay. Protein concentrations are determined by using a commercial kit (Pierce, Rockford, USA) such as the microbicin-chrononic acid assay kit [27].

Plate screening for cellulases and xylanases

Screening for enzyme activity from pure cultures or rumen fluid can be performed by plating cells (or rumen fluid) onto 0.8% (w/v) agarose plates containing 0.2% (w/v) CMC or 0.2% (w/v) oat spelt xylan/birchwood xylan. While both forms of xylan are often used as substrates in xylanase assays, it should be noted that oat spelt xylan is less soluble and more heterogenous than birchwood xylan in terms of composition and linkages. After a 12 h incubation at 39°C, the plates are stained for 10 min with 0.3% (w/v) Congo red for CMC-containing and 0.5% (w/v) Congo red

for xylan-containing plates [30]. The zones of hydrolysis are then revealed by destaining the plates with 1 M NaCl for 10 min at 25°C. Total crude, periplasmic and pure enzymes are screened for their activity by coring a well into the substrate agarose plates [(1% (w/v) agarose, 1% (w/v) substrate (CMC or xylan) in 50 mM Na-citrate (pH 6.0)] and applying 5–10 μl of enzyme into the well. The plates are then incubated at 39°C for 4–12 h and stained with Congo red and washed as mentioned above.

Swollen cellulose preparation

Preparation of H_3PO_4-swollen cellulose (Avicel) and filter paper discs (Whatman No. 1) is performed according to the methods described by Wood [33] and Rasmussen et al. [26], respectively. Avicel or filter paper discs (20 g) prepared with a paper punch are incubated on ice in 100 ml H_3PO_4 for 1 h with occasional stirring. The mixture is then transferred to 8 l of ice-cold distilled H_2O and left on ice for 30 min. The swollen cellulose product is then washed several times with cold distilled H_2O by decantation and then with 1% (w/v) $NaHCO_3$ solution. Finally, a 1% (w/v) suspension of swollen cellulose in distilled H_2O is adjusted to a pH of 6.5–7.0 with 1 N NaOH. The swollen cellulose is then collected by centrifugation at 1000 *g* for 30 min. To estimate the dry weight of H_3PO_4-swollen cellulose, a 0.5 g wet sample is dried in an oven (at 170°C) and re-weighed.

Preparation of [U-^{14}C] cellulose

This technique is based on the method developed by du Preez and Kistner [8] and employs *Acetobacter xylinum*, which synthesizes cellulose during growth and forms a pellicle on the surface of a broth culture. To maintain the organism, transfer the culture at monthly intervals by removing the whole pellicle with a sterile loop and placing it in a fresh broth of GluPPY medium. Cap the tube tightly and shake briefly before removing the pellicle. Incubate the loosely capped tube at about 25°C.

The method for synthesis of [U-^{14}C] cellulose is as follows:

1. Dispense 150 ml sterile Glucose-Phosphate-Peptone-Yeast (GluPPY) medium, pH 6.3 [(g/l): yeast extract (Difco), 5; Peptone (Difco), 5; KH_2PO_4, 6.8; Glucose, 10] into disposable plastic tissue culture flasks with surface area of about 175 cm^2 (e.g. cat. No. 1–56502; A/S Nunc, Kamstrup, DK 4000 Roskilde, Denmark).
2. Dilute required amount of D-[U-^{14}C]-glucose into sufficient glass distilled water to yield 1 ml of glucose solution for each flask. Filter this solution into a sterile container using a sterile membrane with 0.2 μm pores. Aseptically dispense 1 ml of filtered glucose solution into each flask.
3. Immediately after dispensing medium and radiolabel into flasks, inoculate each with a single pellicle from a 5 ml culture that has been incubated for 7–10 d. Incubate flasks in the flat position with loosened caps at 30°C for 17–18 d.
4. Remove pellicles from the flasks and wash with distilled water until the washes are clear. Boil the pellicles in 2 M KOH for 20 min and wash them with distilled water until the washes are neutral. Mascerate in suitable small quantities in a kitchen

blender at high speed for 30 s. Recover the ^{14}C-cellulose after freeze-drying the slurry or use as fresh material in assays.

Assay for total cellulase activity in growing cultures of rumen inoculum using ^{14}C-cellulose

Medium preparation

- In the anaerobic cabinet pipet 3.0 ml of well-blended ^{14}C-cellulose pellicle material into Balch tubes. Ensure the suspension is well mixed during this operation and use cut pipette tip to prevent straining.
- Add 6.0 ml basal medium containing 30% clarified rumen fluid prepared according to medium formulation, stopper, seal and autoclave.
  NB: Pink (oxidized) colour disappears prior to stoppering in the cabinet.
- Weigh out exactly 10–12 g of rumen sample into 300 ml beaker in cabinet and dilute exactly 1/10 with chilled diluent i.e. by weight. Blend for 1.0 min with bar mixer (clean between samples).
- Pipet 1.0 ml into diluent tube (9.0 ml amount) i.e. this is 10^{-2} dilution, using wide-mouth tip, stopper, crimp seal and remove from cabinet
- Prepare remainder of dilution series on bench using syringe and 18 gauge needles. Fresh syringe and needle for each dilution.
- Use 1.0 ml of 10^{-3} dilution to inoculate marked tubes of ^{14}C-cellulose medium. Do this in triplicate and record t_0.

Sampling protocol for ^{14}C-activity

- Adjust sampling times based on cellulase activity in samples. For example, sample at 8, 13, 17, 20, 23, 26, 29, 32 and 36 h after t_0. Optimize sampling schedule to get as many points on the linear increase portion of curve. Use 8.0 h sample as background (normally 20–250 cpm).
- Remove 0.5 ml sample from each tube with 1.0 ml syringe and 18 gauge needle. Ensure sample is homogenous with that in tube. Use new syringe and needle for each sample.
- Centrifuge at 14 000 *g* for 15 min to pellet residual cellulose.
- Remove 400 μl supernatant taking care not to disturb pellet.
- Add 2.5 μl INSTAGEL (Perkin Elmer, USA) to screw-cap counting vial. Close and store in cold/dark until sampling complete i.e. until no residual cellulose.
- Count in scintillation counter: 2 cycles (5 min counting time).
- Mean triplicates for each time and plot.

Specific activity of ^{14}C-cellulose

1. Do DM determinations on tubes of prepared medium or ^{14}C-cellulose suspension (n = 10).
2. Do radioactivity measurements on acid-hydrolyzed cellulose suspension. Calculate cpm/mg cellulose.
3. Use max release of activity from live assay as an approximate analysis for this.

Amylase activity assay

Amylase activity is measured as an indication of starch degrading capacity. A modification of the dextrinogenic assay is used to estimate amylase activity [21]. A 1% solution of soluble starch is prepared in 0.1 M acetate buffer (pH 4.6). To each of two test tubes is added buffered starch substrate (5 ml), 0.1 M acetate buffer (pH 4.6, 3 ml) and 0.5 M $CaCl_2$ (1 ml). A third tube is used as a reagent blank. All tubes are equilibrated at 39°C, enzyme is added to one of the substrate tubes and incubated at 39°C for a further 10 min and then 1 N HCl (2 ml) is added to all three tubes. Enzyme is then added to the reagent blank and the undigested starch control tube. After thorough mixing, an aliquot (0.2 ml) from each tube is placed in volumetric flasks (50 ml) containing 1 N HCl (0.5 ml) and distilled H_2O (40 ml). Colour is developed in each flask by the addition of an iodine solution (0.1 ml; 3% KI or 0.3% I_2). Absorbance of the resultant blue solutions is measured at 620 nm.

One unit of alpha amylase activity is that amount of protein which will hydrolyse 10 mg starch per min. Specific activity is expressed as units per mg protein.

Protease activity assays

Cells for protease assays are separated from culture fluid by centrifugation (7000 *g* for 20 min at 4°C), washed and suspended in 0.1 M Bis-tris (*bis* [2-hydroxy-ethyl]imino-tris[hydroxymethyl]methane, pH 7) and re-centrifuged as described above. The cell pellet is resuspended in 0.01 its original volume and disrupted twice by ultrasonication with an ultrasonic disintegrator (Ultrasonic Industries Pty Ltd, Sydney) at 60 W for 10 min at a time. Centrifuged culture fluid was also assayed for protease activity.

Proteolytic activity in cell associated and extracellular fractions of cultures can be determined spectrophotometrically with azocasein as the substrate [2]. Controls are performed by incubating enzyme samples and the azocasein substrate separately and by combining these solutions at the time of acid addition as described by Cotta and Hespell [5]. One proteolytic enzyme unit equalled 1 μg of azocasein digested per h at 39°C. Extracellular proteolytic activity can be expressed per ml of culture fluid per unit absorbance of culture prior to centrifugation. Cell associated proteolytic activity can be expressed per g protein. Enzyme activities in culture fluid can be expressed per mg of microbial protein.

References

1. Aranka, A., and R. Freter. 1972. Use of anaerobic glove boxes for the cultivation of strictly anaerobic bacteria. *Am. J. Clin. Nutr.* **25:**1329–1334.
2. Brock, F.M., C.W. Forsberg, and J.G. Buchanan-Smith. 1982. Proteolytic activity of rumen microorganisms and effects of proteinase inhibitors. *Appl. Environ. Microbiol.* **44:**561–569.
3. Bryant, M.P. 1972. Commentary on the Hungate technique for culture of anaerobic bacteria. *Am. J. Clin. Nutr.* **25:**1324–1328.
4. Caldwell, D.R., and M.P. Bryant. 1966. Medium without rumen fluid for nonselective enumeration and isolation of rumen bacteria. *Appl. Microbiol.* **14:**794–801.

5. Cotta, M.A., and R.B. Hespell. 1986. Proteolytic activity of the ruminal bacterium *Butyrivibrio fibrisolvens*. *Appl. Environ. Microbiol.* **52:**51–58.
6. Dehority, B.A. 1993. *Laboratory Manual for Classification and Morphology of Rumen Ciliate Protozoa*. CRC Press, Boca Raton, FL.
7. Dehority, B.A., P.A. Tirabasso, and A.P. Grifo. 1989. Most-probable-number procedures for enumerating ruminal bacteria, including the simultaneous estimation of total and cellulolytic numbers in one medium. *Appl. Environ. Microbiol.* **55:**2789–2792.
8. du Preez, P., and A. Kistner. 1986. A versatile assay for total cellulase activity using U-[^{14}C] labelled bacterial cellulose. *Biotechnol. Lett.* **8:**581–586.
9. Hespell, R.B., and M.P. Bryant. 1981. The genera Butyrivibrio, Succinivibrio, Lachnospira and Selenomonas, pp. 1479–1494. *In* M.P. Starr, H. Stolp, H.G. Truper *et al.* (eds.), *The Prokaroytes: A Handbook on Habitats, Isolation and Identification of Bacteria*, Vol. 2. Springer Verlag, Berlin.
10. Hobson, P.N. 1969. Rumen bacteria, pp. 139–149. *In* J.R. Norris and D.W. Ribbons (eds.), *Methods in Microbiology*, Vol. 3B. Academic press, London and New York.
11. Holdeman, L.V., E.P. Cato, and W.E.C. Moore. 1977. *Anaerobe Laboratory Manual*. Virginia Polytechnic Institute, Blacksburg.
12. Hungate, R.E. 1969. A roll tube method for cultivation of strict anaerobes, pp. 117–132. *In* I.R. Norris and E.W. Ribbons (eds.), *Methods in Microbiology*, Vol. 3. Academic Press, New York.
13. Hungate, R.E., and R.J. Stack. 1982. Phenylpropanoic acid: Growth factor for *Ruminococcus albus*. *Appl. Environ. Microbiol.* **44:**79–83.
14. Joblin, K.N. 1981. Isolation, enumeration, and maintenance of rumen anaerobic fungi in roll tubes. *Appl. Environ. Microbiol.* **42:**1119–1122.
15. Kistner, A. 1960. An improved method for viable counts of bacteria of the ovine rumen which ferment carbohydrates. *J. Gen. Microbiol.* **23:**565–576.
16. Leedle, J.A.Z., and R.B. Hespell. 1980. Differential carbohydrate media and anaerobic replica plating technique in delineating carbohydrate utilising subgroups in rumen bacterial populations. *Appl. Environ. Microbiol.* **39:**709–719.
17. Lever, M. 1972. A new reaction for colorimetric determination of carbohydrates. *Anal. Biochem.* **47:**273–279.
18. Lowe, S.E., M.K. Theodorou, A.P.J. Trinci, and R.B. Hespell. 1985 Growth of anaerobic rumen fungi on defined and semi-defined media lacking rumen fluid. *J. Gen. Microbiol.* **131:**2225–2229.
19. Mackie, R.I., F.M.C. Gilchrist, A.M. Robberts, P.E. Hannah, and H.M. Schwartz. 1978. Microbiological and chemical changes in the rumen during the stepwise adaption of sheep to high concentrate diets. *J. Agric. Sci.* (Cambridge) **90:**241–254.
20. Mackie, R.I., and C.A. Wilkins. 1988. Enumeration of anaerobic bacterial microflora of the equine gastrointestinal tract. *Appl. Environ. Microbiol.* **54**:2155–2160.
21. Manning, G.B., and L.L. Campbell. 1961. Thermosatable α-amylase of *Bacillus stearothermophilus*. *J. Biol. Chem.* **236**:2952–2957.
22. McSweeney, C.S., M.A. Pass, and P. Henry. 1983. Changes in rumen contents associated with lantana poisoning of sheep. *Comp. Biochem. Physiol.* **75C:**361–367.
23. McSweeney, C.S., B. Palmer, R. Bunch, and D.O. Krause. 2001. Effect of the tropical forage calliandra on microbial protein synthesis and ecology in the rumen. *J. Appl. Microbiol.* **90**:78–88.
24. Meyer, J.H.F., and R.I. Mackie. 1986. Microbiological evaluation of the intraruminal in sacculus digestion technique. *Appl. Environ. Microbiol.* **51:**622–629.
25. Moench, T.T., and J.G. Zeikus. 1983. An improved preparation method for titanium (III) media reductant. *J. Microbiol. Methods* **1:**199–202.
26. Rasmussen, M.A., R.B. Hespell, B.A. White, and R.J. Bothast. 1988. The inhibitory effects of methylcellulose on cellulose degradation by *Ruminococcus flavefaciens*. *Appl. Environ. Microbiol.* **54:**890–897.
27. Smith, P.K., R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, and D.C. Klenk. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**:76–85.
28. Stewart, C.S., H.J. Flint, and M.P. Bryant. 1997. The rumen bacteria, pp. 10–72. *In* P.N. Hobson and C.S. Stewart (eds.), *The Rumen Microbial Ecosystem*. Chapman and Hall, London.

29. Stewart, C.S., C. Paniagua, D. Dinsdale, K.-J. Cheng, and S.H. Garrow. 1981. Selective isolation and characteristics of *Bacteroides succinogenes* from the rumen of a cow. *Appl. Environ. Microbiol.* **41:**504–510.
30. Teather, R.M., and P.J. Wood. 1982. Use of Congo-red–poysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Appl. Environ. Microbiol.* **43:**777–780.
31. Thatcher, F.S., and D.S. Clarke. 1978. Coliform bacteria, pp.128–139. *In* F.S. Thatcher and D.S. Clarke (eds.), *Microorganisms in Food, Their Significance and Methods of Enumeration*, Vol. 1, 2nd ed. University of Toronto Press, Toronto.
32. Theodorou, M.K., M. Gill, C. King-Spooner, and D.E. Beever. 1990. Enumeration of anaerobic chytridiomycetes as thallus-forming units: Novel method for quantification of fibrolytic fungal populations from the digestive tract ecosystem. *Appl. Environ. Microbiol.* **56:**1073–1078.
33. Wood, T.M. 1988. Preparation of crystalline, amorphous, and dyed cellulase substrates. *Methods Enzymol.* **160:**19–23.
34. Zehnder, A.J.B., and K. Wuhrmann. 1976. Titanium (III) citrate as a non-toxic, oxidation-reduction buffering system for the culture of obligate anaerobes. *Science* **194:**1165–1166.

2.2. Bacteriophages

ATHOL V. KLIEVE
Queensland Department of Primary Industries and Fisheries, Animal Science Yeerongpilly, Queensland 4105, Australia

Introduction

Bacteriophages or phages are bacterial viruses and are present in the rumen in large numbers. They are obligate pathogens of bacteria and are ubiquitous to the rumen ecosystem. Bacteriophages are capable of lysing their bacterial hosts within the rumen and are therefore regarded as contributing to protein recycling within the rumen, a process identified as reducing the efficiency of feed utilization [5, 15, 18, 19]. However, their presence may not be entirely detrimental to the ecosystem, and it has been argued [22] that phages may also be involved in the maintenance of a balanced ecosystem and may play a role in recycling limiting nutrients within the rumen. Furthermore, phage therapy is enjoying a renaissance and the use of phages to control or eliminate detrimental or unwanted microbes from the gastro-intestinal tract, such as Shiga-toxin producing *E. coli* (food-borne disease), *Streptococcus bovis* (acidosis in grain-fed cattle) and methanogens (produce the greenhouse gas methane), is the focus of current investigation [4, 12, 13].

In order to be able to study the interaction between individual bacteriophages and their bacterial hosts, it is necessary to: (a) isolate the phage of interest from other viruses in the source material; (b) to derive stock cultures of known phage concentration; (c) store the isolated phages; and (d) determine basic physical characteristics, such as morphology. These procedures are achieved using classical microbiological procedures and this will be the methodology described in this chapter. It is also necessary to determine nucleic acid characteristics of the phage genome and to fingerprint the phage population in the rumen using molecular biological techniques. These will be described and discussed in Chapter 4.2.

Procedures for phage isolation and storage

Many procedures associated with the isolation, purification and storage of bacteriophages have been adapted from general texts such as Maniatis et al. [17] and modified to take into account the specific needs of the largely anaerobic host bacteria from the rumen.

H.P.S. Makkar and C.S. McSweeney (eds.), Methods in Gut Microbial Ecology for Ruminants, 39–46.
© 2005 *IAEA. Printed in the Netherlands.*

Steps in sample collection, handling and preparation

1. Collect 50 ml of liquid sample or 10 g of solid sample.

   (*Notes:* (a) Phages can be isolated from a wide variety of sources either liquid (e.g. ruminal fluid, abattoir kill-floor runoff, sewage effluent) or solid (e.g. faecal material, soil, compost, silage). (b) Appropriate safety considerations (wearing gloves, face mask, goggles and protective clothing; washing thoroughly and sterilizing used equipment) should be taken during sample collection and throughout phage isolation. Samples that contain phages are likely to contain other viruses that may be pathogenic to humans.)
2. If solid, elute phages present by mixing with an equal volume of phage storage buffer (PSB), mixing thoroughly and allowing to stand at room temperature for 30 min.

PSB: 2 ml 1 M Tris·HCl
20 ml 1 M NaCl
2 ml 1 M $MgCl_2$
0.1 g gelatin
Combine and add high purity water to 100 ml
Warm (50°C) to dissolve gelatin prior to dispensing into required volumes and sterilizing by autoclaving.

3. Centrifuge sample at 15 000 *g* for 15 min at 4°C to remove large particulate matter and bacteria. Retain supernatant on ice and discard pellet.
4. Filter supernatant through a 0.22 or 0.45 μm low-protein-binding membrane filter (e.g. durapore membrane, Millipore).

   (*Notes:* (a) Low-protein-binding membranes are essential as other filters can remove the majority of phages present in the sample [15, 21], particularly if the concentration of phages in the sample is expected to be low. (b) Filters of pore size 0.22 μm are best and result in a completely sterile filtrate. However, the rumen contains some very large bacteriophages [10] that may be excluded at this pore size. If all phages are to be the subject of investigation, then filtration at 0.45 μm is preferable, although it is theoretically possible that bacterial spores and mycoplasmas could pass through membranes of this pore size. In our experience, this has not been problematic.)
5. Filtrates are stored at 4°C until assayed for phages.

   (*Note:* In our experience, filtrates can be stored at 4°C for months or years without apparent loss of phage activity.)
6. If samples are to be used in enrichment procedures (see below), as opposed to direct plating, the samples need to be bubbled with anaerobic gas (CO_2 : H_2 – 95 : 5) for 30 min before use. Sample containers should then be sealed to maintain anaerobic conditions as much as possible and transferred to an anaerobic chamber immediately prior to use.

   (*Note:* Phages do not have a metabolism of their own and their viability is not affected by oxygen. The removal of oxygen from samples is to protect the host bacterium. This is generally not required when samples are directly plated, as the volume of sample plated is very small (10 μl).)

Steps in the soft-agar-overlay technique

The technique used for primary phage isolation, to enumerate phage particles, produce primary stocks of purified phages and establish host range is the soft-agar-overlay technique of Gratia [6], as described by Adams [3]. The method immediately below is for direct plating of phage sample.

1. Within an anaerobic chamber, prepare solid culture medium as agar plates (1.5% agar, approximately 15 ml of molten medium per Petri dish) of an anaerobic rumen fluid (RF) based medium, as detailed in Chapter 2.1.

   (*Note:* Most rumen bacteria will be grown and maintained in an RF based medium. If the prospective bacterial host is maintained in a specialized medium, then this medium is to be used throughout the soft-agar-overlay technique instead of RF.)
2. Under anaerobic conditions, prepare RF medium and dispense into 50 ml serum bottles containing 0.4 g of agar (final concentration is 0.8%). Seal serum bottles and ste-rilize by autoclaving. Prior to use re-melt agar and maintain at 50°C until required.
3. Prepare an overnight culture of the prospective host bacterium in RF broth. Allow 1 ml of culture per assay plate.
4. The remainder of this technique is performed within the anaerobic chamber.
5. Dispense 1 ml aliquots of bacterial culture into pre-sterilized Hungate (or boiling) tubes.
6. Add 10 μl of sample to the bacteria and mix. Allow to stand for 15 min for phages to attach to host cells.

   (*Note:* It is important that the time for attachment of phages to bacterial cells does not exceed 15 min as some phages reproduce very quickly and may complete a lifecycle resulting in the release of new phage particles if this time is exceeded, this is particularly problematic when enumerating phage particles.)
7. To each tube, dispense 3 ml of molten soft-agar (0.8%), mix with contents and immediately pour over a solid agar plate. Swirl to cover the solid agar and allow to set. Once set, the plates should be inverted, sealed with parafilm and incubated anaerobically for 16–24 h at 39°C.
8. After this time, the plates are examined for the presence of plaques, a circular clearing in an otherwise continuous lawn of bacteria.

   (*Note:* Plates of fast growing bacterial species can be examined at a shorter interval than stipulated, e.g. plaques on *E. coli* can be evident within 6 h of plating.)

Enrichment techniques prior to soft-agar-overlay

At times, phages are present at very dilute concentrations within environmental samples, and it is necessary to specifically enrich for them under these circumstances. Two methods are detailed below.

Specific enrichment [20]

1. An aliquot (0.2 ml) of filtered sample is added to a 5 ml broth culture of the host bacterium in the early stages of active growth (culture just visibly turbid) and the culture incubated at 39°C overnight.
2. The culture is centrifuged at 15 000 *g* for 15 min at 4°C to remove cells and cellular debris. The supernatant is retained and the pellet is discarded.
3. The supernatant is assayed for phage using the soft-agar-overlay technique.

Adsorption enrichment [16]

1. Within the anaerobic cabinet, 5 ml of sample is added to an equal volume of an overnight culture of the host bacterium.
2. The mixture is incubated at room temperature for 15 min to allow for phage adsorption prior to transfer to sealable centrifuge tubes that had been allowed to equilibrate to anaerobic conditions within the anaerobic chamber for a period of 2 d.
3. The centrifuge tubes are sealed, removed from the anaerobic chamber, centrifuged at 4000 *g* for 30 min and returned to the anaerobic chamber.
4. The supernatant is discarded and the pellet resuspended in 1 ml of RF broth.
5. Molten soft-agar is then added and the sample is plated as per the soft-agar-overlay technique from step 7.

Steps in phage purification and titration

1. From the primary isolation plate, a well-isolated plaque is selected and an agar plug from the centre of the plaque is removed using a wide-bore automatic pipette tip (200 μl).
2. The agar plug is placed into a microcentrifuge tube containing 500 μl PSB, briefly vortexed and allowed to stand at room temperature for 30 min.
3. From this tube, a series of dilutions at 10^{-1} increments to at least 10^{-6} are made in PSB (100 μl volumes are convenient).
4. From each dilution, 10 μl is plated with the bacterial host using the soft-agar-overlay technique.
5. At a dilution with very few plaques, a well-isolated plaque is again selected and 'picked' using a pipette tip.
6. Steps 1–5 are repeated a total of three times, after which the phage should be a pure isolate and free from contaminants. If there is any doubt (obviously differing plaque morphologies), then this procedure should be repeated.

Enumeration of phage is by titration. A series of dilutions of the sample are plated as in steps 3 and 4 above. At the dilution giving a readily countable number of plaques (50–100 is a good number), plaques are counted and by taking into account the volume of sample plated and the dilution that was counted the number or titre, of phages in the original sample are calculated. Numbers are expressed as plaque forming units (pfu).

Steps for preparation and storage of phage stocks

Once a phage is deemed pure, it is necessary to produce and store stocks for future use.

A. Primary stocks from soft-agar-overlays

1. Following the final plaque pick, in step 6 above, the plaque is again placed in PSB, a dilution series made and plated by soft-agar-overlay.
2. Following lysis, the dilution plate that is completely covered in plaques such that their edges just touch (where lysis is confluent) is selected.

   (*Note:* To achieve the highest titre of phage, it is important to choose the plate where lysis is nearest to confluent but not where lysis has obliterated all the bacteria in the lawn and there is no indication of plaques.)
3. Five millilitre of PSB is added to the plate and a sterile spreader, or similar instrument, is used to macerate the soft-agar layer finely.

   (*Note:* Maceration should be undertaken with due care not to break up the solid agar layer beneath the soft-agar.)
4. The plate is left at room temperature for 30 min.
5. The plate is then tilted so that the liquid can drain from the macerated soft-agar layer. The liquid is collected using an automatic pipette fitted with a large-bore disposable tip into microcentrifuge tubes.
6. The tubes are centrifuged at 15 000 *g* for 2 min to remove contaminating agar. The liquid is transferred into a sterile, sealed, 10 ml serum bottle via the septum using a syringe operated 0.22 or 0.45 μm (if phage are expected to be very large) low-protein-binding filter.

   (*Note:* A standard method of storing phages and phage λ in particular has been to store in PSB over chloroform at 4°C [17]. This method works well with many tailed phages that do not contain lipids. However, if the stability of phages to chloroform is unknown, this is a very easy way of losing phages or not identifying the true diversity present. In our experience, phages of ruminal bacteria are particularly sensitive to chloroform [9, 11], and we recommend the method above as an alternative.)
7. This primary stock can be stored at 4°C.

   (*Note:* Sterile phage lysates can generally be stored in this manner for long periods of time (months to years) but stability will vary, possibly markedly, with different phages and when working with new phages the stability of storage in this manner should be determined experimentally in each case.)

With well-studied phage host systems, such as phage λ and *E. coli*, high titre stocks are produced by adding a defined number of phages to a bacterial culture at a specified density of growth, so that the multiplicity of infection is such that growth of bacterial cells will be maximal at the point that all are infected and will lyse to produce maximal phage yield [17]. With less well-studied phage host systems, as is usual with ruminal bacteria and their phages, this is not possible, as the basic biological attributes of both the phage and the bacterium have not been studied in sufficient detail. The following method will yield high titre phage stocks without the need to define infectivity parameters for each phage.

B. High titre stocks for liquid lysis

1. Prepare a 5 ml overnight broth culture of the host bacterium.
2. Prepare 5 × 50 ml anaerobic RF broths in serum bottles.

3. Using a needle and syringe, anaerobically transfer 1 ml of culture to each serum bottle and incubate bottles at 39°C.
4. Add 100 μl of primary phage stock to each serum bottle in a time series of 0, 2, 4 and 8 h (each bottle only receives one inoculum). The fifth serum bottle receives no phage.

   (*Note:* These time periods appear to work well for most ruminal bacteria, however, for fast growing bacteria, such as *S. bovis*, these times need to be shortened, and we suggest that the intervals in step 4 be halved in these circumstances. Conversely, slow growing bacteria may need longer intervals in the time series.)
5. Incubate serum bottles for a further 16 h.
6. Compare the bottles with the uninfected control bottle for visual density of growth and the presence of cellular debris (usually white stringy material). The bottle where lysis has been most complete, least dense growth and most cellular debris, at the latest time interval that phage was added is selected as having the highest phage titre lysate.
7. The lysate is transferred to centrifuge tubes and cellular debris removed by centrifugation at 15 000 *g* for 15 min at 4°C.
8. The supernatant is filtered (0.22 or 0.45 μm) and can be stored as a high titre stock or for subsequent studies by electron microscopy (see below) or nucleic acid studies (see Chapter 4.2).

Phage storage

Short-term storage at 4°C has been covered above (*A. Primary stocks from soft-agar-overlays,* steps 6 and 7). An easy and effective method of long-term storage has been adapted from methodology for the storage of rumen bacteria at –20°C [23]. In our experience, we have not encountered a phage that this technique was not successful with nor have we ever noted a drop in titre over time (even many years) [11].

1. Prepare RF medium containing 50% glycerol.
2. A volume (usually 5 ml) of filtered phage lysate is injected through the septum into a sterile serum bottle (20 ml capacity) with a syringe and needle.
3. An equal volume of the glycerol RF medium is combined with the lysate and they are mixed.
4. The mixture is then frozen at –20°C.

Procedure for the detection and the isolation of temperate bacteriophages

All the aforementioned procedures are for isolating phages that infect and lyse their bacterial host. A large variety of phages exist as DNA integrated into the host chromosome. These phages are known as temperate and the hosts as lysogens. Bacterial lysogens are common and widespread amongst ruminal bacteria [14]. These phages do infect sensitive bacteria and form plaques but many are found only within genomes of bacteria and sensitive hosts are unknown or may not still exist. These phages can be isolated by use of an inducing agent that causes the prophage DNA to excise from

the bacterial genome and the phage to grow vegetatively, culminating in cell lysis and the release of intact, normally infectious (given that a sensitive host can be found) phage particles. The method below was that of Iverson and Millis [8] as modified by Klieve et al. [14].

1. An aqueous solution of Mitomycin C (Sigma) was prepared by dissolving the contents of the sealed vial with RF broth to a concentration of 50 μg/ml. This solution was stored at 4°C when not in use and only used on the day that it was prepared.

   (*Note:* Appropriate safety precautions should be taken when handling Mitomycin C. The type of medium used to dissolve Mitomycin C is not critical; RF is used here, as it is the growth medium for the bacteria.)
2. An overnight culture of the bacterial species to be examined is prepared.
3. From this culture, 0.1 ml aliquots are dispensed into 5 ml RF broth tubes and the resultant cultures are incubated at 39°C.
4. When cultures reach early to mid-logarithmic growth, that is, when turbidity is first barely visible (usually after 2–4 h incubation), Mitomycin C solution is added to the culture to a final concentration of 1 μg/ml.
5. The cultures are incubated overnight at 39°C.
6. Lysed cultures are then treated in the same manner as other lysates (as above).

Phage morphology (transmission electron microscopy)

The primary characterization of phages is the visual determination of virion or phage particle morphology. The technique of preference is negative staining and observation of particles with a transmission electron microscope. These are standard procedures contained in many textbooks [7].

1. Bacteriophage particles are concentrated from lysates by centrifugation at 30 000 *g* for 2 h at 4°C. The supernatant is discarded and the pellet is resuspended in a minimal volume of PSB (usually between 100 and 200 μl).
2. Prepare a solution of 1% phosphotungstic acid (PTA) in sterile distilled water and adjust pH to 6.5.

   (*Note:* A number of other negative stains are also commonly in usage, such as uranyl acetate, and these can be found in many textbooks. The application of the stains to the grid is generally the same.)
3. Prepare Formvar or Butvar coated electron microscope grids (a specialist electron microscope technician will need to do this or instruct in how it is done). These grids can also be carbon coated for extra stability.
4. A small volume (5 μl) of concentrated phage particles (step 1) is placed onto the grid and allowed to settle for 1 min. Excess sample is then carefully removed from the side of the grid with filter paper.
5. Add 5 μl of PTA to the grid, allow to settle for 1 min and remove excess.
6. Allow to dry momentarily. The specimen is ready for examination.

   (*Note:* A good starting magnification for viewing is 35 000 times. Small 'doughnut-shaped' objects may indicate a viral particle and can be observed at

a higher magnification. There are many good texts with morphological descriptions of phages. Ackermann and DuBow [1, 2] give a comprehensive treatment.)

References

1. Ackermann, H.W., and M.S. Dubow. 1987. *General Properties of Bacteriophages*. CRC Press, Boca Raton.
2. Ackermann, H.W., and M.S. Dubow. 1987. *Natural Groups of Bacteriophages*. CRC Press, Boca Raton.
3. Adams, M.H. 1959. *Bacteriophages*. Interscience Publishers Inc., New York.
4. Bach, S.J., T.A. McAllister, D.M. Veira, V.P.J. Gannon, and R.A. Holley. 2002. Transmission and control of *Escherichia coli* O157 : H7 – a review. *Can. J. Anim. Sci.* **82:**475–490.
5. Firkins, J.L., W.P. Weiss, and E.J. Piwonka. 1992. Quantification of intraruminal recycling of microbial nitrogen using nitrogen-15. *J. Anim. Sci.* **70:**3223–3233.
6. Gratia, A. 1936. Des relations numeriques entre bacteries lysogenes et particules des bacteriophages. *Ann. Inst. Pasteur*. **57:**652–676.
7. Horne, R.W. 1965. Negative staining methods, pp. 328–355. *In* D.H. Kay (ed.), *Techniques for Electron Microscopy*. Blackwell Scientific Publications, Oxford.
8. Iverson, W.J., and N.F. Millis. 1976. Lysogeny in *Streptococcus bovis*. *Can. J. Microbiol.* **22:**853–857.
9. Klieve, A.V., P.A. Bain, M.T. Yokoyama, D. Ouwerkerk, R.J. Forster, and A.F. Turner. 2004. Bacteriophages that infect the cellulolytic ruminal bacterium *Ruminococcus albus* AR67. *Lett. Appl. Microbiol.* **38:**333–338.
10. Klieve, A.V., and T. Bauchop. 1988. Morphological diversity or ruminal bacteriophages from sheep and cattle. *Appl. Environ. Microbiol.* **54:**1637–1641.
11. Klieve, A.V., and T. Bauchop. 1991. Phage resistance and altered growth habit in a strain of *Streptococcus bovis*. *FEMS Microbiol. Lett.* **80:**155–160.
12. Klieve, A.V., G.L. Heck, M.A. Prance, and Q. Shu. 1999. Genetic homogeneity and phage susceptibility of ruminal strains of *Streptococcus bovis* isolated in Australia. *Lett. Appl. Microbiol.* **29:**108–112.
13. Klieve, A.V., and R. Hegarty. 1999. Opportunities for biological control of ruminal methanogenesis. *Aust. J. Agric. Res.* **50:**1315–1319.
14. Klieve, A.V., J.F. Hudman, and T. Bauchop. 1989. Inducible bacteriophages from ruminal bacteria. *Appl. Environ. Microbiol.* **55:**1630–1634.
15. Klieve, A.V., and R.A. Swain. 1993. Estimating ruminal bacteriophage numbers using pulsed field gel electrophoresis and laser densitometry. *Appl. Environ. Microbiol.* **59:**2299–2303.
16. Klishans, A.W., and B.J. Chang. 1986. Isolation and characterization of bacteriophages of *Aeromonas*. *Aust. Microbiol.* **7:**150.
17. Maniatis, T., E.V. Fritsch, and J. Sambrook. 1982. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, New York.
18. Nolan, J.V., and R.A. Leng. 1972. Dynamic aspects of ammonia and urea metabolism in sheep. *Br. J. Nutr.* **27:**177–194.
19. Nolan, J.V., and S. Stachiw. 1979. Fermentation and nitrogen dynamics in Merino sheep given a low quality roughage diet. *Br. J. Nutr.* **42:**63–80.
20. Schmidt, J.M., and R.Y. Stanier. 1965. Isolation and characterization of bacteriophages active against stalked bacteria. *J. Gen. Microbiol.* **39:**95–107.
21. Schwan, W.R., and P.A. Hartman. 1986. A comparison of Selas and membrane filters for the sterilization of bacteriophage preparations. *J. Virol. Methods* **14:**189–191.
22. Swain, R.A., J.V. Nolan, and A.V. Klieve. 1996. Natural variability and diurnal fluctuations within the bacteriophage population of the rumen. *Appl. Environ. Microbiol.* **62:**994–997.
23. Teather, R.M. 1982. Maintenance of laboratory strains of obligately anaerobic rumen bacteria. *Appl. Environ. Microbiol.* **44:**499–501.

2.3. Methanogenic archaea

KEITH N. JOBLIN
Rumen Biotechnology, Grasslands Research Centre, AgResearch, Private Bag 11008, Palmerston North, New Zealand

Introduction

This chapter outlines procedures for enumerating, isolating, culturing and storing methanogens from ruminal digesta. The methanogens, a large and diverse group of *Archaea* [4], have unique features that separate them from the bacteria and the eukaryotes [1, 28]. They are the only recognized ruminal microbes belonging to the *Archaea* and are an integral part of the rumen microbial ecosystem [7, 15, 29]. By scavenging hydrogen gas, methanogens play a key ecological role in keeping the partial pressure of hydrogen low so that fermentation can proceed efficiently [30, 31]. Although about 70 methanogenic species belonging to 21 genera have been identified from anaerobic environments, and a range of different methanogens co-exist in the rumen [9, 21, 25, 27], to date only seven ruminal species have been isolated and purified. These are listed in Table 1. The population densities of methanogens in the rumen appear to be influenced by diet, and in particular by the fibre content of the diet [12]. Sheep and cattle fed diets rich in concentrates contained 10^7–10^8 and 10^8–10^9 ruminal methanogens/g, respectively [17, 13], whereas sheep and dairy cows grazing pasture contained 10^9–10^{10} ruminal methanogens/g (G.N. Jarvis and K.N. Joblin, unpublished data). With careful application, methanogen population densities can readily be determined using culture methods. These appear to be similar to the population densities determined by culture-independent methods (P. Evans and K.N. Joblin, unpublished data).

Experimental approach

Access to a system for flushing gases from culture tubes, a method for removing traces of O_2 from gases, culture tubes or bottles and septa for anaerobic culture, and an awareness of factors affecting growth of obligate anaerobes are necessary. Articles by Hungate [8], Balch and Wolfe [2], Sowers and Noll [23] and Sowers and Schreier [24] are recommended for reading.

Careful collection and handling of samples from the rumen prior to culture is a key step in the enumeration and the isolation of methanogens because of their

H.P.S. Makkar and C.S. McSweeney (eds.), Methods in Gut Microbial Ecology for Ruminants, 47–53.
© 2005 *IAEA. Printed in the Netherlands.*

Table 1. Methanogen species cultured from the rumen

Genus and species	Morphology	Host	References
Methanobacterium	Long rods, filaments		
formicicum	Gram variable	Bovine, ovine	[9, 18], G.N. Jarvis and K.N. Joblin unpublished data
bryantii	Gram variable	Bovine	P. Evans and K.N. Joblin, unpublished data
Methanobrevibacter	Coccobacilli	Bovine	[14, 16]
ruminantium	Gram +ve	Bovine, ovine, corvine	[22], G.N. Jarvis and K.N. Joblin, unpublished data
smithii	Gram +ve	Ovine	K.N. Joblin and D.M. Pacheco, unpublished data
Methanomicrobium	Motile curved rods		
mobile	Gram −ve	Bovine	[9, 20]
Methanosarcina	Pseudosarcina	Caprine, bovine	[3, 19]
barkeri		Bovine	[9]
Methanoculleus	Irregular cocci		
olentangyi	Gram −ve	Cervine	G.N. Jarvis, L.C. Skillman and K.N. Joblin, unpublished data

high sensitivity to oxygen. For enumeration, digesta samples should contain both liquid and solid material and be representative of the digesta under study. Samples should be collected quickly into screw-top glass containers, which have been flushed with O_2-free CO_2 and autoclaved. Plastic containers are not recommended because of their O_2 porosity. Samples should be maintained near 38°C. Where this is not possible, samples are best retained at room temperature rather than cooled or chilled. Culture procedures should be applied as soon as possible after sample collection.

It is recommended that a growth medium containing rumen fluid is used. This is a simple means of providing essential cofactors and micro-nutrients, and assists in poising media at the low redox potential necessary for methanogen growth. Semi-defined media can be used [6, 14] but growth may be strain-dependent. The nutrient requirements of methanogens include trace metals, branched chain fatty acids and coenzyme M [1, 14]. A culture medium based on a 30% rumen fluid medium [10, 16] under a H_2/CO_2 headspace has proved successful in the isolation and culturing of a range of different methanogen species (Table 1), and this is recommended as an initial medium. As substrate, H_2/CO_2 is preferable, but sodium formate (0.5% w/v) can be used as an alternative if H_2 andCO_2 or a gasing system is not available. Although most ruminal methanogens grow on formate, some such as *Methanobacterium bryantii* do not, and there may be some selection from using formate. The antibiotics used to inhibit growth of bacteria during enumeration or isolation of methanogens include penicillin, streptomycin, vancomycin, clindamycin, gentamicin and cephalothin. Antibiotics are discontinued once methanogens are in culture.

Solutions

Solution A
Dissolve NaCl (6.0 g), KH_2PO_4 (3.0 g), $(NH_4)_2SO_4$ (1.5 g), $CaCl_2 \cdot 2H_2O$ (0.79 g) and $MgSO_4 \cdot 7H_2O$ (1.2 g) in distilled water and make up to 1 l. Store at 4°C.

Solution B
Dissolve $K_2HPO_4 \cdot 3H_2O$ (7.86 g) in distilled water and make up to 1 l. Store at 4°C.

Anaerobic solution
Mix Solution A (170 ml); Solution B (170 ml); $NaHCO_3$ (5 g); 5 drops of resazurin (0.1% w/v) and distilled water (700 ml), boil for 2 min under O_2-free CO_2 and cool. Add L-cysteine·HCl (500 mg), mix and dispense 9 ml aliquots into Hungate tubes flushed with O_2-free CO_2 and autoclave.

Reducing agent
Dissolve L-cysteine·HCl (2.5 g) in distilled H_2O (50 ml), adjust to pH 10 with NaOH and make to 205 ml with distilled H_2O. Boil under O_2-free N_2 for 3 min, add $Na_2S \cdot 9H_2O$ (2.5 g), mix well and cool (ice bath) before dispensing aliquots (10 ml) into tubes flushed with O_2-free N_2. Stopper, autoclave and store at room temperature.

Vitamins
Boil 1.05 l of distilled H_2O under O_2-free CO_2 and cool (ice bath) under O_2-free CO_2. Add separately and dissolve pyridoxine·HCl (10.0 mg), L-ascorbic acid (5.0 mg), calcium pantothenate (5.0 mg), lipoic acid (5.0 mg), nicotinamide (5.0 mg), nicotinic acid (5.0 mg), *p*-aminobenzoic acid (5.0 mg), pyridoxal·HCl (5.0 mg), riboflavin (5.0 mg), thiamin·HCl (5.0 mg), D-biotin (2.0 mg), folic acid (2.0 mg), and cyanocobolamin (0.1 mg). Remove 10–12 ml into a 20 ml sterile syringe flushed with O_2-free CO_2 and filter sterilize through a pre-flushed 0.45 μm filter into sterile Hungate tubes containing O_2-free CO_2. Ensure that the back pressure generated during the addition does not rupture the filter membrane**.** Add reducing solution (0.1 ml) to each tube. Store frozen.

Trace elements [1]
Add nitrilotriacetic acid (1.5 g) to 500 ml of distilled water, dissolve by adjusting pH to 6.5 with KOH and bring to 1 l with distilled water. Add $MgSO_4 \cdot H_2O$ (3 g), $MnSO_4 \cdot H_2O$ (0.5 g), NaCl (1 g), $FeSO_4 \cdot 7H_2O$ (0.1 g), $CoCl_2 \cdot 6H_2O$ (0.1 g), $CaCl_2$ (0.1 g), $ZnSO_4 \cdot 7H_2O$ (0.1 g), $CuSO_4 \cdot 5H_2O$ (10 mg), $AlK(SO_4)_2 \cdot 12H_2O$ (10 mg), H_3BO_3 (10 mg), $Na_2MoO_4 \cdot 2H_2O$ (10 mg), $NiSO_4 \cdot 6H_2O$ (30 mg), Na_2SeO_3 (20 mg) and $Na_2WO_4 \cdot 2H_2O$ (20 mg), mix thoroughly and store as frozen aliquots.

Lincomycin and vancomycin
Add lincomycin·HCl (20 mg) or vancomycin·HCl (20 mg) to a sterile culture tube flushed with sterile O_2-free CO_2, cap and add sterile anaerobic salts (10 ml) by syringe. Store frozen.

Penicillin/streptomycin
Add Solution A (17 ml), Solution B (17 ml), resazurin (2 drops) and $NaHCO_3$ (0.5 g) to 71 ml distilled water, boil for 2 min and cool (ice bath) under O_2-free CO_2. Add benzyl penicillin (2×10^7 IU), streptomycin sulphate (2.0 g) and cysteine·HCl (50 mg), mix and dispense 10 ml aliquots into sterile CO_2-filled Hungate tubes. Using a syringe, add 6 ml of the stock solution to 9 ml of sterilized anaerobic salts solution in Hungate tubes under O_2-free CO_2 to give a working solution of penicillin/streptomycin. Store frozen.

Dimethyl sulphoxide
Add 5 ml dimethyl sulphoxide to a tube containing 4.8 ml sterile anaerobic salts solution and inject reducing agent (0.2 ml). Store at room temperature.

BY medium
Mix Solution A (170 ml), Solution B (170 ml), $NaHCO_3$ (5 g), 8 drops of resazurin (0.1% w/v), yeast extract (1 g), centrifuged rumen fluid (300 ml), trace elements (10 ml) and distilled water (360 ml) and boil for 2 min under O_2-free CO_2. Cool (ice bath) and add L-cysteine·HCl (500 mg), mix, dispense 9 ml aliquots into Hungate tubes flushed with O_2-free CO_2 and autoclave. Add sterile vitamins (0.1 ml) to each tube after autoclaving.

Roll tubes
To prepare roll tubes, add agar (4.5 g) to freshly prepared BY medium (300 ml) and boil carefully under O_2-free CO_2 for 3 min with mixing to melt the agar. Cool the mixture to 47–50°C (water bath) under O_2-free CO_2, add L-cysteine·HCl (150 mg), mix, transfer 4.3 ml to Hungate tubes flushed with O_2-free CO_2 and autoclave.

Procedures

All preparations and transfers are carried out under an O_2-free CO_2 atmosphere using the anaerobic procedures and the culture techniques described by Hungate [8]. Cultures are grown at 38–39°C in 16 ml screw-top Hungate tubes (Bellco Glass Inc., NJ, USA) under H_2/CO_2 (80/20). Gases are passed over copper filings at 350°C to remove traces of oxygen. If formate, instead of H_2/CO_2, is the substrate, sodium formate (0.5% w/v) is added directly to the medium before tubing up and autoclaving.

Enumeration of methanogens

1. Weigh 40 g of ruminal digesta directly into a Waring blender vessel flushed with O_2-free CO_2, add 160 ml sterile BY medium, blend contents for 10 s and then for 50 s.
2. Because of fibrous material, the initial transfer is carried out with a pipette. Cut the ends of pipette tips to allow solids to enter, flush tips several times with O_2-free CO_2 and transfer aliquots (4 ml) of blended contents into each of two Hungate

tubes containing 4 ml sterile BY medium. Flush tubes with sterile O_2-free CO_2 during the transfer, replace septa and caps after the transfer and mix contents to provide two 10^{-1} dilutions. These are the inocula for two primary dilution series.

3. For further dilutions, transfer 1 ml by syringe into 9 ml BY medium, mix well and continue to a final dilution of 10^{-11}. Use a new sterile syringe flushed with O_2-free CO_2 for each transfer.
4. For most probable number (MPN) determination, inoculate triplicate tubes of BY medium (9 ml) containing penicillin/streptomycin solution (0.1 ml) with 0.5 ml from each of the 10^{-6} to 10^{-11} dilutions. Repeat for the duplicate of the primary dilution.
5. Pressurize inoculated tubes and uninoculated controls to 2 atm with H_2/CO_2 and incubate with shaking for 14 d or stand for 3 weeks.
6. Measure the presence or absence of methane by GC analysis of headspace gases [20, 26].
7. Calculate MPN values from methane-positive cultures using the method of Clarke and Owens [5].

Isolation, characterization and storage of methanogens

1. Prepare agar roll tubes containing 4.3 ml of BY agar medium and autoclave.
2. Melt agar medium by immersion of tubes in a boiling water, transfer tubes to a water bath at 42°C and add vitamin solution (0.1 ml) and penicillin/streptomycin solution (0.1 ml) to each tube.
3. From each of the 10^{-6}–10^{-11} primary dilutions prepared above, inoculate a roll tube with 0.5 ml, mix carefully and immediately roll under cool water to solidify the agar.
4. Pressurize tubes to 2 atm with H_2/CO_2 and incubate for 2–4 weeks.
5. Examine tubes under a dissecting microscope to locate colonies and pick discrete colonies from tubes containing <30 colonies and transfer to BY broth. If mycoplasmas [11], a common contaminant with fried-egg type colonies, are present in the roll tubes, add lincomycin (0.1 ml) or vancomycin (0.1 ml) to broth cultures.
6. Pressurize tubes with H_2/CO_2 and incubate with shaking for 2–3 weeks.
7. Transfer an inoculum from each methane-positive culture through a dilution series of roll tubes containing antibiotics.
8. Pressurize with H_2/CO_2, incubate for 2–4 weeks and pick discrete colonies to BY medium as before to yield isolates for study.
9. Confirm isolate purity by phase-contrast microscopy and by examination of Gram-stain preparations. Use culture morphology as an indicator of the identity of the isolate (Table 1).
10. Determine species by isolating the 16S rRNA gene, sequencing and comparing the sequence with known sequences in databases [9, 21, 25, 27].
11. Determine phenotypic characteristics, such as optimal growth pH, optimal growth temperature and substrate specificities using broth cultures [1, 9].

12. For culture preservation, inject anaerobic dimethyl sulphoxide into viable cultures to a final concentration of 5%, mix well and pressurize with H_2/CO_2. Stand for 2 h at 4°C and transfer tubes to −80°C freezer.
13. To regenerate methanogens, warm frozen cultures quickly to 39°C and inoculate 0.1, 0.2 and 0.3 ml into BY broth, pressurize with H_2/CO_2 and incubate with shaking for 2 weeks.

References

1. Balch, W.E., G.E. Fox, L.J. Magrum, C.R. Woese, and R.S. Wolfe. 1979. Methanogens: re-evaluation of a unique biological group. *Microbiol. Rev.* **43:**260–296.
2. Balch, W.E., and R.S. Wolfe. 1976. New approach to cultivation of methanogenic bacteria: 2-mercaptoethanesulphonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. *Appl. Environ. Microbiol.* **32:**781–791.
3. Beijer, W.H. 1952. Methane fermentation in the rumen of cattle. *Nature* **170:**576–577.
4. Boone, D.R., W.B. Whitman, and P. Rouvière. 1993. Diversity and taxonomy of methanogens, pp. 35–80. *In* J.G. Ferry (ed.), *Methanogenesis: Ecology, Physiology, Biochemistry and Genetics*. Chapman and Hall, New York.
5. Clarke, K.R., and N.J.P. Owens. 1983. A simple and versatile micro-computer program for the determination of "Most Probable Number". *J. Microbiol. Methods* **1:**133–137.
6. Cote, R.J., and R.L. Gherna. 1994. Nutrition and media, pp. 155–178. *In* P. Gerhardt, R.G.E. Murray, W.A. Wood, and N.R. Krieg (eds.), *Methods for General and Molecular Microbiology*. American Society of Microbiology, Washington.
7. Hungate, R.E. 1966. *The Rumen and Its Microbes*. Academic Press, New York.
8. Hungate, R.E. 1969. A roll-tube method for cultivation of strict anaerobes. *Methods Microbiol.* **3b:**117–132.
9. Jarvis, G.N., C. Strompl, D.M. Burgess, L.C. Skillman, E.R.B. Moore, and K.N. Joblin. 2000. Isolation and identification of ruminal methanogens from grazing cattle. *Curr. Microbiol.* **40:**327–332.
10. Joblin, K.N., G.E. Naylor, and A.G. Williams. 1990. Effect of *Methanobrevibacter smithii* on xylanolytic activity of anaerobic ruminal fungi. *Appl. Environ. Microbiol.* **56:**2287–2295.
11. Joblin, K.N., and G.E. Naylor. 2002. The ruminal mycoplasmas: a review. *J. Appl. Anim. Res.* **21:**161–179.
12. Kirchgessner, M., W. Windisch, and H.L. Muller. 1995. Nutritional factors affecting methane production by ruminants, pp. 333–343. *In* W.V. Engelhardt, S. Leonhard-Marek, G. Breves, and D. Giesecke (eds.), *Ruminant Physiology: Digestion, Metabolism, Growth and Reproduction*. Ferdinand Enke Verlag, Stuttgart.
13. Leedle, J.A.Z., and R.C. Greening. 1988. Postprandial changes in methanogenic and acidogenic bacteria in the rumens of steers fed high or low forage diets once daily. *Appl. Environ. Microbiol.* **54:**502–506.
14. Lovley, D.K., R.C. Greening, and J.G. Ferry. 1984. Rapidly growing rumen methanogenic organism that synthesizes co-enzyme M and has a high affinity for formate. *Appl. Environ. Microbiol.* **48:**81–87.
15. Miller, T.L. 1995. Ecology of methane production and hydrogen sinks in the rumen, pp. 317–331. *In* W.V. Engelhardt, S. Leonhard-Marek, G. Breves, and D. Giesecke (eds.), *Ruminant Physiology: Digestion, Metabolism, Growth and Reproduction*. Ferdinand Enke Verlag, Stuttgart.
16. Miller, T.L., M.J. Wolin, Z. Hongxue, and M.P. Bryant. 1986. Characteristics of methanogens isolated from bovine rumen. *Appl. Environ. Microbiol.* **51:**201–202.
17. Morvan, B., F. Bonnemoy, G. Fonty, and P. Gouet. 1996. Quantitative determination of H_2-utilizing acetogenic and sulfate-reducing bacteria and methanogenic archaea from digestive tract of different mammals. *Curr. Microbiol.* **32:**129–133.
18. Oppermann, R.A., W.O. Nelson, and R.E. Brown. 1957. *In vitro* studies on methanogenic rumen bacteria. *J. Dairy Sci.* **40:**779–788.

19. Patterson, J.A., and R.B. Hespell. 1979. Trimethylamine and methylamine as growth substrates for rumen bacteria and *Methanosarcina barkeri*. *Curr. Microbiol.* **3:**79–83.
20. Paynter, M.J.B., and R.E. Hungate. 1968. Characterization of *Methanobacterium mobilis*, sp. n., isolated from the bovine rumen. *J. Bacteriol.* **95:**1943–1951.
21. Sharp, R., C.J. Ziemer, M.D. Stern, and D.A. Stahl. 1998. Taxon-specific associations between protozoal and methanogen populations in the rumen and a model rumen system. *FEMS Microbiol. Ecol.* **26:**71–78.
22. Smith P.H., and R.E. Hungate. 1958. Isolation and characterization of *Methanobacterium ruminantium* n. sp. *J. Bacteriol.* **75:**713–718.
23. Sowers, K.R., and K.M. Noll. 1995. Techniques for anaerobic growth, pp. 15–47. *In* K.R. Sowers and H.J. Schrier (eds.), *Methanogens: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, USA.
24. Sowers, K.R., and H.J. Schreier. 1995. *Methanogens: A Laboratory Manual*, p. 540. Cold Spring Harbor Laboratory Press, USA.
25. Tajima, K., T. Nagamine, H. Matsui, M. Nakamura, and R.I. Aminov. 2001. Phylogenetic analysis of archaeal 16S rRNA libraries from the rumen suggests the existence of a novel group of archaea not associated with known methanogens. *FEMS Microbiol. Lett.* **200:**67–72.
26. Wallace, R.J., and K.N. Joblin. 1985. Proteolytic activity of a rumen anaerobic fungus. *FEMS Microbiol. Lett.* **29:**19–25.
27. Whitford, M.F., R.M. Teather, and R.J. Forster. 2001. Phylogenetic analysis of methanogens from the bovine rumen. *BMC Microbiol.* **1**, http://www.biomedcentral.com/1471–2180/1471/1475.
28. Woese, C.R., O. Kandler, and J.L. Whellis. 1990. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria and Eucarya. *Proc. Natl Acad. Sci. U.S.A.* **87:**4576–4579.
29. Wolin, M.J. 1979. The rumen fermentation: a model for microbial interactions in anaerobic ecosystems, pp. 49–77. *In* M. Alexander (ed.), *Advances in Microbial Ecology*, Vol. 3. Plenum Press, New York and London.
30. Wolin, M.J. 1982. Hydrogen transfer in microbial communities, pp. 323–356. *In* A.T. Bull and J.H. Slater (eds.), *Microbial Interactions and Communities*, Vol. 1. Academic Press, London.
31. Wolin, M.J., T.L. Miller, and C.S. Stewart. 1997. Microbe–microbe interactions, pp. 467–491. *In* P.N. Hobson and C.S. Stewart (eds.), *The Rumen Microbial Ecosystem*, Chapman and Hall, London.

2.4. Anaerobic fungi

MICHAEL K. THEODOROU[1], JAYNE BROOKMAN[1] and ANTHONY P.J. TRINCI[2]
[1]*Institute of Grassland and Environmental Research, Plas Gogerddan, Aberystwyth, SY23, 3EB, UK*
[2]*School of Biological Sciences, 1800 Stopford Building, University of Manchester, Manchester, M13 9PL, UK*

Introduction

Although the rumen represents one of the most thoroughly investigated of all microbial ecosystems, more information is required about the size, diversity and function of the various cultivatable and non-cultivatable subgroups that constitute the rumen microflora. While microbial, molecular methodologies are developing at a considerable pace, and this will ultimately assist in the description of non-cultivatable forms, there is still a need to study the cultivatable forms, and to do this we need to grow and maintain their viability in axenic laboratory culture.

Anaerobic techniques and culture media

Many of the culture techniques used in rumen microbiology were first described by Hungate [15, 16]. Subsequently, Bryant [6], Miller and Wolin [24] and Balch and Wolfe [3] modified and added to them. With relatively few exceptions, these techniques, together with modifications of the anaerobic plating procedures of Leedle and Hespell [18], and the continuous-flow culture methodologies of Zhu et al. [37] are now used to culture the anaerobic fungi.

Anaerobic fungi are generally grown in small batch cultures in thick-walled glass tubes, serum tubes or serum bottles, sealed with butyl rubber stoppers. Cultures are grown with or without agitation, on soluble as well as insoluble carbon sources, under a head-space gas of 100% CO_2 or 70% CO_2 : 30% N_2. Mixtures of head-space gas are generally used to study the growth of anaerobic fungi in co-culture with methanogens [26]. Culture volumes generally range from 7 to 100 ml amounts, and colonized particulate material, as opposed to free zoospores, is preferred as the starting inoculum, as this leads to more vigorous culture growth and a substantial reduction in culture lag phase. In addition to growth in liquid and sloppy agar media [4, 20, 21, 28], agar roll tubes and Petri dish cultures have also been used to culture the anaerobic fungi [17, 19, 25].

The culture media used to grow anaerobic fungi are prepared using anaerobic procedures, and based on those used for the cultivation of rumen bacteria; for the

H.P.S. Makkar and C.S. McSweeney (eds.), Methods in Gut Microbial Ecology for Ruminants, 55–66.
© 2005 *IAEA. Printed in the Netherlands.*

most part and with the notable exception of the rumen fluid free medium of Lowe et al. [19], they are complex, non-defined media and contain rumen fluid. The media usually contain phosphate and/or bicarbonate buffers, the chemical reducing agents, sodium sulphide and/or L-cysteine hydrochloride, and resazurin, which is included as a redox indicator. Air is displaced from the medium by boiling and/or bubbling with O_2-free CO_2 gas. When using agar media and Petri dishes, air can be excluded during manipulations by use of an anaerobic chamber. Syringe and needle methods (see in what follows) are routinely used to transfer liquid and cultured materials from one container to another during inoculation and other manipulation procedures.

Medium C (modified Orpin's medium)

Medium C is used as a routine medium for maintenance of anaerobic fungi, as it supports good growth and is relatively simple to make. Use of a complex particulate carbon source (such as milled wheat straw), rather than just simple sugars (such as glucose or cellobiose), is advocated for routine cultures, as this provides a more vigorous and consistent growth and promotes greater stability of the fungal strains.

Solutions and reagents required
Clarified rumen fluid is prepared by squeezing rumen fluid obtained from fistulated cattle or sheep through muslin cloth and centrifugation at 25 000 *g* for 30 min at 4°C; the supernatant is retained. This rumen fluid is then stored at −20°C until required.
Salts solution I 3 g K_2HPO_4 in 1 l distilled H_2O. Store at 4°C in dark.
Salts solution II 3 g KH_2PO_4, 6 g $(NH_4)_2SO_4$, 6 g NaCl, 0.6 g $MgSO_4 \cdot 7H_2O$, 0.6 g $CaCl_2 \cdot 2H_2O$ in 1 l distilled H_2O; each component should be allowed to dissolve before addition of the next salt. Store at 4°C in dark.
Cysteine hydrochloride, Yeast extract, bacto-casitone and ammonium carbonate (see in what follows for amounts).
Resazurin solution (1000× stock solution). Dissolve 1 g in 1 l distilled H_2O. Store at 4°C in dark. The solution is blue under aerobic conditions and becomes pink then colourless with decreasing redox potential. Media are not used if they assume a pinkish tinge during preparation or storage. Inoculation sometimes gives a momentary pink colouration to the culture medium, but this should disappear within a few minutes.

Bottles and tubes. Serum bottles of 60 or 125 ml capacity for 45 and 90 ml media volumes and crimp-sealed serum tubes or screw-capped 'Hungate' tubes for smaller volumes (7–20 ml) are used. Serum bottles and serum tubes are sealed with butyl rubber stoppers plus crimped aluminium seals, whereas plastic caps are used for 'Hungate' tubes.

Carbon sources, such as glucose or cellobiose, should be added to the liquid medium at 4.5 and 5 g/l. Particulate substrates such as milled wheat straw should be placed in the tubes (1 g) or bottles (5/10 g) before dispensing of the liquid medium to give ∼10% (w/v) final concentration.

Preparation of medium C

1. Add 150 ml salt solution I; 150 ml salt solution II; 150 ml clarified rumen fluid; 2.5 g yeast extract; 10 g bacto-casitone; 6 g $NaHCO_3$ and 1 ml resazurin solution to a 2 l conical flask and make up to 1 l with distilled H_2O.
2. Place onto a heated stirrer and heat to 60°C with stirring (up to 1 h). Displace dissolved O_2 in the solution with O_2-free CO_2 bubbled vigorously through a submerged wide-bore (2 mm) stainless steel needle.
3. If using cellobiose or glucose as a carbon source, add to the medium.
4. Add 1 g cysteine hydrochloride to the medium to give a final reduction.
5. Prior to dispensing, flush tubes or bottles, with O_2-free CO_2 for ~1 min, then dispense medium, under a stream of CO_2 gas. After dispensing, gas tubes for a further minute (tubes) or two (bottles) to ensure that the head space is filled with CO_2 and then stopper firmly.
6. Sterilize media immediately by autoclaving at 115°C for 20 min.

Defined or semi-defined media are prepared in the same manner, and Lowe et al. [19] describe the composition of such media. Defined media are useful for experimentation where conditions need to be closely controlled such as radiolabelling studies or in work concerned with the addition of specific reagents and/or inhibitors. Medium C is perfectly compatible with most downstream procedures required from cultures, such as genomic DNA, RNA and protein extraction. Biomass for nucleic acid extractions is routinely produced by subculture of a wheat straw culture into a serum tube with cellobiose as the carbon source. After 3 d of incubation, the cellobiose culture is used to inoculate a 125 ml serum bottle with cellobiose as the carbon source, which is incubated for growth of biomass for 60 h for DNA preparation. Three time points are routinely used throughout the growth cycle of 20–24 h, 40–44 h and 60 h for RNA and/or protein samples.

Maintenance of cultures

Although Yarlett et al. [36] have developed cryopreservation methods for the long-term storage of anaerobic fungi, laboratory cultures usually require frequent subculturing in order to retain their viability. According to Milne et al. [25], most batch cultures of anaerobic fungi remain viable for 5 or 15 d in media containing glucose or wheat straw, respectively. Twice-weekly subculture is recommended, as described in what follows, although with experience, visual inspection of the cultures will determine the optimum frequency of subculture [20, 28, 29]. Fermentation gases will be produced during incubation of maintenance cultures grown on wheat straw, and this will raise the plug of wheat straw from the bottom of the tube to the top of the liquid phase. With increasing incubation, the fungal culture will enter stationary phase and the floating wheat straw plug will begin to sink to the bottom of the tube. The optimum time for use of the fungal culture as an inoculum for subculture is after the plug has reached the top of the liquid phase and before it begins to sink back again. These cultures are actively growing and approaching stationary phase, and they contain a

relatively high concentration of zoospores (ca. 10^3 zoospores/ml, [20]) or nucleated thalli. Slower growing cultures may take longer than the 3 d recommended above to be suitable for subculture.

Growth of cultures

Items required
A static incubator set at 39°C and CO_2 gassing apparatus are required for all subsequent procedures in this chapter but will not be specifically referred to below.
Tubes or bottles containing growth media, such as medium C, pre-warmed to 39°C for at least 1 h.
Hypodermic needles 4 cm long, wide-bore (0.2 mm) plus sterile plastic syringes (2 or 5 ml).

Inoculation
Inoculations are carried out on the bench using the Miller and Wolin [24] modifications to the method of Hungate [16] as below:
1. Flame the butyl rubber stoppers of both the inoculum culture and the fresh tube to be inoculated with 100% ethanol prior to insertion of the needle.
2. Shake the inoculum culture vigorously to disperse the fungal material within the culture. Tip the tube upside down and insert the needle into the tube. Withdraw 1 ml for inoculation of tubes or 2–10 ml for inoculation of serum bottles. Ensure that the inoculum is withdrawn at a rate which avoids the needle becoming blocked with particles of colonized wheat straw.
3. Inject the inoculum into the recipient tube or bottle and shake gently.
4. Incubate cultures at 39°C without agitation.

Preparation of long-term liquid nitrogen stocks

For long-term maintenance of anaerobic fungi, cultures are stored in liquid nitrogen using anaerobic glycerol as a cryoprotectant.

Items required
Cryovials, usually 1.8 ml (A/S Nunc, Roskilde, Denmark).
Sterile glass Pasteur pipettes and sterile disposable plastic loops.
10% glycerol: The 10% glycerol in double-distilled H_2O solution should be saturated with O_2-free CO_2 by bubbling for 10 min and then sterilized by autoclaving at 115°C for 20 min.

Preparation of stocks
1. Cultures for stock production are grown in tubes for 48 h with milled wheat straw as the major carbon source.
2. Under a stream of CO_2 and using aseptic technique, remove the plant/fungal plug from the culture tube using a sterile plastic disposable loop.

3. Transfer into a cryovial and cover in 1 ml 10% glycerol solution. Seal the vial and freeze by immersion in liquid nitrogen.
4. Cultures stored under liquid nitrogen remain viable for a considerable period of time; strains have been successfully resurrected after 10 years storage.

Reviving of cultures from liquid nitrogen stores

Items required
Tubes or bottles containing medium C growth media plus wheat straw, pre-warmed to 39°C for at least 1 h. To prevent bacterial contamination; the medium should be supplemented with chloramphenicol (5 mg/ml stock solution; 5 ml/l to give 0.025 mg/ml final concentration).
Sterile glass Pasteur pipettes and sterile disposable plastic loops.
1. Thaw vials at room temperature. As soon as the glycerol is melted, open the vial and remove the plug with a sterile glass Pasteur pipette.
2. Rapidly transfer the plant/fungal biomass into a pre-warmed serum tube (under a stream of O_2-free CO_2).
3. Gas the tube, replace the stopper and then incubate at 39°C without shaking.
4. Check the revival of the culture daily. Fermentation gases should be visible within 24–48 h of inoculation. Check the medium for bacterial contamination, which is manifested by clouding.
5. Subculture as above after 3–5 d.

Isolation procedures

The distribution of anaerobic fungi is restricted to the digestive tract of ruminant and non-ruminant herbivores where they are prevalent in fore-gut (rumen) and hind-gut digesta; they can also be isolated from saliva and faeces and from various other organs of the digestive tract, although their role in these situations remains unclear but is likely to be related to host transfer [8, 9, 23, 25].

Techniques to obtain fungal from these environments generally involve the use of antibacterial antibiotics to reduce bacterial numbers and some form of physical separation, such as the growth of isolated colonies on agar medium, to separate fungal colonies from bacterial contamination. Some procedures benefit by including a stage of enrichment culture, to increase the size of the fungal population relative to that of the bacterial and protozoal populations, prior to isolation. The single colony isolation methods of Bauchop and Mountfort [5] and Lowe et al. [19, 20] also ensure that axenic cultures are derived from just one fungal thallus or zoospore.

Sloppy agar media have been used for isolation procedures. For example, Orpin [28] overlaid particles of rumen digesta onto sloppy agar media and after incubation discarded the upper portion of the culture that contained fungal rhizoids while the lower portion, which contained migrating zoospores, was transferred to fresh sloppy agar medium. These new cultures were incubated and the earlier-mentioned process was repeated until successive subcultures yielded anaerobic fungi that were free from

contaminating bacteria. Orpin and his co-workers used the earlier-mentioned procedure to obtain *Neocallimastix*, *Piromyces* and *Caecomyses* isolates, and these fungi quickly became the subject of extensive research, from which many of the defining features and characteristics of the gut fungi were determined.

Joblin [17] developed a relatively straightforward procedure for the isolation of anaerobic fungi from rumen fluid. The method involved straining rumen fluid through muslin, preparing a dilution series of the filtrate and then mixing the appropriate, higher dilutions with a molten agar medium containing antibiotics. The inoculated agar medium was then used to prepare roll tubes [16], from which isolated fungal colonies could be obtained after a period of incubation. This technique was also used by Milne et al. [25], following enrichment culture, to obtain anaerobic fungi from faeces. The method outlined in what follows is a modified version of the procedure described by Joblin [17].

Isolation of axenic cultures of anaerobic gut fungi

Solutions and reagents required

Medium C agar: medium C containing 4.5 g/l glucose as a carbon source plus 18 g/l bacteriological agar.

Medium C with wheat straw and antibiotics: medium C as above plus 0.025 mg/ml chloroamphenicol from stock as above.

Medium C with cellobiose.

Sterile loops.

Rumen digesta or faeces: a source of fungi, such as rumen digesta contents from fistulated animals or a small amount of faecal material.

To isolate anaerobic fungi

1. Dispense 2 ml medium C agar into tubes under CO_2, seal and autoclave at 115°C for 20 min. Store at 4°C until required.
2. Inoculate source material into pre-warmed tubes containing medium C plus wheat straw and antibiotics. Incubate at 39°C for 3 d.
3. Melt medium C agar tubes at 50°C.
4. Add 0.5 ml mixed wheat straw culture to each agar tube. Immediately, roll the tube under cold water tap to give a thin layer of inoculated agar around the inside surface of the tube.
5. Incubate at 39°C for 2 d. Inspect for individual fungal colonies.
6. Open tube under CO_2 and pick individual colonies into medium C with cellobiose tubes.
7. Incubate at 39°C for 3 d.
8. Add 0.5 ml cellobiose culture to each agar tube. Immediately, roll the tube under cold water tap to give a thin layer of inoculated agar around the inside surface of the tube.
9. Repeat steps 5–8 again (on two occasions more) to obtain axenic, isolated cultures.

Enumeration procedures

Although it is difficult to estimate fungal biomass *in vivo*, counting procedures for the enumeration of fungal zoospores are relatively straightforward. By serially diluting samples of strained rumen fluid through antibiotic-containing molten agar in roll tubes and counting the thalli present after 20 h of incubation, Joblin [17] estimated that rumen fluid contained 2×10^4 viable zoospores/ml. This value was in good agreement with the zoospore population density of 3.5×10^4/ml as determined by counting zoospores with the aid of a microscope [30].

Theodorou et al. [33] developed most probable numbers (MPN) procedure for enumeration of anaerobic fungi as thallus forming units (TFUs). This technique, which relies on MPN statistical tables to provide a viable cell count, involves squeezing rumen contents through muslin and preparing a serial dilution of the filtrate in an anaerobic, antibiotic-containing, basal medium. Appropriate dilutions are then transferred to culture tubes containing a carbon source, incubated for up to 9 d and scored periodically for the presence or absence of anaerobic fungi. This procedure can be used with digesta and faecal samples, and the values obtained are generally equal to or higher than those recorded using either of the two zoospore-counting procedures described earlier. The centre of the raft region of the bovine rumen, for example, was found to contain 10^4–10^5 TFU/ml of rumen fluid while rumen fluid contained significantly lower populations.

Determining growth curves in batch culture

Optical density procedures have been used extensively as a method of choice for rapid and non-destructive determination of growth for microorganisms cultured on soluble substrates. However, where it is necessary to determine the growth of filamentous microorganisms on particulate substrates, optical density methods are often inappropriate. This is because of the spatial heterogeneity of the filamentous biomass and/or interference caused by the light scattering properties of the particulate substrates upon which the microorganisms are growing. This is a problem of considerable significance in the study of microbial ecology where it is often the norm for microorganisms to grow on, or at the expense of, particulate substrates.

Theodorou et al. [34] introduced a new technique to follow the growth of anaerobic fungi grown in batch culture on soluble and particulate substrates. This procedure uses a pressure transducer to measure and release the fermentation gases accumulating in the head space of culture bottles as a consequence of fungal growth in the liquid of the incubating cultures. The technique is straightforward and the equipment relatively inexpensive (Fig. 1), and as the method is non-destructive, it is possible to obtain an entire growth curve from individual culture bottles. Results from this technique are precise and reproducible [34], and the method has been used to demonstrate unique differences in the substrate colonization strategies adopted by rumen bacteria (surface erosion strategy) and rumen fungi (particle invasion strategy) [10]. The method

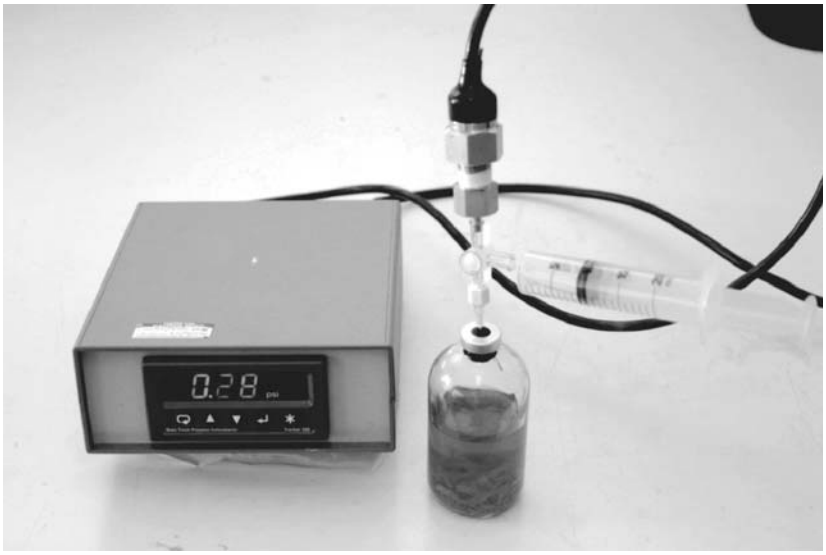


Figure 1. The pressure transducer assembly and a digital display unit for measurement of head-space gas pressure and volume (see Ref. [34] for further details).

can also be used as a screening technique to rank anaerobic fungi relative to their ability to degrade particular substrates [27] or to identify a particular time in the fungal growth cycle when cultures should be harvested, for example, for optimal nucleic acid extraction.

Continuous-flow culture

The rumen is an open ecosystem providing a relatively stable (steady-state) environment in which microorganisms grow at relatively high concentrations of digesta dry matter, within the region of 120 g/l [32]. For microorganisms to survive at such high substrate concentrations, it is essential to prevent the build-up of toxic end products in the rumen and reduce the development of adverse physiological conditions, including dramatic pH decline. This is achieved by salivary flow, the absorption of fermentation end products across the rumen epithelium and the flow of digesta to the lower tract. Under these conditions, and in the absence of any dramatic dietary perturbation, anaerobic fungal populations remain relatively constant, with an equilibrium between fungal biomass production in the rumen and the loss of fungi by death and/or passage from the rumen [11, 33].

In the laboratory, however, because of the difficulty of establishing rumen-like culture systems, anaerobic fungi are usually grown in closed batch cultures on particulate substrate concentrations of only 5–10 g/l dry matter, markedly below those found in the rumen. At these concentrations, growth of the fungus is initially rapid and not inhibited by the accumulation of fermentation end products [22]. As growth

proceeds, anaerobic fungi are subjected to a changing environment and ultimately fail to survive for more than a few days unless they are subcultured into fresh medium [21, 22, 25, 33].

A number of continuous culture systems have been used to grow rumen microorganisms. In general, they involve quite complicated pieces of apparatus in which rumen-like conditions are developed by using dialysis, sequestration or filtration techniques to remove culture liquid and preferentially retain plant and microbial biomass [1, 2, 12, 14, 35]. Although these systems have been used to grow rumen bacteria and protozoa, they have not been used to grow anaerobic fungi. The rumen simulation apparatus, Rusitec [7], has been used to grow anaerobic fungi on fragments of wheat straw in the presence of rumen bacteria [13]. A semi-continuous culture system has also been used for anaerobic fungi [31]; in this system, culture fluids were removed continuously while plant and fungal biomass were selectively retained.

Little attention has been given to the growth of anaerobic fungi on substrate concentrations approximating those found in the rumen. In a study by Zhu et al. [37], a simple continuous-flow culture system was used to investigate and compare the growth of *Neocallimastix hurleyensis*, on increasing concentrations of wheat straw. A unique feature of the continuous-flow system was its ability to operate up to 20 cultures at the same time, enabling comparative investigations of fungal growth against increasing levels of substrate concentrations (Fig. 2). Although not representative

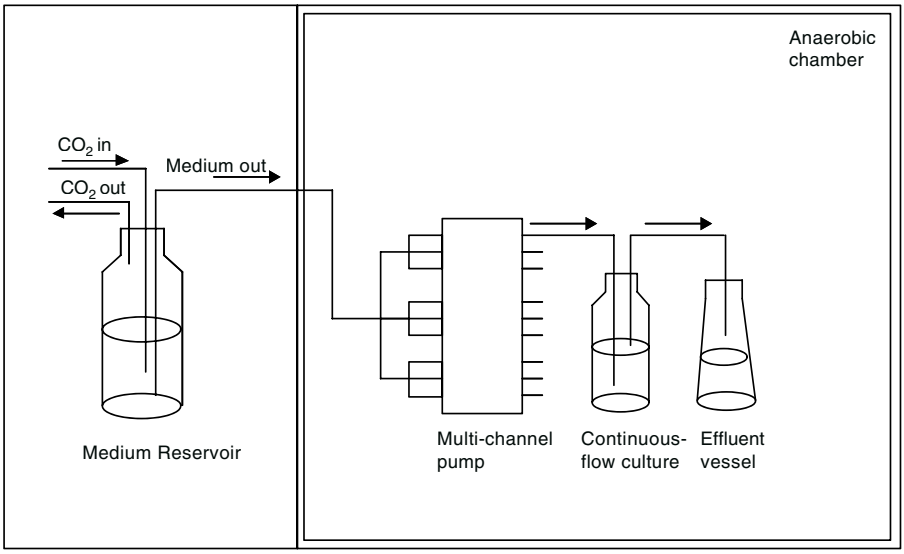


Figure 2. A schematic diagram of the continuous-flow culture system. A 20 l capacity medium reservoir is positioned external to an anaerobic chamber and constantly flushed with a stream of CO_2. Reduced medium from the reservoir is pumped by a multi-channel peristaltic pump to the continuous-flow cultures which contain various amounts of substrate. The culture vessels are operated at constant volume (100 ml), and effluent from the cultures collected individually in effluent collection vessels is kept inside the anaerobic chamber (see Ref. [37] for further details).

of a conventional continuous culture, continuous-flow culture provides a simple and effective means of growing anaerobic fungi on high concentrations of plant biomass approximating those found in the rumen.

Conclusions

The anaerobic fungi have stimulated considerable interest since the original communications of Orpin [28] and Bauchop [4]. Prompted by the initial-colonizer hypothesis of Bauchop [4] and by the uniqueness of the fungal anaerobes themselves, several research groups have developed the methodology for working with anaerobic fungi *in vitro* and *in vivo.* In this chapter, we have described some of the conventional techniques used for the isolation and maintenance of anaerobic fungi in laboratory culture alongside methods for estimating their growth and enumeration. This chapter is intended to provide a general overview of the methods employed to isolate, grow and maintain anaerobic fungi in axenic culture. It was also our intention to add some historical background to the subject area. While we recognize that the newer molecular methodologies add considerable new insight to the study of microbial ecology, it is currently impossible to elaborate phenotypic characteristics from genetic information alone. Consequently, the need to cultivate and study anaerobic fungi in axenic culture can never be completely replaced. Hence, this chapter is intended as a companion article to be read alongside other chapters in this manual which describe the subject area using more modern, molecular approaches and methodologies.

References

1. Abe, M., and F. Kumeno. 1973. In-vitro simulation of rumen fermentation: apparatus and effects of dilution rate and continuous dialysis on fermentation and protozoal population. *J. Anim. Sci.* **36:** 941–969.
2. Abe, M., and Y. Kurihara. 1984. Long-term cultivation of certain rumen protozoa in a continuous fermentation system supplemented with sponge materials. *J. Appl. Bacteriol.* **56:**201–213.
3. Balch, W.E., and R.S. Wolfe. 1976. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. *Appl. Environ. Microbiol.* **32:**781–791.
4. Bauchop, T. 1979. Rumen anaerobic fungi of cattle and sheep. *Appl. Environ. Microbiol.* **38:**148–158.
5. Bauchop, T., and D.O. Mountfort. 1981. Cellulose fermentation by a rumen anaerobic fungus in both the absence and presence of rumen methanogens. *Appl. Environ. Microbiol.* **42:**1103–1110.
6. Bryant, M.P. 1972. Commentary on the Hungate technique for culture of anaerobic bacteria. *Ann. J. Clin. Nutr.* **25:**1324–1328.
7. Czerkawski J.W., and G. Breckenridge. 1977. Design and development of a long-term rumen simulation technique (Rusitec). *Br. J. Nutr.* **38:**371–385.
8. Davies, D.R., M.K. Theodorou, A.E. Brooks, and A.P.J. Trinci. 1993. Influence of drying on the survival of anaerobic fungi in rumen digesta and faeces of cattle. *FEMS Microbiol. Lett.* **106:**59–64.
9. Davies, R.D., M.K. Theodorou, M.I.G. Lawrence, and A.P.J. Trinci. 1993. Distribution of anaerobic fungi in the digestive tract of cattle and their survival in faeces. *J. Gen. Microbiol.* **139:**1395–1400.
10. France, J., M.S. Dhanoa, M.K. Theodorou, S.J. Lister, D.R. Davies, and D. Isac. 1993. A model to inteprit gas accumulation profiles associated with *in vitro* degradation of ruminant feeds. *J. Theor. Biol.* **163:**99–111.

11. France, J., M.K. Theodorou, and D.R. Davies. 1990. Use of zoospore concentrations and life cycle parameters in determining the population of anaerobic fungi in the rumen ecosystem. *J. Theor. Biol.* **147:**413–422.
12. Gijzen, H.J., K.B. Zwart, P.T. van Gelder, and G.D. Vogels. 1986. Continuous cultivation of rumen microorganisms, a system with possible application to the anaerobic degradation of lignocellulosic waste materials. *Appl. Microbiol. Biotechnol.* **25**:155–162.
13. Hillaire, M.C., and J.P. Jouany. 1989. Effects of rumen anaerobic fungi on the digestion of wheat straw and the end products of microbial metabolism studies in a semi-continuous *in vitro* system, pp. 269–272. *In* J.V. Nolan, R.A. Leng, and D.I. Demeyer (eds.), *The Role of Protozoa and Fungi in Ruminant Digestion,* OECD/UNE International Seminar, Plenambul Books, Armidale, Australia.
14. Hoover, W.H., B.A. Crooker, and C.J. Sniffen. 1976. Effects of differential solid–liquid removal rates on protozoa numbers in continuous cultures of rumen content. *J. Anim. Sci.* **58:**692–700.
15. Hungate, R.E. 1966. *The Rumen and Its Microbes*. Academic Press, London.
16. Hungate, R.E. 1969. A roll tube method for the cultivation of strict anaerobes, pp. 117–132. *In* J.R. Norris and D.W. Ribbons (eds.), *Methods in Microbiology*, Vol. 3B. Academic Press, London.
17. Joblin, K.N. 1981. Isolation, enumeration and maintenance of rumen anaerobic fungi in roll tubes. *Appl. Environ. Microbiol*. **42:**1119–1122.
18. Leedle, J.A.Z., and R.B. Hespell. 1980. Differential carbohydrate media and anaerobic replica plating techniques in delineating carbohydrate-utilizing subgroups in rumen bacterial populations. *Appl. Environ. Microbiol.* **39:**709–719.
19. Lowe, S.E., M.K. Theodorou, A.P.J. Trinci, and R.B. Hespell. 1985. Growth of anaerobic rumen fungi on defined and semi-defined media lacking rumen fluid. *J. Gen. Microbiol.* **131:**2225–2229.
20. Lowe, S.E., G.W. Griffiths, A. Milne, M.K. Theodorou, and A.P.J. Trinci. 1987. Life cycle and growth kinetics of an anaerobic rumen fungus. *J. Gen. Microbiol.* **133:**1815–1827.
21. Lowe, S.E., M.K. Theodorou, and A.P.J. Trinci. 1987. Cellulases and xylanases of an anaerobic rumen fungus grown on wheat straw, wheat straw holocellulose, cellulose and xylan. *Appl. Environ. Microbiol.* **53:**1216–1223.
22. Lowe, S.E., M.K. Theodorou, and A.P.J. Trinci. 1987. Growth and fermentation of an anaerobic rumen fungus on various carbon sources and effect of temperature on development. *Appl. Environ. Microbiol.* **53:**1210–1215.
23. McGranaghan, P., J.C. Davies, G.W. Griffith, D.R. Davies, and M.K. Theodorou. 1999. The survival of anaerobic fungi in cattle faeces. *FEMS Microbiol. Ecol*. **29:**293–300.
24. Miller, T.L., and M.J. Wolin. 1974. A serum bottle modification of the Hungate technique for cultivating obligate anaerobes. *Appl. Microbiol.* **27:**985–987.
25. Milne, A., M.K. Theodorou, M.G.C. Jordan, C. King-Spooner, and A.P.J. Trinci. 1989. Survival of anaerobic fungi in faeces, in saliva and in pure culture. *Exp. Mycol.* **13:**27–37.
26. Mountfort, D.O., R.A. Asher, and T. Bauchop. 1982. Fermentation of cellulose to methane and carbon dioxide by a rumen anaerobic fungus in tri-culture with *Methanobrevibacterium* sp. Strain RA1 and *Methanosarcina barkari. Appl. Environ. Microbiol*. **44:**128–134.
27. Nielsen, B.B., W.-Y. Zhu, M.S. Dhanoa, A.P.J. Trinci, and M.K. Theodorou. 2002. Comparison of growth kinetics of anaerobic gut fungi on wheat straw in batch culture. *Anaerobe* **8:**216–222.
28. Orpin, C.G. 1975. Studies on the rumen flagellate *Neocallimastix frontalis*. *J. Gen. Microbiol.* **91:**249–262.
29. Orpin, C.G. 1976. Studies on the rumen flagellate *Sphaeromonas communis*. *J. Gen. Microbiol.* **94:**270–280.
30. Orpin, C.G. 1977. The occurance of chitin in the cell walls of the rumen organisms *Neocallimastix frontalis, Piromonas communis and Sphaeromonas communis*. *J. Gen. Microbiol.* **99:**215–218.
31. Teunissen, M.J., R.J.S. Baerends, R.A.G. Knelissen, H.J.M. Op den Camp, and G.D. Vogels. 1992. A semi-continuous culture system for production of cellulolytic and xylanolytic enzymes by the anaerobic fungus *Piromyces* sp. strain E2. *Appl. Microbiol. Biotechnol.* **38:**28–33.
32. Theodorou, M.K., S.E. Lowe, and A.P.J. Trinci. 1992. Anaerobic fungi and the rumen ecosystem, pp. 43–72. *In* G.C. Carroll and D.T. Wicklow (eds.), *The Fungal Community, Its Organization and Role in the Ecosystem*, 2nd ed. Marcel Dekker, Inc., NY.

33. Theodorou, M.K., M. Gill, C. King-Spooner, and D.E. Beever. 1990. Enumeration of anaerobic chytridiomycetes as thallus forming units: novel method for quantification of fibrolytic fungal populations from the digestive tract ecosystem. *Appl. Environ. Microbiol.* **56:**1073–1078.
34. Theodorou, M.K., D.R. Davies, B.B. Nielsen, M.I.G. Lawrence, and A.P.J. Trinci. 1995. Determination of the growth of anaerobic fungi on soluble and cellulosic substrates using a pressure transducer. *Microbiology* **141:**671–678.
35. Weller R.A., and A.F. Pelgrim. 1974. Passage of protozoa and volatile fatty acids from the rumen of the sheep and from a continuous *in vitro* fermentation system. *Br. J. Nutr.* **32:**341–352.
36. Yarlett, N.C., N. Yarlett, C.G. Orpin, and D. Lloyd. 1986. Cryopreservation of the anaerobic fungus *Neocallimastix patriciarum*. *Lett. Appl. Microbiol.* **3:**1–3.
37. Zhu, W.-Y., M.K. Theodorou, A.C. Longland, B.B. Nielsen, J. Dijkstra, and A.P.J. Trinci. 1996. Growth and survival of anaerobic fungi in batch and continuous-flow cultures. *Anaerobe* **2:**29–37.

2.5. Ciliate protozoa

BURK A. DEHORITY
Department of Animal Sciences, Ohio Agricultural Research and Development Center, The Ohio State University, 1680 Madison Avenue, Wooster, OH 44691, USA

Introduction

Gruby and Delafond [10] discovered the rumen protozoa in 1843 and suggested that their digestive activity was the primary means by which ruminants could survive on an all-plant diet. However, subsequent studies have clearly shown that bacteria actually play a prominent role in the fermentation of plant materials, and fungi are also involved [8, 13, 20].

The rumen ciliates range in size from 18 to 500 μm and can be enumerated and identified microscopically at relatively low magnifications. On the basis of cell morphology, they have been classified into at least five families containing 24 different genera [8]. Although new species are still being reported yearly [6, 9, 11, 14], the last summary was compiled in 1992 by Williams and Coleman [22] and listed 257 separate species.

Ciliate protozoa belonging to different families are found in most other herbivorous mammals. This includes those animals in which fermentation occurs in the hindgut, such as the horse, elephant, rhinoceros and capybara, as well as in the non-ruminant foregut fomenters, i.e. camelids, hippopotamus and kangaroo [4]. These ciliates can be enumerated and identified using the methods and techniques presented in this chapter, presuming appropriate procedures are used for obtaining representative samples from the animal.

Enumeration of total protozoa

Sampling rumen contents

When taking samples from the rumen, it is highly desirable to obtain a thoroughly mixed homogeneous sample of the total contents. Many studies reported in the literature are based on protozoal concentrations per milliliter of rumen fluid, generally obtained by straining whole rumen contents through cheesecloth. However, Dehority [3] found that the concentration of protozoa can be significantly lower in the fluid fraction than in whole contents, and this difference varied with the time of sampling

H.P.S. Makkar and C.S. McSweeney (eds.), Methods in Gut Microbial Ecology for Ruminants, 67–78.
© 2005 *IAEA. Printed in the Netherlands.*

and procedure used to separate the solid and fluid fractions. In addition, generic distribution was also affected. Essentially, there are three ways in which samples of rumen contents can be obtained and each one has its advantages and disadvantages.

1. By stomach tube from a normal animal: To obtain samples from a large number of animals or from animals that do not belong to you, this is the only method available. However, it is probably the least accurate of the procedures. In general, samples obtained by stomach tube probably represent the more fluid portion of the contents. One of the disadvantages of this method is that little control can be used on the area sampled within the rumen. Folding of the flexible tubing can occur, and the length of tubing swallowed is not an indication of sampling site in the rumen. More rigid tubes are difficult for the animal to swallow and can injure the mouth and throat. With coarser feeds, the end of the tube tends to become plugged, again yielding a more fluid sample. In addition, many times the sample is diluted or contaminated with saliva and sometimes with blood. However, if pliability and diameter of the tube are considered in relation to size of the animal and a suitable source of suction is used, it is possible to obtain a representative sample, particularly if the animal is consuming a ground or finely chopped feed.
2. Through a rumen fistula: A rigid tube or small container, depending on the size of the opening, can be used to obtain samples through a rumen fistula. Rumen contents can be mixed with the rigid tube in smaller animals like sheep, or by hand in cattle. Samples should be taken from various sites within the rumen and obviously, a larger sample gives better representation of the total contents. Considerable time and effort is required to fistulate and maintain animals; however, for studies requiring frequent samples taken over time, a fistulated animal is the only practical solution.
3. At slaughter: This allows one to obtain a well-mixed subsample of the total rumen contents; however, the obvious drawback is that the animal is sacrificed and further samples are not available. It is the method normally used for sampling wild ruminants, and samples from domestic ruminants can be collected at a slaughterhouse.

Preservation of rumen contents for subsequent counting

Subsampling of rumen contents for preservation can be accomplished in several ways. If the rumen contents are quite fluid without large pieces of particulate matter, as occurs many times in samples from sheep, a cut-off 10 ml measuring pipette (8 mm inside diameter) can be used. The rumen contents are transferred to a suitable container, such as a 20×150 mm^2 culture tube, and 10 ml of 50% formalin are added. The tube is closed with a rubber stopper. The 50% formalin is prepared by diluting commercial 37% formaldehyde with an equal volume of distilled water, giving a final concentration of 18.5% formaldehyde. Thus, the final concentration of formaldehyde in the preserved sample is 9.25% formaldehyde.

If there are larger particles of feed in the sample of rumen contents, a small plastic cup, 10–15 ml capacity, is filled to the brim with a thoroughly mixed sample of whole contents. The contents are transferred to a small beaker, a cup full of 50% formalin is added, the contents mixed and transferred to a suitable container for storage. Since this is a 1 : 2 dilution of the whole rumen contents, it is not necessary to know the

volume of the sampling cup. In those instances where very large particles of hay or other plant parts such as leaves are present in the sample, a larger cup or 50 ml beaker is used as the sampling container.

Subsampling, staining and dilution of rumen contents

An aliquot of 1 ml of the preserved sample is pipetted into two separate 16 × 150 mm^2 culture tubes, using a 1.0 ml wide-orifice (3 mm) pipette (Bellco Glass Inc., no. 1231-01001). If the 1 : 2 dilution of fixed rumen contents is still somewhat dry or contains many larger feed particles, additional volumes of 25% formalin can be added, using the same container as in the original dilution. This, then, results in a 1 : 3 or 1 : 4 dilution of the original whole rumen contents and maintains the same concentration of formaldehyde.

The following counting procedure was adapted from Purser and Moir [21] as modified by Dehority [3]. Two drops of brilliant green dye are added to the 1.0 ml sample in the tube, and the contents are mixed and allowed to stand for at least 4 h. Standing overnight, generally, results in a more intense and uniform staining of the protozoa cells.

After the staining period, 9 ml of a 30% glycerol solution are added, giving a 1 : 20 dilution of whole rumen contents (if further dilutions were made as described earlier, i.e. 1 : 3 or 1 : 4, final dilutions would be 1 : 30 or 1 : 40, respectively). The 30% glycerol solution is used because it has a high-enough viscosity to prevent rapid settling of the protozoa during the process of pipetting subsamples for counting or further dilution. However, the protozoan cells will settle to the bottom of the counting chamber in a short time, i.e. 5–10 min.

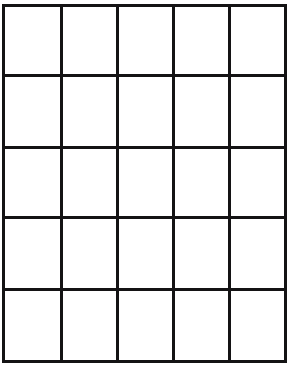
Procedures involved in filling the Sedgewick-Rafter chamber used to count protozoa

A Sedgewick-Rafter chamber (Arthur Thomas no. 9851-C20) is used to count rumen protozoa. The chamber, formed by four glass strips cemented on a 34 × 76 mm^2 slide, is 50 mm × 20 mm × 1 mm and contains 1.0 ml. Using a wide-bore pipette, approximately 1.1–1.2 ml of the 1 : 20 dilution (or other dilution if applicable) is pipetted into the Sedgewick-Rafter chamber. The counting chamber is calibrated to contain 1.0 ml, and any excess volume is squeezed out as the cover slip is put in place. Starting at one end of the cell, the cover slip is slowly slide over the cell, to prevent any currents that would cause movement and possible accumulation of the protozoa at one end of the cell. If insufficient fluid is present or gets siphoned out by the cover slip, an air bubble will be formed in the cell. In that case, the cell should be emptied, washed and refilled as before. The 30% glycerol solution is water soluble, and the cell can quickly be washed in water and dried.

Calibration of eyepiece grid used to count protozoa

Counts are made microscopically using a 10× eyepiece and 10× objective for a total magnification of 100×. The 45× objective cannot be used with the Sedgewick-Rafter

cell because of its total 4.1 mm thickness. A 0.5 mm square counting grid is used in the eyepiece. Actual dimensions of the grid are determined using a stage micrometer, and subsequent calculations are modified if the dimensions differ from 0.5 mm square. A diagram of the grid is shown in what follows:



As a standard practice, any protozoan cells touching the two solid outside lines (top and left side) are counted, whereas any cells touching the dotted outside lines (bottom and right side) are not counted.

Counting the protozoa

Using a wide-bore pipette, a 1 ml aliquot of the 1 : 20 dilution is pipetted into the Sedgewick-Rafter chamber, and two to five grids are quickly counted at a magnification of 100×. The approximate total number of protozoa that would be counted in 50 fields is calculated, and any dilutions required to bring this total into the range of 100–200 protozoa per 50 grids are made. For example, if there were 43 protozoa in five grids, the total would be about 430 in 50 grids. Diluting 1 : 3 would bring the total count down to ∼143. Using a 5 ml wide mouth pipette, 3 ml of the 1 : 20 dilution are pipetted into a 16 × 150 mm^2 tube, and 6 ml of 30% glycerol are added. The final dilution is 1 : 20 × 1 : 3 = 1 : 60. The duplicate tube is also diluted to the same final dilution.

The Sedgewick-Rafter cell is filled with the appropriate dilution and using a calibrated microscope stage, 50 grids evenly spaced over the 20 mm × 50 mm chamber are counted. The counting sites are marked on the graduated scales located on the stage of the microscope. The slide is then rotated 180°, and a second 50-grid count is made. The average of these two counts is used to calculate the protozoan concentration. The duplicate tube is counted in the same manner.

If the counts between duplicate samples differ by >15%, both duplicates are counted a second time and all four values are averaged for the calculation of protozoal concentration. The difference between duplicates is calculated by subtracting the smaller value from the larger, dividing that number by the smaller value and multiplying by 100. For example, if the 50-grid average for duplicate one is 113 and for duplicate two is 129, the difference would be 129 − 113 = 16. (16/113) × 100 = 14.2%,

which is within the suggested range. Thus, the average of the two values, (113 + 129)/2 = 121 would be used to calculate protozoal concentration.

Calculations

Assuming the size of the eyepiece counting grid is 0.5 mm × 0.5 mm (the actual size has been determined using the stage micrometer and may differ from this, but the author has found that most grids are these dimensions), the area of the grid is 0.5 mm × 0.5 mm = 0.25 mm^2. If 50 grids are counted, then 0.25 mm × 50 mm = 12.5 mm^2 were actually counted. The total area of the slide surface is 20 mm × 50 mm = 1000 mm^2. Since all cells settle to the bottom of the chamber, the depth does not need to be included in the calculation. Thus, we have counted 1/80th (1000/12.5 = 80) of the total area. Multiplying the average of 50 grids times 80 gives the number of protozoa per millilitre of diluted rumen contents. We, then, multiply times the dilution factor to determine the concentration per millimetre of rumen contents.

A sample calculation would be as follows:

> Average count per 50 grids = 121.
> Final dilution = 1 : 40.
> Therefore, 121 × 80 × 40 = 387 200 or 38.72 × 10^4/ml rumen contents.

Identification

Subfamily and generic level

With a little practice, it is possible to determine subfamily/generic composition while doing total counts. A lab counter, with nine counting units, greatly facilitates making this differential count. The genera *Entodinium*, *Epidinium*, *Ophryoscolex*, *Isotricha* and *Dasytricha* can be identified at 100× magnification with the Sedgewick-Rafter counting cell. However, those protozoa in the genera that are in the subfamily Diplodiniiae cannot be distinguished at that magnification. They are simply counted as being in the subfamily. If needed, they can be identified to the generic level by observing the sample at 450× on a regular slide with cover slip. It may also be necessary to use differential stains (to be described later) to classify them into the individual genera. In general, these are the principle subfamily/genera, which will be encountered in domestic and many wild ruminants. Possible exceptions would be the genera *Buetschlia* and *Charonina*, which, if present, generally occur only in very low concentrations. The genera *Epiplastron* and *Opisthotrichum* are found only in wild African ruminants.

Diagrammatic sketches of the common subfamily/genera found in the rumen are shown in Figs. 1 and 2, with the various morphological features and cell orientation listed. A simplified key for classification to the subfamily/generic level is in what follows.

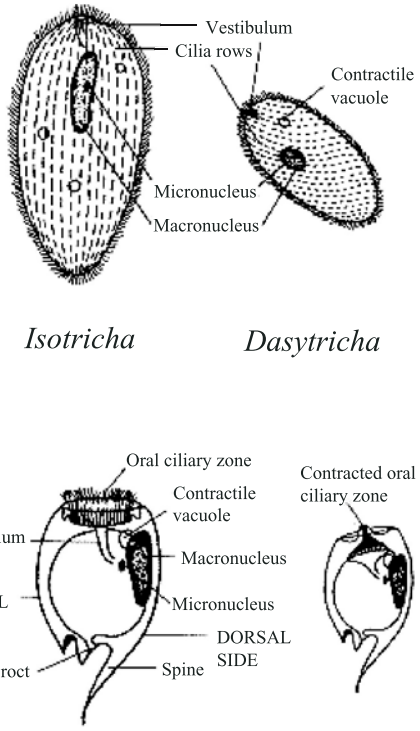


Figure 1. Schematic drawings of rumen protozoa. Approximate size ranges in μm [22]: *Isotricha* – length (L) = 80–200; width (W) = 45–150; L/W = 1.65–2.55. *Dasytricha* – L = 35–75; W = 20–40; L/W = 1.70–2.70. *Entodinium* – L = 18–120; W = 10–90; L/W = 1.0–2.0.

Key for identification of rumen protozoa to the subfamily/generic level

1. With cilia over entire body surface..... 2
   Adoral (oral) zone of cilia located at anterior end of body..... 3
   Adoral (oral) zone of cilia at anterior end plus
   a second ciliary zone at a different location..... 4
2. Cilia in longitudinal rows parallel to the long body axis;
   body usually over 100 μm in length..... *Isotricha*
   Longitudinal rows of cilia that spiral around the long body
   axis; usually between 50 and 75 μm in length..... *Dasytricha*
3. Adoral zone of cilia surrounds the mouth at anterior end of
   body; only one contractile vacuole; macronucleus lies next to
   dorsal body wall; micronucleus to ventral side of macronucleus..... *Entodinium*
4. Second or dorsal ciliary zone in same transverse plane as adoral
   ciliary zone at anterior end of body; operculum present; two or
   more contractile vacuoles; body size can range from

50 to 350 μm; micronucleus between macronucleus and dorsal body wall; skeletal plates may be present..... Subfamily Diplodiniinae
Second (dorsal) ciliary zone displaced toward posterior end of cell..... 5

5. Dorsal ciliary zone a short band located just slightly toward the posterior end of cell. Cell shape is quite slender, L/W ratio generally ≥ 2..... *Epidinium*
Dorsal ciliary zone is a band of cilia which encircles three-fourths of the cell approximately one-third of the distance towards the posterior end..... *Ophryoscolex*

Identification of species

To identify protozoa to the species level, it is necessary to use higher magnifications, 450× or 1000× (oil immersion), as well as using differential stains. Acidified

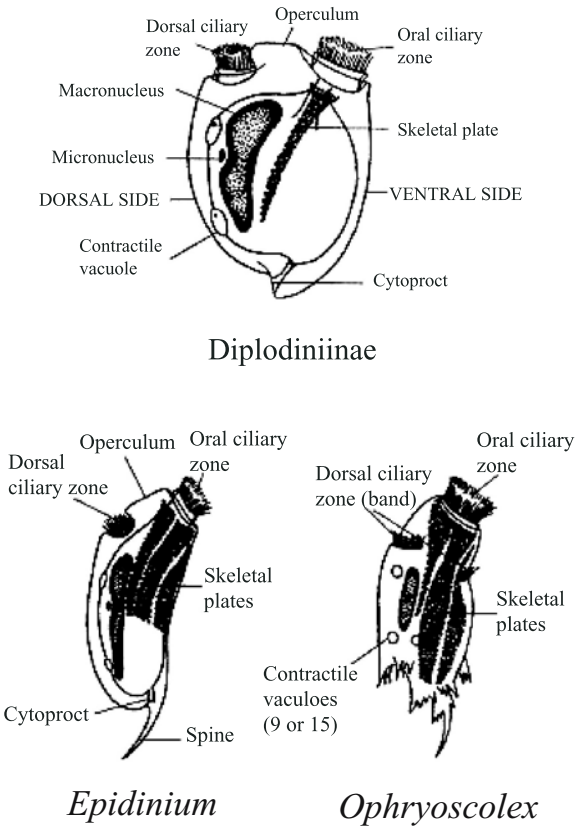


Figure 2. Schematic drawings of rumen protozoa. Approximate size ranges in μm [22]: Diplodiniinae – length (L) = 20–500; width (W) = 0–240; L/W = 1.2–2.0. *Epidinium* – L = 50–230; W = 20–120; L/W = 1.6–2.9. *Ophryoscolex* – L = 120–215; W = 60–110; L/W = 1.6–2.0.

methylene blue is commonly used as a nuclear stain, and Lugol's iodine can be used to stain skeletal plates. Composition and preparation of the stains are described in what follows.

Staining with methylene blue

A sample of rumen contents, preserved in 50% formalin, is filtered through a single layer of cheesecloth in order to remove any large particles which would prevent the cover slip from lying flat on the slide. Two or three drops of the acidified methylene blue dye are added to a 1 ml aliquot of the filtered sample, the contents mixed and allowed to stand at least 4–6 h before observing under the microscope. Allowing to stand overnight is sometimes beneficial for staining the larger ciliates. One or two drops of the stained sample are placed on a glass slide and covered with a glass cover slip. The slide is placed under the microscope, and the protozoa are examined either at 450× or 1000× (oil immersion). Methylene blue will stain both the macro- and micronucleus a deep blue in comparison to the rest of the cell.

The cell length and width, length of the macronucleus and length of spine(s), where applicable, are measured with a calibrated ocular scale. Divisions on the ocular scale are calibrated with a stage micrometer. For uniformity, the length of the cell is designated as the distance on the longitudinal axis from the most anterior point of the cell (oral area or operculum) to the posterior end of the cytoproct or rectum. Width is the greatest distance between the sides, usually near the middle of the cell. Length of the macronucleus is measured as the distance on a straight line between the anterior and posterior ends.

Staining with Lugol's iodine

One millilitre of the strained 50% formalin sample is stained with a 0.5 ml of Lugol's iodine. The mixture is allowed to stand for 15–30 min and examined microscopically as mentioned earlier. Skeletal plates will be stained a deep orange to brown.

Criteria for identification

In general, the following criteria are used to classify and identify rumen protozoa:

a) Cell is covered with cilia, or cilia only present in one or more distinct zones.
b) Number and location of ciliary zones.
c) Overall shape of cell and measurements, including the length-to-width ratio.
d) If present, location, size and number of skeletal plates.
e) Number and location of contractile vacuoles.
f) Spines should be noted but are generally not considered to be reliable characteristic.

With this information, several different references can be consulted to identify individual species [5, 15, 16, 17, 19, 22]. Reference [5] has keys for identifying the protozoa most commonly found in domestic ruminants.

Isolation of individual protozoan cells

Rumen fluid is diluted anaerobically somewhere between 1 : 10 and 1 : 100 in anaerobic dilution solution (ADS) [1]. The dilution tubes are held in a 39°C water bath under CO_2. A small volume (0.01 ml) of the diluted sample is placed on a glass slide and observed microscopically at 100×. Attempts are made to draw individual cells into a capillary pipette and transfer them to a drop of ADS on a second slide. Pasteur pipettes can be drawn out to a very small diameter for this purpose, probably about 0.5–1.0 mm in diameter. If only one cell appears to be present, it is drawn into the capillary pipette and transferred to a culture tube containing medium and substrate. If more than one cell is present, it can be drawn up and transferred to a second drop of ADS to make sure that no other cells are present. This procedure can be repeated until only one cell is observed. If possible, it is desirable to view the capillary pipette under the microscope to be sure that only one cell is present. For isolating a particular species, it is advisable to inoculate several tubes, some with single cells and others with two or three cells. Many times, growth from a single cell is difficult to obtain.

Anaerobic dilution solution (ADS) 300 ml:

45.0 ml Mineral solution I
45.0 ml Mineral solution II
0.3 ml of 0.1% Resazurin solution
197.0 ml distilled water

Heat flask carefully over burner and gas with O_2 free CO_2. When solution is fairly well reduced, (changes from pink to colorless), add:

7.5 ml of 12% Na_2CO_3, and
5.0 ml of 3% cysteine-HCI

Transfer 9.0 ml aliquots anaerobically under CO_2 into 16 × 150 mm culture tubes or stopper the flask with a rubber stopper and wire in place. Autoclave the flask or tubes in racks for 20 min at 15 pounds per square inch pressure.

Min solution I:
0.3% K_2HPO_4

Min Solution II:
0.3% KH_2PO_4
0.6% $(NH4)_2SO_4$
0.6% NaCl
0.06% $MgSO_4$
0.06% $CaCl_2$

Cultivation

Using the earlier-mentioned technique for isolating single species or clone cultures and the medium and procedures described in what follows, the author has successfully cultured *Entodinium caudatum*, *E. exiguum*, *Ophryoscolex purkynjei*, *Eudiplodinium maggii*, *Epidinium caudatum*, *Metadinium affine* and *Ostracodinium gracile.* All cultures are grown in a 39°C incubator, with the tubes incubated at an angle of about

10–15°. This gives a larger surface area for the substrate that falls to the bottom of the tube.

Media preparation, feeding and transferring of cultures are all carried out using the anaerobic techniques described by Hungate [12] and modified by Dehority [2]. The basal medium, medium M (Table 1), used to culture protozoa is given in what follows.

The protozoa are fed daily a suspension containing ground wheat and ground orchardgrass in distilled water (see in what follows). Each day, a tube of substrate is removed from the freezer and placed in a 39°C water bath to thaw. The protozoa cultures are removed from the incubator and also placed in the water bath for feeding. Each culture is opened under anaerobic conditions (CO_2), and 0.1 ml of the substrate suspension is added. This scheme of feeding is required to maintain the cultures, since adding more feed at one time provides substrate for the bacteria, which will ferment it to acids that lower the pH and inhibit protozoal growth.

For maintaining stock cultures, 5 ml of the culture is transferred every 3 or 4 d to a new tube containing 5 ml of medium M plus 0.1 ml of substrate suspension. To increase the number of cultures for experimental studies, two new cultures can be initiated from each stock culture.

Table 1. Medium M for culturing protozoa [7]

Ingredient	Percentage in medium (vol/vol)
Mineral mix M[a]	50.0
Sodium acetate, 1.5%	5.0
Rumen fluid(1000 × *g* supernatant)[b]	10.0
Sodium bicarbonate, 6%	8.33
Distilled water	26.0
Cysteine HCl, 3%[c]	0.67

[a]Mineral mix M: 6.0 g NaCl, 0.2 g $MgSO_4$, 0.26 g $CaCl_2 \cdot 2H_2O$ and 2.0 g KH_2PO_4/l.
[b]Supernatant obtained by straining rumen contents through a double layer of cheesecloth and centrifuging the filtrate at 1000 *g* for 10 min.
[c]Small aliquots were tubed anaerobically under nitrogen, sterilized in the tube and stored until needed.

Cryopreservation and storage of protozoal cultures

Just recently, Nsabimana et al. [18] published a two-step freezing procedure for the cryopreservation of rumen ciliates. Dimethyl sulfoxide was used as a cryoprotectant, at concentrations ranging from 3% to 6%, depending on the species. After adding the cryoprotectant, cultures were held at ~25°C for 5 min and then cooled at a rate between 7°C and 10°C /min to the extracellular ice nucleation temperature and then at a rate between 1.2°C and 2.5°C down to the holding temperature of –30°C. Survival after 1 year in liquid nitrogen ranged from 59% for *E. maggii* to 100% for *Isotricha prostoma.* The other species studied included *Polyplastron multivesiculatum*, *E. caudatum*, *Dasytricha ruminantium* and *E. caudatum.*

The author has used some very simple procedures to try and cryopreserve protozoal cultures. Essentially, dimethyl sulfoxide was added to the culture at a level of 4%, 2 ml aliquots were incubated at 39°C for 15 min, 2°C for 1 h, –29°C for 1 h and then placed into –72°C low-temperature deep freeze. To revive, the cultures were removed from the freezer, placed in a 39°C water bath for several minutes and 8 ml of medium M plus substrate were added. Recovery of cultures was poor and not reproducible. *Epidinium caudatum* was recovered after 1 year, but most cultures were not viable.

A freezing apparatus called the Nalgene Cryo 1°C Freezing Container is available from The Nalge Company (PO Box 20365, Rochester, NY 14602-0365, USA), which has a controlled rate of cooling at –1°C/min. The author has not tried this method; however, Dr. Jamie Newbold has had some success in recovering frozen rumen ciliates with this procedure (personal communication).

Stains and medium for cultivation

Stains

Brilliant green

Brilliant green – 2.0 g.
Acetic acid – 2.0 ml of glacial acetic acid.
Distilled water – 100 ml volume.

Acidified methylene blue

Methylene blue – 0.5 g.
Acetic acid – 2.0 ml of glacial acetic acid.
Distilled water – 100 ml volume.

Lugol's iodine

Iodine – 1.0 g.
KI – 2.0 g.
Distilled water – 300 ml.
Dissolve KI in distilled water and then add iodine.

Substrate for feeding

1.5% Ground wheat.
1.0% Ground orchard grass.

The feedstuff suspension is gassed with O_2-free CO_2 for 15–20 min and 3 ml aliquots are tubed anaerobically into 16 × 150 mm^2 tubes. The tubes are closed with rubber stoppers and stored in the freezer.

In preparing the medium, all ingredients except cysteine are added to a 500 ml round-bottom flask and the medium is gassed with O_2-free CO_2 for 15–20 min. Cysteine is then added and gassing continued for at least additional 10 min. At that time, pH is measured, and if needed, adjusted within the range of 6.5–6.8 with either NaOH or HCl. The medium is tubed anaerobically (under CO_2) into 16 × 150 mm

culture tubes, in 5.0 and 10.0 ml aliquots. The tubes are closed with rubber stoppers, and the medium is autoclaved in clamp-type racks for 20 min at 121°C.

References

1. Bryant, M.P., and L.A. Burkey. 1953. Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. *J. Dairy Sci.* **36:**205–217.
2. Dehority, B.A. 1969. Pectin-fermenting bacteria isolated from the bovine rumen. *J. Bacteriol.* **99:**189–196.
3. Dehority, B.A. 1984. Evaluation of subsampling and fixation procedures used for counting rumen protozoa. *Appl. Environ. Microbiol.* **48:**182–185.
4. Dehority, B.A. 1986. Protozoa of the digestive tract of herbivorous mammals. *Insect Sci. Appl.* **7:**279–296.
5. Dehority, B.A. 1993. *Laboratory Manual for Classification and Morphology of Rumen Ciliate Protozoa*. CRC Press, Inc., Boca Raton, FL.
6. Dehority, B.A. 1995. Rumen ciliates of the pronghorn antelope (*Antilocapra americana*), mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*) and elk (*Cervus canadensis*) in the northwestern United States. *Arch. Protistenkd.* **146:**29–36.
7. Dehority, B.A. 1998. Generation times of *Epidinium caudatum* and *Entodinium caudatum*, determined *in vitro* by transferring at various time intervals. *J. Anim. Sci.* **76:**1189–1196.
8. Dehority, B.A. 2003. *Rumen Microbiology*. Nottingham University Press, Nottingham, UK.
9. Göçmen, B., B. Falakali Mutaf, and M. Tosuno lu. 2001. New rumen ciliates from Turkish domestic cattle (*Bos taurus* L.): IV. *Eudiplodinium dehorityi* n. sp. *Acta Parasitol. Turc.* **25:**305–307.
10. Gruby D., and O. Delafond. 1843. Recherches sur des animacules se développant dans l'estomac et dans les intestins pendant la digestion des animaux herbivores et carnivores. *C. R. Acad. Sci.* **17:**1304–1308.
11. Guirong, Su N., Z.X. Hua, S. Zhu, and S. Imai. 2000. Rumen ciliated protozoan fauna of the yak (*Bos grunniens*) in China with the description of *Entodinium monuo* n. sp. *J. Eukaryot. Microbiol.* **47:**178–182.
12. Hungate, R.E. 1950. The anaerobic mesophilic cellulolytic rumen bacteria. *Bacteriol. Rev.* **14:**1–49.
13. Hungate R.E. 1966. *The Rumen and its Microbes*. Academic Press, Inc., New York.
14. Ito, A., N. Arai, Y. Tsutsumi, and S. Imai. 1997. Ciliate protozoa in the rumen of sassaby antelope, *Damaliscus lunatus lunatus,* including the description of a new species and form. *J. Eukaryot. Microbiol.* **44:**586–591.
15. Kofoid, C.A., and R.F. MacLennan. 1930. Ciliates from *Bos indicus* Linn. I. The genus *Entodinium* Stein. *Univ. Calif. Publ. Zool.* **33:**471–544.
16. Kofoid, C.A., and R.F. MacLennan. 1932. Ciliates from *Bos indicus* Linn. II. A revision of *Diplodinium* Schuberg. *Univ. Calif. Publ. Zool.* **37:**53–152.
17. Kofoid, C.A., and R.F. MacLennan. 1933. Ciliates from *Bos indicus* Linn. III. *Epidinium* Crawley, *Epiplastron gen. nov.,* and *Ophryoscolex* Stein. *Univ. Calif. Publ. Zool.* **39:**1–34.
18. Nsabimana E., S. Kišidayová, D. Macheboeuf, C.J. Newbold, and J.P. Jouany. 2003. Two-step freezing procedure for cryopreservation of rumen ciliates, an effective tool for creation of a frozen rumen protozoa bank. *Appl. Environ. Microbiol.* **69:**3826–3832.
19. Ogimoto, K., and S. Imai. 1981. *Atlas of Rumen Microbiology*. Japan Scientific Societies Press, Tokyo.
20. Orpin, C.G. 1977. The occurrence of chitin in the cell walls of the rumen organisms *Neocallimastiz frontalis, Piromonas communis* and *Sphaeromonas communis. J. Gen. Microbiol.* **99:**107–117.
21. Purser, D.B., and R.J. Moir. 1959. Ruminal flora studies in the sheep. IX. The effect of pH on the ciliate population of the rumen *in vivo*. *Aust. J. Agric. Res.* **10:**555–564.
22. Williams, A.G., and G.S. Coleman. 1992. *The Rumen Protozoa*. Springer-Verlag, New York.

PART THREE

PCR-based methods for analysis of populations and gene expression

3.1. Nucleic acid extraction, oligonucleotide probes and PCR methods

ZHONGTANG YU[1] and ROBERT J. FORSTER[2]
[1]*The MAPLE Research Initiative, Department of Animal Sciences, The Ohio State University, Columbus, Ohio, USA*
[2]*Agriculture and Agri-Food Canada. Lethbridge Research Centre, Lethbridge, Alberta, Canada*

Introduction

Complex microbiomes of rumen and gastrointestinal tracts

Bacteria, fungi and protozoa, present in rumen and gastrointestinal (GI) tracts, interact with feed, with each other, and with their host animals, resulting in a complex symbiotic microbiota of distinctive composition and structure [11, 20, 32, 39, 46]. Such microbiota is dynamic and highly responsive to a variety of biotic and abiotic factors, such as diet, feed additives, age, health and physiological status of the host animal, geographical locations, season and feeding regimen (reviewed in Ref. [39]). This symbiotic microbiota has been the focus of microbial research for over half a century in search for improved ruminant nutrition. Before the advent of molecular biology techniques, microorganisms in rumen and GI tracts, as in other habitats, were studied with cultivation-based techniques, which only allows for the isolation and characterization of a limited number of readily culturable species.

As estimated, there are more than 400 species of bacteria and up to 100 species of protozoa and fungi inhabiting rumen and GI tracts [32, 39, 46]. In human GI tracts, as much as 60% of these members cannot be isolated on agar plates and, thus, remain unknown [12, 47]. In ruminants, although it is not known, the culturable species of the microbiota are probably in the same range. Even among the culturable species, probably only some of them have been isolated and described.

The application of cultivation-independent, more sensitive and accurate molecular techniques to the study of ruminal and GI microorganisms provided an alternative to directly examining the diversity and the community structure of ruminal and GI microbiota on the basis of genotypes, instead of phenotypes [41, 45]. Both polymerase chain reaction (PCR)-based methods, such as denaturing gradient gel electrophoresis (DGGE), ribosomal intergenic spacer analysis, terminal restriction fragment length polymorphism, cloning and sequencing of PCR amplicons and amplified 16S ribosomal DNA restriction analysis, and hybridization-based methods, such as RNA-targeted hybridization, fluorescence *in situ* hybridization (FISH), and microarray, have been employed. The application of these molecular techniques has

H.P.S. Makkar and C.S. McSweeney (eds.), Methods in Gut Microbial Ecology for Ruminants, 81–104.
© 2005 *IAEA. Printed in the Netherlands.*

been changing our perspectives about ruminal and GI microbiota. Except for FISH, all these methods analyse DNA or RNA extracted from samples collected from rumen or GI tracts. Therefore, reliable and efficient DNA/RNA extraction is the pre-requisite of molecular ecological studies of ruminal microbiota.

Sampling for molecular analyses

A number of issues need to be addressed when rumen and GI tracts are sampled for microbial analyses. First, digesta samples have higher DNase and RNase activities than other environmental samples due to the presence of dense populations of diverse organisms. Thus, DNA and RNA need to be protected from degradation before and during DNA/RNA extraction procedures. Freezing immediately right after sampling is an effective way to preserve DNA and RNA in digesta samples. Chemical preservation using formalin or RNAprotect Bacteria Reagent (QIAGEN, Inc., Valencia, CA, USA) also works well for some applications. Second, physical segregation within rumen and GI tracts creates different habitats. For instance, in rumen, microbes may be 'free-living', loosely associated with digesta particles or tightly adherent to digesta particles. The microbes in these three different microhabitats have their own niches: structural-carbohydrate-hydrolysing microbes are likely to adhere to digesta particle; the microbes fermenting monomers, such as simple sugars and amino acids, are predominant in the liquid fraction while syntrophic microbes of the adherent microbes are probably found associated with digesta particles. Third, the ruminal and GI tract wall selects microbes that are oxygen-tolerant or microaerophilic, and thus, lumen and mucosa harbour different microbial communities. Fourth, ruminal and GI tracts function in a plug-flow mode and samples are often variable in consistency. Thereby, different sampling methods have to be used to obtain representative samples of interest for molecular analyses.

DNA and RNA extraction

Genomic community DNA and RNA can be extracted either from microbial cells previously isolated from digesta (indirect extraction) or directly from digesta samples (direct extraction). Indirect extraction yields cleaner nucleic acids free from inhibitory substances, but it is more laborious, time consuming and may bias against those microbes tightly attached to digesta particles. Direct extraction of DNA and RNA from digesta samples in the presence of undigested feed is rapid, less biased and more efficient [5, 28]. However, the DNA and RNA extracted by this approach may contain inhibitory substances that interfere in subsequent enzymatic manipulations unless removed. Potential inhibitors can be readily removed by a purification step incorporated into the extraction procedures or following the extraction. Currently, direct extraction of DNA and RNA is the preferred approach in most microbial ecological studies.

There are three main steps in DNA or RNA extraction: (i) cell lysis to release DNA and RNA into the lysis buffer, (ii) separation of DNA and/or RNA by enzymatic

manipulations, differential precipitation or binding to a solid matrix and (iii) recovery of DNA and/or RNA into a suitable buffer (often TE: 10 mM Tris–HCl and 1 mM EDTA, pH 8.0). Typically, cell lysis is the most important step to achieve efficient DNA and RNA extraction. Cell lysis can be achieved by physical means such as freeze–thaw, bead beating or French Press; or enzymatic disruption of cell wall and membrane by enzymes, such as lysozyme, mutanolysin, lysostaphin, proteinase K, and detergents such as sodium dodecyl sulphate (SDS) and sodium lauryl sarcosine (SLS). Most protocols combine two or more of the earlier-mentioned mechanisms to achieve maximal cell lysis efficiency.

DNA extraction

Direct DNA extraction

Ruminal and GI tract microbial communities are complex and contain numerous hard-to-lyse microorganisms. Variable consistency and variable endogenous and dietary components also make the DNA extraction difficult [26]. Consequently, community DNA is often extracted directly from digesta samples without cell isolation, using enzymo-chemical or mechano-chemical lysis. Mechano-chemical lysis, especially bead beating in the presence of a detergent, is most commonly used due to its robustness and high efficiency in cell lysis [51]. A few commercial kits (such as the FastDNA Spin Kit from QBIOgene, Inc.,Carlsbad, CA, USA and the QIAamp DNA Stool Mini Kit from QIAGEN) employing the above lysis mechanisms have been commonly used in DNA extraction from ruminal and GI tract samples [26, 43]. However, we found that the DNA recovery of these kits was 5–6 folds lower than the RBB+C method that we developed recently [51]. The RBB+C method permits efficient extraction of PCR-quality community DNA from digesta and mucosa samples collected from rumen and GI tracts. Its detailed procedures are described in what follows.

Materials

- Lysis buffer: 500 mM NaCl, 50 mM Tris–HCl (pH 8.0), 50 mM EDTA, and 4% SDS. Add SDS to the lysis buffer after autoclaving.
- Zirconia beads (0.1 and 0.5 mm in diameter, BioSpec Products, OK, USA).
- 10 M ammonium acetate.
- Isopropanol.
- 70% Ethanol.
- 100% or 95% Ethanol (molecular biology grade).
- TE buffer (10 mM Tris–Hcl, 1 mM EDTA, pH 8.0).
- DNase-free RNase (10 mg/ml).
- Proteinase K (QIAGEN, Inc., Valencia, CA, USA).
- Buffer AE, AL, AW1, and AW2 (QIAGEN).
- QIAamp spin column for genomic DNA extraction (QIAGEN).
- A Mini-Beadbeater-8 or a Mini-Beadbeater (BioSpec Products, OK, USA).
- A water bath (up to 70°C).
- A microcentrifuge (up to 16 000 *g*).

Procedures of RBB+C method

A. Cell lysis:

1. Transfer 0.25 g of digesta sample into a sterile 2 ml screw-cap tube. Add 1.0 ml of lysis buffer and 0.4 g of sterile zirconia beads (0.3 g of 0.1 mm and 0.1 g of 0.5 mm in diameter).
2. Homogenize for 2–3 min at maximum speed on a Mini-Beadbeater-8.
3. Incubate at 70°C for 15 min, with gentle end-over-end inversion by hand every 5 min.
4. Centrifuge at 16 000 *g* for 15 min at 4°C. Transfer the supernatant to a fresh 2-ml Eppendorf tube.
5. Add 300 μl of fresh lysis buffer to the lysis tube and repeat steps 2–4. Pool the supernatant.

B. Precipitation of nucleic acids:

6. Add 260 μl of 10 M ammonium acetate to each lysate tube and mix completely. Incubate on ice for 5 min.
7. Centrifuge at 16 000 *g* for 10 min at 4°C.
8. Transfer the supernatant to two 1.5-ml Eppendorf tubes. Add 1.0 volume of isopropanol and mix completely. Incubate at room temperature for 30 min.
9. Centrifuge at 16 000 *g* for 15 min at 4°C.
10. Decant the supernatant. Add 1 ml of 70% ethanol. Invert the tube end-over-end several times. Centrifuge at 16 000 *g* for 5 min at 4°C.
11. Decant the supernatant. Dry the pellet under vacuum for 2–3 min.
12. Dissolve the above nucleic acids pellet in 100 μl of TE. Pool the two nucleic acid solutions from the same digesta sample.

C. Removal of RNA, protein and DNA purification:

13. Add 2.0 μl of DNase-free RNase, mix and then incubate at 37°C for 15 min.
14. Add 15 μl of proteinase K and 200 μl of buffer AL. Mix and incubate at 70°C for 10 min.
15. Add 200 μl of ethanol and mix.
16. Transfer to a QIAamp column and centrifuge at 16 000 *g* for 1 min.
17. Discard the flow-through. Add 500 μl of buffer AW1 and centrifuge for 1 min at room temperature.
18. Discard the flow-through. Add 500 μl of buffer AW2 and centrifuge for 1 min.
19. Place the column in a fresh 2 ml collection tube. Centrifuge the column at room temperature for 1 min to dry it.
20. Add 100 μl of pre-warmed (60°C) buffer AE or TE into each column. Incubate at room temperature for 2 min.
21. Centrifuge at room temperature for 1 min to elute the DNA.
22. Repeat steps 20 and 21 to repeat the elution into the same tube.
23. Aliquot the DNA solution into four tubes. Run 2 μl on a 0.8% gel to check the DNA quality.
24. Store the DNA aliquots at –20°C.

The DNA extracted using the RBB+C is sheared to some extent. However, for molecular ecological analyses targeting *rrn* genes, the sizes of the resultant DNA fragments are sufficiently large. The resultant DNA is clean enough for PCR amplification, endonuclease digestion or hybridization. In analyses involving PCR amplification, however, the inclusion of bovine serum albumin at a concentration of 670 ng/μl in PCR reactions is recommended to safeguard and ensure robust amplification reaction.

Indirect DNA extraction

In some cases, such as ecological studies of microbes present in different fractions of digesta samples, DNA may need to be extracted from isolated microbial cells. In what follows, we describe isolation of bacterial cells and protozoan cells from ruminal samples and subsequent indirect DNA extraction.

Procedures to isolate bacterial cells

Materials

- Sterile cheesecloth.
- Anaerobic phosphate buffered saline (PBS, pH 7.5).
- Coleman buffer: (per litre) 5.0 g K_2HPO_4, 4.0 g KH_2PO_4, 0.52 g NaCl, 70 mg $MgSO_4 \cdot 7H_2O$, 35 mg $CaCl_2$, 5.9 g $NaHCO_3$ and 17.4 mg cysteine hydrochloride, and 0.15% (vol/vol) Tween 80.
- HiTE buffer (50 mM Tris–HCl, 5.0 mM EDTA, pH 8.0).
- TE (10 mM Tris–HCl, 1.0 mM EDTA, pH 8.0).

Isolation of bacteria from liquid fraction

1. Lay two layers of sterile cheesecloth onto a sterile funnel.
2. Place a 250 ml centrifuge bottle underneath the funnel.
3. Transfer 200 g or ml of crude rumen sample into the funnel, and let the rumen fluid drain down to the centrifuge bottle. Keep the retained solid on ice.
4. Centrifuge the filtrate at 16 000 *g* for 10 min at 4°C.
5. Discard the supernatant. Add 10 ml of HiTE buffer to resuspend the pellet.
6. Repeat steps 4 and 5 to wash the bacterial pellet.
7. Aliquot 1.0 ml to each 2 ml screw-cap tube. Centrifuge at 16 000 *g* for 10 min at 4°C to pellet the cells.
8. Discard the supernatant and freeze the pellet at –20°C for DNA extraction.

Isolation of bacteria from associated fraction

9. Wrap and squeeze the rumen solid retained in the cheesecloth to remove residual rumen fluid.
10. Transfer the retained solids into a fresh 250 ml centrifuge bottle. Add 150 ml anaerobic PBS. Shake the bottle by hand vigorously for 30 s.
11. Centrifuge the bottle at 350 *g* for 15 min at 4°C.
12. Transfer the supernatant carefully without disturbing the pellet to a fresh centrifuge bottle. Keep the solid on ice.
13. Pellet the cells at 16 000 *g* for 10 min at 4°C.
14. Discard the supernatant. Resuspend the pellet in 10 ml of HiTE buffer.

15. Repeat steps 13 and 14 to wash the cell pellet.
16. Aliquot 1.0 ml into each 2 ml screw-cap tubes. Centrifuge at 16 000 *g* for 10 min at 4°C.
17. Discard the supernatant and freeze the pellet at –20°C for DNA extraction.

Isolation of bacteria from adherent fraction

18. Transfer the solid on two layers of cheesecloth. Wrap and squeeze the solid to remove the trapped liquid.
19. Transfer the solid into a 250 ml centrifuge bottle. Add 150 ml of Coleman buffer. Shake vigorously by hand for 30 s. Incubate on ice on a rocking platform for 2.5 h.
20. Centrifuged at 500 *g* for 15 min at 4°C.
21. Transfer the supernatant carefully without disturbing the pellet to a fresh centrifuge bottle.
22. Pellet the cells at 16 000 *g* for 10 min at 4°C.
23. Discard the supernatant. Resuspend the pellet in 10 ml of HiTE buffer.
24. Repeat steps 22 and 23 to wash the cell pellet.
25. Aliquot 1.0 ml into each 2 ml screw-cap tubes. Centrifuge at 16 000 *g* for 10 min at 4°C.
26. Discard the supernatant and freeze the pellet at –20°C for DNA extraction.
    This protocol may not completely detach tightly adherent cells. Direct DNA extraction from the solid (from step 18) using the RBB+C method described earlier can be used alternatively to obtain more representative genomic DNA from the adherent fraction.

Alternate method to isolate bacterial cells from rumen content

Materials

Phosphate rinsing buffer (1 l)

– K_2HPO_4, 5.23 g; KH_2PO_4, 2.72 g; $NaHCO_4$, 3.0 g; 0.5 ml 0.1% resazurin.
– Degas by boiling with anaerobic CO_2 sparging.
– While still warm, transfer to anaerobic chamber and add 20 ml of 2.5% cysteine HCl.

Methylcellulose release buffer

– Phosphate buffer plus 0.2% methylcellulose (400 cpoise).
– Add the warm buffer to pre-weighed methylcellulose in a screw cap media bottle, cap and shake to disperse.
– Remove from chamber and refrigerate.
– When cooled, shake to dissolve methylcellulose. Methylcellulose disperses well in warm buffer but only dissolves in cold buffer. If added directly to cold buffer, it will form a lump.

Anaerobic glove bag and preparation

– Use an Aldrich Two-hand AtmosBag (cat. no. Z10 608-9).
– Insert an electrical extension cord through one port and use electrical tape to seal.

– Attach a tygon tube for anaerobic CO_2 addition, tape to seal.
– Attach a vacuum cleaner crevice probe to a third port, tape to seal.
– Use a rubber stopper to seal the crevice tool between evacuations.
– Place equipment and supplies in anaerobic bag and seal.
– Using a vacuum cleaner, evacuate the bag.
– Fill the bag with anaerobic CO_2.
– Evacuate and refill five times.

Procedures to separate bacteria cells in liquid and solid phases of rumen content

1. Collect whole rumen content samples in 250 ml centrifuge bottles.
2. In the anaerobic glove bag, place ~100 ml in a pre-weighed 250 ml beaker and reweigh.
3. Squeeze contents with Bodum coffeemaker plunger, pour off some liquid into a 50 ml beaker and take 4 × 1 ml samples of the liquid in 2 ml tubes using a pipetor with a cut off tip.
4. Discard the remainder of the liquid, add ~100 ml phosphate rinse buffer and stir with a spatula. The phosphate buffer should be used at room temperature, as bacteria may prematurely release in cold buffer.
5. Squeeze contents with Bodum coffeemaker plunger and discard liquid.
6. Repeat steps 4 and 5 another two times, to rinse the fibre a total of three times.
7. Add ~100 ml cold phosphate/methylcellulose release buffer and weigh.
8. Stir and pour beaker contents into a Braun hand blender chopper attachment and homogenize with 3 × 20 s bursts, pausing 10 s between bursts. Return homogenate to beaker.
9. Squeeze contents with Bodum coffeemaker plunger, pour off some liquid into a 50 ml beaker and take 4 × 5 ml samples in 15 ml Falcon tubes.
10. Weigh beaker with squeezed residue.
11. Remove all the samples from the anaerobic glove bag.
12. Centrifuge the 1 ml rumen liquid samples for 10 min at 10 000 *g* in a microcentrifuge and discard the liquid.
13. Centrifuge the 5 ml M/C released samples for 10 min at 10 000 *g* in a high-speed centrifuge and discard the liquid.
14. Add 1.4 ml ASL stool lysis buffer from Qiagen (if using Qiagen stool kit, if not, use lysis buffer of preferred method) to each pellet and resuspend with an ultrasound bath and vortexing.
15. Transfer suspended samples to 2 ml Eppendorf tubes (except the rumen liquid samples already in 2 ml Eppendorf tubes) and store in –80°C freezer.

DNA extractions from isolated bacterial cells

Community DNA can be extracted from the isolated bacterial cells using a number of protocols. We recommend the RBB+C method, because it is rapid, efficient and can minimize differences in DNA extraction between fractionated and whole digesta samples. However, if large molecular genomic DNA is desired, the following protocols can be followed.

Materials
- Lysis buffer: 50 mM Tris–HCl (pH 8.0), 100 mM NaCl, 100 mM EDTA, lysozyme (5 mg/ml), lysostaphin (100 U/ml), mutanolysin (500 U/ml) and RNase (40 μg/ml). The enzymes should be added to the buffer right before use.
- Proteinase K (20 mg/ml).
- SDS (10%).
- Phenol : chloroform : isoamyl alcohol (25 : 24 : 1, PCI).
- 3 M potassium acetate, pH 4.8.
- Ethanol (100% or 95%, molecular biology grade).
- 70% ethanol.
- TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0).

Procedures to extract large molecular-weight DNA
1. To the isolated cell pellet, add 0.5 ml of lysis buffer and resuspend the cells by flicking the tube. Incubate at 37°C for 1 h.
2. Freeze at –80°C for 10 min.
3. Thaw at 60°C for 5 min. Add 100 ml of SDS and 10 μl of proteinase K. Mix and incubate at 75°C for 15 min.
4. Add 0.6 ml of PCI and mix by end-over-end inversion. Centrifuge for 3 min.
5. Transfer the supernatant to a fresh tube containing 0.6 ml of PCI. Mix and centrifuge for 3 min.
6. Transfer the supernatant to a fresh tube. Add 0.1 volume of potassium acetate and 2 volumes of ethanol. Mix well and incubate on ice for 30 min or at −20°C for 15 min.
7. Centrifuge at 16 000 *g* for 15 min at 4°C.
8. Decant the supernatant. Wash the DNA pellet with 1 ml of 70% ethanol.
9. Dry the DNA pellet under vacuum or on bench.
10. Add 100 μl of TE to dissolve the DNA. Aliquot and store the DNA at –20°C, and run 2 μl on a 0.8% gel to check the DNA quality.

DNA extraction from protozoan cells isolated from rumen content

Ruminal protozoa mainly exist in the liquid fraction. Thus, protozoan DNA is represented in the DNA extracted from the liquid fraction and rumen content. If only the ruminal protozoan DNA is desired, however, protozoan cells can be readily separated by taking advantage of their large sizes. The following procedures are essentially the same as described by Sylvester et al. [40] with minor modifications.

Materials
- Sterile cheesecloth.
- Coleman buffer.
- Formaldehyde
- Nylon filter (10 μm pore size).
- HiTE buffer (50 mM Tris–HCl, 5.0 mM EDTA, pH 8.0).
- Lysis buffer: 50 mM Tris–HCl (pH 8.0), 500 mM EDTA, 1.5% SLS, and 40 μg/ml DNase-free RNase.
- Proteinase K (20 mg/ml).

– PCI: phenol : chloroform : isopropanol (25 : 24 : 1).
– 3 M potassium acetate, pH 4.8.
– Ethanol (100% or 95%, molecular biology grade).
– 70% ethanol.

Isolation of ruminal protozoan cells

1. Take 250 ml or g of representative fresh rumen contents. Filter through two layers of cheesecloth to collect the rumen fluid. Keep the rumen fluid at 39°C.
2. Add 100 ml of Coleman buffer (pre-warmed to 39°C) to the solid and mix. Collect the liquid by filtering again.
3. Combine the two filtrates. Incubate at 39°C for 45 min.
4. Remove and discard the scum layer on the surface. Add formaldehyde to the filtrate to a final concentration of 1% and mix.
5. Transfer the filtrate to a centrifuge bottle and centrifuge at 500 *g* for 5 min.
6. Discard the supernatant. Transfer the protozoa pellet to a filter of nylon mesh (10 μm pore size).
7. Wash the retained protozoa with 400 ml of HiTE.
8. Collect the washed protozoa in 2–5 ml of HiTE buffer, depending on the size of the protozoa paste.
9. Aliquot, collect the pellet by centrifugation, and store at –80°C.

Genomic DNA extraction from ruminal protozoan cells

10. Add 0.5 ml of lysis buffer to one protozoa aliquot (from step 9 above). Resuspend the cells and incubate at 65°C for 10 min.
11. Add 50 μl of proteinase K. Mix and incubate at 50°C for 30 min.
12. Freeze at –80°C for 10 min and then thaw at 65°C for 10 min.
13. Centrifuge at 16 000 *g* for 5 min at 4°C.
14. Transfer the supernatant to a fresh 1.5 ml Eppendorf tubes. Add 1.0 volume of PCI. Mix by end-over-end inversion.
15. Centrifuge at 16 000 *g* for 3 min at 4°C.
16. Transfer the supernatant to a fresh 1.5 ml Eppendorf tube.
17. Add 0.1 volume of potassium acetate and 2 volumes of ethanol. Mix by end-over-end inversion. Incubate on ice for 30 min.
18. Centrifuge at 16 000 *g* for 15 min at 4°C to pellet the genomic DNA.
19. Wash the DNA pellet with 70% ethanol and dry the pellet under vacuum for 3 min.
20. Add 100 μl of TE to dissolve the DNA.
21. Run 2 μl on a 0.8% agarose gel to check the DNA quality.
22. Aliquot and store at –20°C.

RNA extraction

Both DNA and RNA can be extracted from the same sample. After cell lysis, both DNA and RNA are precipitated. Then, in separate aliquots, RNase and DNase treatment will lead to DNA and RNA preparations, respectively. For total RNA isolation from

pure cultures, several commercial kits, such as the RiboPure™-Bacteria Kit (cat. no. 1925, www.ambion.com), the RNeasy Protect Bacteria Mini Kit (cat. no. 74524, www.qiagen.com), the TRIzol® Max™ Bacteria RNA Isolation Kit (cat. no. 16096, www.invitrogen.com) and the UltraClean™ Microbial RNA Isolation Kit (cat. no. 15800, www.mobio.com) are available. Here, we only describe a protocol to isolate total microbial RNA directly from digesta samples. This protocol is adapted from the 'two-bird method' developed for simultaneous DNA and RNA extraction from activated sludge samples [49]. When both DNA and RNA are analysed for RNA : DNA ratio (a direct measure of metabolic status), simultaneous extraction of DNA and RNA is advantageous because DNA and RNA are extracted with the same cell lysis efficiency and nucleic acid recovery.

Materials

- Diethyl pyrocarbonate (DEPC).
- Lysis buffer: 500 mM NaCl, 50 mM Tris–HCl, pH 8.0, 50 mM EDTA and 4% SDS. Make the buffer with DEPC-treated water. Add SDS to the lysis buffer after autoclave.
- Zirconia beads (0.1 mm and 0.5 mm in diameter, BioSpec Products, OK, USA). Autoclave the beads in DEPC-treated water and dry them in an oven at 105°C.
- 10 M ammonium acetate made in DEPC-treated water.
- Isopropanol (molecular biology grade).
- 70% ethanol (made in DEPC-treated water).
- Ethanol (100% or 95%, molecular biology grade).
- TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) made in DEPC-treated water.
- RNase-Free DNase set (QIAGEN).
- DNase-free RNase (10 mg/ml).
- Proteinase K (molecular biology grade).
- RNeasy Protect Bacteria Mini Kit (QIAGEN).
- A Mini-Beadbeater-8 or a Mini-Beadbeater (BioSpec Products, OK, USA).
- A water bath (up to 70°C).
- A microcentrifuge (up to 16 000 *g*).

Procedures to extract total RNA

RNA stabilization

1. Transfer 0.25 g of fresh digesta sample into a sterile 2 ml screw-cap tube. Add 0.5 ml of RNAprotect Bacteria Reagent.
2. Immediately mix by vortexing for 1 min. Incubate for 5 min at room temperature.
3. Centrifuge at 16 000 *g* for 10 min at 4°C.
4. Decant the supernatant and leave tubes inverted on a paper towel for 10 s. At this stage, the pellet can be stored at –80°C for up to 4 weeks.

Cell lysis

5. Add 1 ml of lysis buffer, 0.4 g of sterile zirconia beads (0.3 g of 0.1 mm and 0.1 g of 0.5 mm in diameter) and 30 μl of DEPC.
6. Homogenize for 3 min at maximum speed on a Mini-Beadbeater-8.

7. Incubate at 70°C for 15 min, with gentle shaking by hand every 5 min.
8. Centrifuge at 16 000 *g* for 5 min at 4°C. Transfer the supernatant to a fresh 2 ml Eppendorf tube.
9. Add 300 μl of fresh lysis buffer to the lysis tube and repeat steps 2–4. Pool the supernatant.

Precipitation of nucleic acids

10. Add 260 μl of 10 M ammonium acetate to each lysate tube and mix completely. Incubate on ice for 5 min.
11. Centrifuge at 16 000 *g* for 10 min at 4°C.
12. Transfer the supernatant to two 1.5 ml Eppendorf tubes. Add 1.0 volume of isopropanol and mix completely. Incubate on ice for 30 min.
13. Centrifuge at 16 000 *g* for 15 min at 4°C. Remove the supernatant. Wash the nucleic acid pellet with 70% ethanol and dry the pellet under vacuum for 2 min.
14. Dissolve the above nucleic acid pellet in 100 μl of TE. Pool, mix and then divide into two aliquots (100 μl each).

Purification of RNA

15. Add 350 μl Buffer RLT and mix thoroughly.
16. Add 250 ml of ethanol and mix thoroughly.
17. Transfer to an RNeasy mini column and centrifuge for 15 s. Discard the flow-through.
18. Add 500 μl of Buffer RW1 into the RNeasy mini column and centrifuge for 15 s. Discard the flow-through.
19. Add 10 μl of DNase I stock solution to 70 μl of Buffer RDD and mix.
20. Add the above DNase I incubation mix directly onto the RNeasy silica-gel membrane of the RNeasy mini column and incubate at room temperature for 15 min.
21. Add 350 μl of Buffer RW1 into the RNeasy mini column and centrifuge for 15 s. Discard the flow-through.
22. Wash the column twice with 500 μl of buffer RPE with centrifugation for 15 s each.
23. Dry the column by centrifuging the column in a fresh collection tube for 1 min.
24. Add 30–50 μl of RNase-free water directly on the RNeasy silica-gel membrane. Incubate for 1 min. Centrifuge for 1 min to elute the RNA. If the expected RNA yield is >30 μg, use another 30–50 μl water to repeat the elution into the same tube.
25. Aliquot the RNA solution. Run 1 μl on a 1% agarose gel containing 0.2% iodoacetic acid sodium salt as RNase inhibitor to check the RNA quality.
26. Store the RNA aliquots at –20°C.

Purification of DNA

27. Add 100 μl TE to one aliquot from step 14.
28. Follow steps 13–24 of the RBB+C method.

This protocol increases cell lysis efficiency due to the use of bead-beating. If the procedures described for the RNeasy Protect Bacteria Mini Kit is used, the following two modifications are recommended: (1) double the amount of lysozyme and (2) add a

bead-beating step after the lysozyme digestion (step 2 of the procedures in the RNeasy Protect Bacteria Mini Kit handbook, version 3).

Primers and probes for total and major groups of rumen microorganisms (bacterial, fungal, and protozoan)

The primers and probes most commonly used for the detection and quantification of rumen and GI microbes have been based on 16S rRNA gene sequences. This is mainly because of the large number of 16S rRNA sequences in public databases. Unfortunately, the complex secondary structure associated with this gene, and the distribution of variable or conserved regions is sometimes not amenable to optimum assay design. The design of primers and probes begins with a well-aligned file of sequences that includes not only the target sequences, but also representative sequences of major groups and nearest neighbour matches [determined by a search of GenBank or the Ribosomal Database Project, RDP (http://rdp.cme.msu.edu)]. One particularly useful program for primer and probe design is ARB [23]. The ARB program allows you to produce well-aligned sequences and then select sequences of interest to determine potential probe or primer sites. The resulting sequences must also be scrutinized with a primer design program (Oligo or Primer3 are examples), to look for problems such as potential to form primer dimers or complicated secondary structure. Unfortunately, ARB has a relatively steep learning curve and is available only for Linux/Unix operating systems.

On-line resources should be investigated before designing *de novo* probes. One such comprehensive database is ProbeBase (http://www.microbial-ecology.de/ probebase). This database provides details on over 1000 probes, including probes that have been tested for use in FISH assays. The database provides information on the coverage of published group-specific probes and has links that allow the user to check probe coverage using the Probe Match program of the RDP or display an ARB difference alignment. Probe information includes Tm, target position, G+C content and reference information.

A new probe selection program, the ARB Probe Library Client, has been released by the Arb Project (http://www2.mikro.biologie.tu-muenchen.de/arb/probelib.html). This java-based program is platform independent and currently uses a 20 000 sequence small subunit rRNA alignment library that includes many sequences determined from the rumen and GI tract environment. The program displays a phylogenetic tree (based on an ARB parsimony procedure) with branches labelled with the number of exact sequence probe matches (blue) or a percentage label in pink, indicating the percentage of the selected branch that is targeted by the best-existing probe. When the branch is right clicked, the probe server is contacted and a list of possible probes appears on the right of the screen. Selecting a probe results in the targeted sequences in the tree being labelled yellow. The list of probes can be sorted based on Tm, G+C content and number of hits. This program has the potential to greatly speed up probe design and analysis; however, it is limited, at this time, to published sequences.

Tables 1 and 2 list the probes that have been used for rumen and GI tract studies or that have potential in these environments. 16S rRNA hybridization probes may be used for semi-quantitative studies or for FISH assays (see Chapters 6.2 and 6.3). For accurate quantification, real-time PCR-based assays must be used.

PCR-based assays

PCR has been extensively used in ecological studies of rumen and GI microbiomes (see Chapters 3.2, 4.1, 4.4 and 4.5). Universal primers [21, 50] have been frequently used in cloning and PCR–DGGE analysis of 16S rRNA genes present in entire microbiomes. Species- and genus-specific primers (Tables 3 and 4) have also been designed to detect and enumerate individual populations.

PCR-based assays have the potential to be used as the basis for quantitative assays when a fluorescent reporter dye (such as SYBR Green) is used in conjunction with a real-time PCR machine. These assays are far cheaper to develop and run than 5′ nuclease-based assays, but are also prone to error caused by formation of primer dimer and non-specific products. The existing assays have product lengths that vary substantially and are beyond what are recommended for efficient amplifications (75–150 bp up to 300 bp). Additionally, very few robust general assays have been developed, resulting in the available assays targeting less than 90 sequences of the more than 600 available rumen sequences in GenBank. The available assays are best used to track very specific populations and are not generally used to evaluate overall changes in microbial ecology.

5′Nuclease (TaqMan) assays

TaqMan, or 5′ nuclease, assays use a combination of PCR primers and an internal probe labelled with a fluorescent reporter and a quencher (Table 4). When amplification takes place, the fluorescent reporter is cleaved from the probe and the signal is detected by the real-time PCR machine. The initial copy number of the target is directly proportional to the threshold cycle (Ct), which is the cycle at which the reported fluorescence is elevated above a selected background level. Real-time PCR assays are becoming the golden standard by which DNA and RNA abundance changes are evaluated. Unfortunately, there is a paucity of assays available for use with rumen or GI tract samples. The following three assays (Table 4) have been used to study the establishment and effect of a *Megasphaera elsdenii* probiotic bacterial strain on ruminal acidosis [16, 33].

Profiling of gene expression using reverse transcription-PCR

Gene expression can be sensitively analysed by amplification of RNA using RT–PCR. RT–PCR requires the activities of reverse transcriptase (RTase) in making the

Table 1. Useful 16S rRNA hybridization probes for individual groups of rumen microorganisms

Probe (5′→3′)	Target	T_m (°C)	Short name	Reference
GTGCTCCCCCGCCAATTCCT	Archaea		Arc915	[34]
Covers all rumen methanoarchaea				
CTTGTCTCAGGTTCCATCTCCG	*Methanobacteriaceae*	59	MB1174	[34]
Covers all rumen Methanobacteria, including *Methanobrevibacter*				
GGCTCGCTTCACGGCTTCCCT	*Methanosarcinaceae*	60	MSMX860	[34]
Covers cultured rumen *Methanosarcina*, but does not hybridize to a related group identified by 16S rRNA cloning studies				
CGGATAATTCGGGGCATGCTG	*Methanomicrobiales*	56	MG1200	[34]
Covers rumen *Methanomicrobium*				
GTACACACAATGAAGTGCATAAAGG	Rumen fungi	53		[6]
GCTGCCTCCCGTAGGAGT	Bacteria	54	Eub338	[1]
Universal bacterial probe				
AAGGGGCATGATG	Low G+C Gram-positive	34		[24]
Covers many low G+C Gram-positive bacteria, including most rumen Gram-positive bacteria. Demonstrates large variation in hybridization intensities.				
GTTTTRTCAACGGCAGTC	*Clostridium leptum*	46	Clept1240	[36]
Covers Clostridium cluster III, including *Ruminococcus albus* and *R. flavefaciens*				
AAAGCCCAGTAAGCCGC	Cluster III of *Clustridium* sub-phylum, similar coverage to *C. leptum* probe, includes *Ruminococcus*	53	Rfla729	[10b]
89% coverage. Misses three cloned sequences within *R. flavefaciens* cluster				
RTTATGRGGTATTMCA	*Ruminococcus*	34		[19]
CCGCGCGTTGGCACGG	*Fibrobacter*	56	Fibro	[22]
Covers all *Fibrobacter*				
CCAATGTGGGGGACCTT	*Cytophaga-Bacteroides*	49	Bac303	[25]
Covers most rumen *Bacteroides-Prevotella*				
TGGTCCGTGTCTCAGTAC	*Cytophaga-Bacteroides*	50	Cf 319	[25]
Covers most rumen *Bacteroides-Prevotella*				
GCAAGCTAAGGTCCTCTCGA	*Synergistes jonesii*	54		[27]

Note: Published sequence 5′-TGK TAA TAC CYC ATA AY-3′ is the target DNA sequence, which covers most rumen *Ruminococcus*.

Table 2. Useful 16S rRNA hybridization probes for particular species of rumen microorganisms

Probe (5′→3′)	Target	T_m (°C)	Short name	Reference
AACGGCAGTCCTGCTAG	*R. albus*	49	RAL1176	[31]
Hits 7 strains of *R. albus*				
TTCCCTCTGTTAACAGCCAT	*R. albus*	50	RAL1269	[31]
Hits 6 of 7 *R. albus* strains				
GTCATGCGGCTTCGTTAT	*R. albus*	48	RAL196	[31]
Hits 5 of 7 *R. albus* strains, some non-specific hybridization				
AACGGCAGTCCCTTTAG	*R. flavefaciens*	47	Rfl1176	[31]
Hits 17 of 21 *R. flavefaciens*				
TTCTCTTTGTTAATTGCCAT		44	Rfl1269	[31]
Hits 11 of 21 *R. flavefaciens*, also hybridizes to *R. albus* and other non-related strains				
CACTGCAATCGTTGAGCGA	*R. albus*	51	Rfl615	[38]
Hybridizes to 6 *R. albus* strains and some non-related strains				
CCATCCGAAAACTTCCTTCT (78–97)*	*Clostridium aminophilum*			[18]
Species specific				
GGCACCGACCTTTGACAG (824–840)	*Clostridium sticklandii*			[18]
Species specific				
GCACCGATAAGACGCGCTCG (63–78)	*Peptostreptococcus anaerobius* strain C			[18]
Species specific				
CACAAAATCATGCGATTCCGT (187–207)	*Butyrivibrio fibrisolvens* 4 strains: 49, VV1, UC 12254, H17c	55		[9]
Limited scope, only hybridizes to four strains				
CACGTTGTCATGCAACATCGT (187–207)	*Butyrivibrio fibrisolvens* 7 strains	55		[9]
Limited scope, hybridizes to strains isolated from white-tailed deer				

*Numbers in parentheses indicate the probe hybridization region (*E. coli* numbering).

Table 3. Primers used for PCR-based assays of microbial groups

Probe (5′→3′)	Target	Products size (bp)	T_m (°C)	Reference
FW, GGTTATCTTGAGTGAGTT RV, CTGATGGCAACTAAAGAA	12 of 12 strains tested, *Prevotella ruminicola*	485	53	[42]
FW, ACTGCAGCGCGAACTGTCAGA RV, ACCTTACGGTGGCAGTGTCTC	5 of 5 strains tested, *Prevotella bryantii*	540	68	[42]
FW, CAGACGGCATCAGACGAGGAG RV, ATGCAGCACCTTCACAGGAGC	1 of 1 strain tested, *Prevotella albensis*	861		
FW, GGTATGGGATGAGCTTGC RV, GCCTGCCCCTGAACTATC	10 of 10 strains tested, *Fibrobacter succinogenes*	445	60–62	[17, 42]
FW, CAACCAGTCGCATTCAGA RV, CACTACTCATGGCAACAT	2 of 2 strains tested, *Ruminobacter amylophilus*	642	57	[42]
FW, TGCTAATACCGAATGTTG RV, TCCTGCACTCAAGAAAGA	15 of 18 strains tested, *Selenomonas ruminantium-Mitsuokella multiacida*	513	53	[42]
FW, CTAATACCGCATAACAGCAT RV, AGAAACTTCCTATCTCTAGG	9 of 12 strains tested, *Streptococcus bovis*	869	57	[42]
FW, AGTCGAGCGGTAAGATTG RV, CAAAGCGTTTCTCTCACT	1 of 4 strains tested, *Treponema bryantii*	421	57	[42]
FW, GCTTCTGAAGAATCATTTGAAG RV, TCGTGCCTCAGTGTCAGTGT	Type strain, *Eubacterium ruminantium*	671	57	[42]
FW, TGGGTGTTAGAAATGGATTC RV, CTCTCCTGCACTCAAGAATT	3 of 4 strains tested, *Anaerovibrio lipolytica*	597	57	[42]

FW, TGGGAAGCTACCTGATAGAG RV, CCTTCAGAGAGGTTCTCACT	Type strain, *Succinivibrio dextrinosolvens*	854	57	[42]
FW, GGACGATAATGACGGTACTT RV, GCAATCYGAACTGGGACAAT	20 of 20 strains tested, *Ruminococcus flavefaciens*	835	62	[42]
FW, TCTGGAAACGGATGGTA RV, CCTTTAAGACAGGAGTTTACAA	19 of 20 strains tested, *Ruminococcus flavefaciens*	295	55	[17]
FW, CCCTAAACAGTCTTAGTTCG RV, CCTCCTTGCGGTTAGAACA	7 of 7 strains tested, *Ruminococcus albus*	175	55	[17]
FW, CTCCTACGGGAGGCG RV, GCAAGCTAAGGTCCTCTCGA	*Synergistes jonesii*	678	58	[15]
FW, GAGTTTGATCCTGGCTCAG RV, CTGAATGCCTATGGCACCCAA	Specific strain, *Clostridium proteoclasticum*	830	62	[35]
FW, CAYGTCTAAKTATAAATAACTAC RV, CTCTAGGTGATWWGRTTTAC	Ruminal ciliates (*Trichostomatia*)	1535	55	[40]
FW, GCTTTCGWTGGTAGTGTATT RV, ACTTGCCCTCYAATCGTWCT	Ruminal ciliates (*Trichostomatia*)	234	54	[40]

Table 4. 5′ Nuclease (Taqman) assays

Primer (5′→3′)	Target	Product size (bp)	T_m (°C)	Reference
FW, GACCGAAACTGCGATGCTAA RV, CGCCTCAGCGTCAGTTGTC Probe, TCCAGAAAGCCGCTTTCGCCACT	13 of 15 strains tested, *Megasphaera elsdenii*	129	72	[33]
FW, ATGTTAGATGCTTGAAAGGAGCAA RV, CGCCTTGGTGAGCCGTTA Probe, CTCACCAACTAGCTAATA CAACGCAGGTCCA	9 of 12 strains tested, *Streptococcus bovis*	90	72	[16]
FW, ACACACCGCCCGTCACA RV, TCCTTACGGTTGGGTCACAGA Probe, TCGGGCATTCCCAACTCCCATG	38 strains tested, *Pseudobutyrivibrio*	63	72	[16]

complementary DNA (cDNA) and *Taq* DNA polymerase to subsequently amplify the resultant cDNA. The r*Tth* polymerase is a thermal-stable enzyme possessing both the activities. Thereby, it allows for RT–PCR in a single tube and in a single buffer at elevated temperature. This minimizes contamination and facilitates cDNA synthesis from difficult mRNA that has extensive secondary structure. However, this polymerase has lower fidelity than other RTase and DNA polymerases [4]. For this reason, most RT–PCR uses two separate enzymes: an RTase and a DNA polymerase, if the cDNA is to be cloned and sequenced. In this chapter, only two-enzyme-mediated RT–PCR will be described. Those interested in RT–PCR mediated by r*Tth* should consult the papers by Ka et al. [14], Muttray and Mohn [30] and Chadwick et al. [4].

Gene expression can be analysed by RT–PCR either in pure cultures or in microbial communities; and global gene expression or expression of particular genes can be analysed using RT–PCR. However, no general protocols can be applied to all situations. RT–PCR has not been used frequently in studies of gene expression in bacteria of animal GI origin. Here, we provide a general guideline of RT–PCR methods for studies of prokaryotic gene expression in general.

RT–PCR using specific primers targeting particular genes

One-step RT–PCR. One-step RT–PCR systems, such as the QIAGEN OneStep RT–PCR Kit (cat. no. 210210, www.qiagen.com), the BD TITANIUM^{TM} OneStep RT–PCR Kit (cat. no. K14030-1, www.bdbiosciences.com) and the Titan One Tube RT–PCR Kit (cat. no. 1939823, www.roche-applied-science.com), provide the convenience to perform RT–PCR in the preferable one-tube fashion. All these kits provide the enzyme mix (RTase and *Taq* DNA polymerase), buffer and other reagents. Only the primers of choice (both forward and reverse) and RNA template are needed. Set up the RT–PCR reaction according to the manufacturer's recommendation. Include an RT step (usually 37–42°C for 30–60 min) to make the cDNA and a denaturation step (typically 95°C for 10 –15 min) to inactivate the RTase while activating the *Taq* DNA polymerase, before the PCR cycles.

This one-step RT–PCR works well with RNA extracted from pure cultures. If total community RNA is used as the template, however, non-specific products often result. Then, the following two-step RT–PCR can eliminate formation of non-specific products.

Two-step semi-nested RT–PCR for improved specificity. Three specific primers are required in this two-step semi-nested RT–PCR: one forward primer and two reverse primers (one of the reverse primers anneals further downstream of the other reverse primer). In the RT step, only the downstream reverse primer is included in the RT mix to prime the cDNA synthesis. Either Moloney murine leukemia virus RTase (M-MLV-RT) or avian myeoblastosis virus RTase can be used in the RT step. The RT reaction is incubated at 37°–42°C for 30–60 min, depending on the vendors of RTase. After inactivation of the RTase at 70°C for 10 min, a small portion of the RT mix (directly or after ethanol precipitation) can be used as the template in subsequent PCR, in which the forward primer and the upstream reverse primer are used. The volume of the RT reaction should be $<1/5$ of the PCR volume in order to reduce potential non-specific

products formed from the reverse primer that is carried over from the RT reaction. More RNA templates (up to 10 μg) may be needed if community RNA serves as the template.

This two-step RT–PCR can also be used when one-step RT–PCR results in non-specific bands.

Quantitative real-time RT–PCR. The abundance of particular mRNA can be quantified by real-time RT–PCR. Either one-step or two-step RT–PCR can be carried out real-time. The following considerations serve as guidelines in setting up real-time RT–PCR:

1. The external real-time RT–PCR standard should be mRNA also to ensure similar amplification efficiency between the standard and the template. Such RNA standard can be generated by *in vitro* transcription of cloned gene or gene fragment of interest [14, 30]. However, to compare relative expression levels among different physiological conditions, cloned gene or gene fragment can be used directly as the external standard. In that case, real-time RT–PCR does not permit determination of absolute mRNA copies in the samples, but DNA equivalents.
2. In order to minimize variations derived from RNA preparation among samples, an internal standard should be included. The mRNA of a housekeeping gene or 16S rRNA can serve as the internal standard. The determined abundance of the mRNA (mRNA copies or DNA equivalents) among different samples should be first normalized against the internal standard and then compared to each other.
3. Either SYBR Green or an internal probe can be used in real-time RT–PCR. Internal probes improve accuracy because it adds another layer of specificity. SYBR Green assay is cost effective, though its accuracy can be compromised by potential formation of non-specific products or primer dimers. In our laboratory, using carefully designed primers and hot start permitted us to reliably quantify mRNA from *Ruminococcus albus*.

Differential display using RNA arbitrarily primed PCR

Owing to the lack of polyadenylation of prokaryotic mRNA, the differential-display PCR (DD-PCR), originally developed for studies of eukaryotic gene expression, cannot be used to examine gene expression in prokaryotes. The derivative of the DD-PCR, RNA arbitrarily primed-PCR (RAP-PCR), developed also for studies of eukaryotic gene expression [44] and was adapted to studies of gene expression in prokaryotes [48]. The RAP-PCR utilizes an arbitrary primer at a low annealing temperature for the cDNA synthesis during RT reaction. The resultant cDNA is then amplified by PCR using the same arbitrary primer [3, 37] or together with another arbitrary primer [7, 8]. cDNA synthesis can also be primed with random hexamers. In that case, one or a pair of arbitrary primers is used in the subsequent PCR amplification of the cDNA [2, 10]. In what follows is a general protocol to set up a RT reaction of RAP-PCR.

Materials

– 1× M-MLV-RTase buffer.
– 200–400 μM each dNTP.

– 5–10 mM dithiothreitol.
– 0.4–6 μM random hexamers or arbitrary primer.
– 40 U of RNase inhibitor.
– 10 ng–10 μg of total RNA.
– 5–200 U of M-MLV Rtase.

In a 20 μl RT reaction, mix all the above reagents. Perform the RT reaction in a thermocycler using the following temperature ramping: decrement from 50°C to 30°C over 15 min, then incubation at 37°C or 42°C for 1 h and finally 95°C for 5 min.

PCR amplification of the resultant cDNA can be carried out as in regular PCR with $\leq$1/10 volume of the RT reaction as templates. Up to 6% of dimethyl sulphoxide can be included in the PCR to facilitate amplification of cDNA with high G+C content. After electrophoresis (typically, on 6% sequencing gel), the RT–PCR products can be visualized by silver staining. For improved sensitivity, 1–2.5 μCi ($\alpha-^{33}$p)dATP or ($\alpha-^{32}$p)dCTP can be included in the PCR reaction to label the RT–PCR products.

The RAP-PCR bands of interest can be excised, re-amplified, cloned using a TOPO-TA cloning kit (QIAGEN) and sequenced [7, 13]. RNA slot blot or Northern analyses should be used to confirm the differential expression of the genes [8, 13].

To obtain desirable transcription profiling, different primers may have to be tested in RT and PCR reactions. Arbitrary primers designed using the approach of Fislage et al. [7] should improve transcription profiling and improve the chance to identify differential gene expressions.

It should be noted that in most total RNA preparations, rRNA accounts for up to 85% of total RNA. Thus, if rRNA is removed from total RNA, mRNA will be substantially enriched. Consequently, RAP-PCR analysis will be significantly improved in terms of sensitivity and the probability to identify differentially expressed genes. The new MICROB*Express* Kit from Ambion (cat no. 1905), which can remove up to 95% of the 16S and 23S rRNA from total RNA, can be used to enrich mRNA to improve differential profiling by RAP-PCR analysis.

References

1. Amann, R.I., K. Lee, and D.A. Stahl. 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J. Bacteriol.* **172:**762–770.
2. Benson, N.R., R.M.-Y. Wong, and M. McClelland. 2000. Analysis of the SOS response in *Salmonella enterica* Serovar Typhimurium using RNA fingerprinting by arbitrarily primed PCR. *J. Bacteriol.* **182:**3490–3497.
3. Brzostowicz, P.C., K.L. Gibson, S.M. Thomas, M.S. Blasko, and P.E. Rouviere. 2000. Simultaneous identification of two cyclohexanone oxidation genes from an environmental *Brevibacterium* isolate using mRNA differential display. *J. Bacteriol.* **182:**4241–4248.
4. Chadwick, N., A.J. Wakefield, R.E. Pounder, and I.J. Bruce. 1998. Comparison of three RNA amplification methods as sources of DNA for sequencing. *BioTechniques* **25:**818–822.
5. Cullen, D.W., and P.R. Hirsch. 1998. Simple and rapid method for direct extraction of microbial DNA from soil for PCR. *Soil Biol. Biochem.* **30:**983–993.
6. Faichney, G.J., C. Poncet, B. Lassalas, J.P. Jouany, L. Millet, J. Doré, and A.G. Brownlee. 1997. Effect of concentrates in a hay diet on the contribution of anaerobic fungi, protozoa, and bacteria to nitrogen in rumen and duodenal digesta in sheep. *Anim. Feed Sci. Technol.* **64:**193–213.

7. Fislage, R., M. Berceanu, Y. Humboldt, M. Wendt, and H. Oberender. 1997. Primer design for a prokaryotic differential display RT–PCR. *Nuclecic Acids Res.* **25:**1830–1835.
8. Fleming, J.T., W.-H. Yao, and G.S. Sayler. 1998. Optimization of differential display of prokaryotic mRNA: application to pure culture and soil microcosms. *Appl. Environ. Microbiol.* **64:**3698–3706.
9. Forster, R., J. Gong, and R. Teather. 1997. Group-specific 16S rRNA hybridization probes for determinative and community structure studies of *Butyrivibrio fibrisolvens* in the rumen. *Appl. Environ. Microbiol.* **63:**1256–1260.
10. Hanna, M.N., R.J. Ferguson, Y.-H. Li, and D.G. Cvitkovitch. 2001. *uvr*A is an acid-inducible gene involved in the adaptive response to low pH in *Streptococcus mutans*. *J. Bacteriol.* **183:**5964–5973.
10b. Harmsen, H.J.M., G.C. Raangs, T. He, J.E. Degener, and G.W. Welling. 2002. Extensive set of 16S rRNA-based probes for detection of bacteria in human feces. *Appl. Environ. Microbiol.* **68:**2982–2990.
11. Hespell, R.B., D.E. Akin, and B.A. Dehority. 1997. Bacteria, fungi, and protozoa of the rumen, pp. 59–141. *In* R.I. Mackie, B.A. White, and R.E. Isaacson (eds.), *Gastrointestinal Microbiology*, Vol. 2. Chapman and Hall, New York, NY.
12. Holdeman, L.V., I.J. Good, and W.E.C. Moore. 1976. Human fecal flora: variation in bacterial composition within individuals and possible effect of emotional stress. *Appl. Environ. Microbiol.* **31:**359–375.
13. Holmstrom, K., and L. Gram. 2003. Elucidation of the *Vibrio anguillarum* genetic response to the potential fish probiont *Pseudomonas fluorescens* AH2, using RNA-arbitrarily primed PCR. *J. Bacteriol.* **185:**831–842.
14. Ka, J.O., Z. Yu, and W.W. Mohn. 2001. Monitoring the size and metabolic activity of the bacterial community during biostimulation of fuel-contaminated soil using competitive PCR and RT–PCR. *Microbiol. Ecol.* **42:**267–273.
15. Klieve, A.V., D. Ouwerkerk, A. Turner, and R. Roberton. 2002. The production and storage of a fermentor-grown bacterial culture containing *Synergistes jonesii*, for protecting cattle against mimosine and 3-hydroxy-4(1H)-pyridone toxicity from feeding on *Leucaena leucocephala*. *Aust. J. Agric. Res.* **53:**1–5.
16. Klieve, A.V., D. Hennessy, D. Ouwerkerk, R.J. Forster, R.I. Mackie, and G.T. Attwood. 2003 Establishing populations of *Megasphaera elsdenii* YE 34 and *Butyrivibrio fibrisolvens* YE 44 in the rumen of cattle fed high grain diets. *J. Appl. Microbiol.* **95:**621–630.
17. Koike, S., and Y. Kobayashi. 2001. Development and use of competitive PCR assays for the rumen cellulolytic bacteria: *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*. *FEMS Microbiol. Lett.* **204:**361–366.
18. Krause, D., and J. Russell. 1996. An rRNA approach for assessing the role of obligate amino acid-fermenting bacteria in ruminal amino acid deamination. *Appl. Environ. Microbiol.* **62:**815–821.
19. Krause, D., B. Dalrymple, W. Smith, R. Mackie, and C. McSweeney. 1999. 16S rDNA sequencing of *Ruminococcus albus* and *Ruminococcus flavefaciens*: design of a signature probe and its application in adult sheep. *Microbiology* **145:**1797–1807.
20. Krause, D.O., and J.B. Russell. 1996. How many ruminal bacteria are there? *J. Dairy Sci.* **79:**1467–1475.
21. Lane, D.J. 1991. 16S/23S rRNA sequencing, pp. 115–175. *In* E. Stackebrandt and M.D. Goodfellow (eds.), *Nucleic Acid Techniques in Bacterial Systematics*. John Wiley and Sons, New York, NY.
22. Lin, C., and D.A. Stahl. 1995. Taxon-specific probes for the cellulolytic genus *Fibrobacter* reveal abundant and novel equine-associated populations. *Appl. Environ. Microbiol.* **61:**1348–1351.
23. Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Forster, I. Brettske, S. Gerber, A.W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. Konig, T. Liss, R. Lussmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K.H. Schleifer. 2004. ARB: a software environment for sequence data. *Nucleic Acids Res.* **32:**1363–1371.
24. MacGregor, B.J., S. Toze, E.W. Alm, R. Sharp, C.J. Ziemer, and D.A. Stahl. 2001. Distribution and abundance of Gram-positive bacteria in the environment: development of a group-specific probe. *J. Microbiol. Methods* **44:**193–203.

25. Manz, W., R. Amann, W. Ludwig, M. Vancanneyt, and K.H. Schleifer. 1996. Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum *cytophaga-flavobacter-bacteroides* in the natural environment. *Microbiology* **142**(Pt 5)**:**1097–1106.
26. McOrist, A.L., M. Jackson, and A.R. Bird. 2002. A comparison of five methods for extraction of bacterial DNA from human fecal samples. *J. Microbiol. Methods* **50:**131–139.
27. McSweeney, C.S., R.I. Mackie, A.A. Odenyo, and D.A. Stahl. 1993. Development of an oligonucleotide probe targeting 16S rRNA and its application for detection and quantitation of the ruminal bacterium *Synergistes jonesii* in a mixed-population chemostat. *Appl. Environ. Microbiol.* **59:**1607–1612.
28. Miller, D.N., J.E. Bryant, E.L. Madsen, and W.C. Ghiorse. 1999. Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. *Appl. Environ. Microbiol.* **65:**4715–4724.
29. Muttray, A.F., and W.W. Mohn. 2000. Quantitation of the population size and metabolic activity of a resin acid degrading bacterium in activated sludge using slot-blot hybridization to measure the rRNA:rDNA ratio. *Microbiol. Ecol.* **38:**348–357.
30. Muttray, A.F., Z. Yu, and W.W. Mohn. 2001. Population dynamics and metabolic activity of *Pseudomonas abietaniphila* BKME-9 within pulp mill wastewater microbial communities assayed by competitive PCR and RT–PCR. *FEMS Microbiol. Ecol.* **38:**21–31.
31. Odenyo, A.A., R.I. Mackie, D.A. Stahl, and B.A. White. 1994. The use of 16S rRNA-targeted oligonucleotide probes to study competition between ruminal fibrolytic bacteria: pure-culture studies with cellulose and alkaline peroxide-treated wheat straw. *Appl. Environ. Microbiol.* **60:** 3697–3703.
32. Orpin, C.G., and K.N. Joblin. 1998. The rumen anaerobic fungi, pp. 140–195. *In* P.N. Hobson and C.S. Stewart (eds.), *The Rumen Microbial Ecosystem*, 2nd ed. Blackie Academic and Professionals, New York, NY.
33. Ouwerkerk, D., A.V. Klieve, and R.J. Forster. 2002. Enumeration of *Megasphaera elsdenii* in rumen contents by real-time *Taq* nuclease assay. *J. Appl. Microbiol.* **92:**753–758.
34. Raskin, L., J.M. Stromley, B.E. Rittmann, and D.A. Stahl. 1994. Group-specific 16S rRNA hybridization probes to describe natural communities of methanogens. *Appl. Environ. Microbiol.* **60:**1232–1240.
35. Reilly, K., and G.T. Attwood. 1998. Detection of *Clostridium proteoclasticum* and closely related strains in the rumen by competitive PCR. *Appl. Environ. Microbiol.* **64:**907–913.
36. Sghir, A., G. Gramet, A. Suau, V. Rochet, P. Pochart, and J. Dore. 2000. Quantification of bacterial groups within human fecal flora by oligonucleotide probe hybridization. *Appl. Environ. Microbiol.* **66:**2263–2266.
37. Shepard, B.D., and M.S. Gilmore. 1999. Identification of aerobically and anaerobically induced genes in *Enterococcus faecalis* by random arbitrarily primed PCR. *Appl. Environ. Microbiol.* **65:**1470–1476.
38. Simek, K., J. Armengol, M. Comerma, J.C. Garcia, P. Kojecka, J. Nedoma, and J. Hejzlar. 2001. Changes in the epilimnetic bacterial community composition, production, and protist-induced mortality along the longitudinal axis of a highly eutrophic reservoir. *Microbiol. Ecol.* **42:** 359–371.
39. Stewart, C.S., H.J. Flint, and M.P. Bryant. 1998. The rumen bacteria, pp. 10–72. *In* P.N. Hobson and C.S. Stewart (eds.), *The Rumen Microbial Ecosystem*, 2nd ed. Blackie Academic and Professionals, New York, NY.
40. Sylvester, J.T., S.K.R. Karnati, Z. Yu, M. Morrison, and J.L. Firkins. 2004. Development of an assay to quantify rumen ciliate protozoal biomass in cows using real-time PCR. *J. Nutr.* 134:3378–3384.
41. Tajima, K., R.I. Aminov, T. Nagamine, K. Ogata, M. Nakamura, H. Matsui, and Y. Benno. 1999. Rumen bacterial diversity as determined by sequence analysis of 16S rDNA libraries. *FEMS Microbiol. Ecol.* **29:**159–169.
42. Tajima, K., R.I. Aminov, T. Nagamine, H. Matsui, M. Nakamura, and Y. Benno. 2001. Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. *Appl. Environ. Microbiol.* **67:**2766–2774.

43. Walter, J., G.W. Tannock, A. Tilsala-Timisjarvi, S. Rodtong, D.M. Loach, K. Munro, and T. Alatossava. 2000. Detection and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primers. *Appl. Environ. Microbiol.* **66:**297–303.
44. Welsh, J., K. Chada, S.S. Dalal, R. Cheng, D. Ralph, and M. McClelland. 1992. Arbitrarily primed PCR fingerprinting of RNA. *Nuclecic Acids Res.* **20:**4965–4970.
45. Whitford, M.F., R.J. Forster, C.E. Beard, J. Gong, and R.M. Teather. 1998. Phylogenetic analysis of rumen bacteria by comparative sequence analysis of cloned 16S rRNA genes. *Anaerobe* **4:**153–163.
46. Williams, A.G., and G.S. Coleman. 1998. The rumen protozoa, pp. 73–139. *In* P.N. Hobson and C.S. Stewart (eds.), *The Rumen Microbial Ecosystem*, 2nd ed. Blackie Academic and Professionals, New York, NY.
47. Wilson, K., and R. Blitchington. 1996. Human colonic biota studied by ribosomal DNA sequence analysis. *Appl. Environ. Microbiol.* **62:**2273–2278.
48. Wong, K.K., and M. McClelland. 1994. Stress-inducible gene of *Salmonella typhimurium* identified by arbitrarily primed PCR of RNA. *PNAS* **91:**639–643.
49. Yu, Z., and W.W. Mohn. 1999. Killing two birds with one stone: simultaneous extraction of DNA and RNA from activated sludge biomass. *Can. J. Microbiol.* **45:**269–272.
50. Yu, Z., and M. Morrison. 2004. Comparisons of different hypervariable regions of *rrs* genes for use in fingerprinting of microbial communities by PCR-denaturing gel electrophoresis. *Appl. Environ. Microbiol.* **70:**4800–4806.
51. Yu, Z., and M. Morrison. 2004. Improved extraction of PCR-quality community DNA from digesta and fecal samples. *BioTechniques* **36:**808–812.

3.2. Quantitative (real-time) PCR

STUART E. DENMAN and CHRISTOPHER S. MCSWEENEY
CSIRO Livestock Industries, 306 Carmody Rd, St Lucia, Queensland 4067, Australia

Introduction

Many nucleic acid-based probe and PCR assays have been developed for the detection tracking of specific microbes within the rumen ecosystem [4–6, 8–11, 14, 15]. Conventional PCR assays detect PCR products at the end stage of each PCR reaction, where exponential amplification is no longer being achieved. This approach can result in different end product (amplicon) quantities being generated [3]. In contrast, using quantitative, or real-time PCR, quantification of the amplicon is performed not at the end of the reaction, but rather during exponential amplification, where theoretically each cycle will result in a doubling of product being created [13]. For real-time PCR, the cycle at which fluorescence is deemed to be detectable above the background during the exponential phase is termed the cycle threshold (Ct). The Ct values obtained are then used for quantitation, which will be discussed later.

Quantitative PCR allows the researcher to view the entire reaction and product being generated throughout all stages of the reaction. In its simplest and cheapest form, real-time PCR employs the DNA-binding dye, SYBR Green. SYBR Green binds to the minor groove of double-stranded DNA and fluoresces at a much higher intensity when bound to double-strand DNA when compared with the dye in free solution. As the amplification reaction proceeds and more double-stranded amplicons are produced, the SYBR Green dye fluorescence signal will increase and can be detected. As SYBR Green will bind indiscriminately to any double-stranded piece of DNA, it is important that the assay is completely optimized so that only the specific target of interest is amplified and that non-specific products or primer dimers are excluded. Dissociation curve analysis is performed at the completion of the amplification cycles to reveal the purity of the amplicon produced for each reaction. The dissociation curve is produced by monitoring the loss of fluorescence signal, as the temperature is slowly raised from 60°C to 95°C causing the double-stranded amplicon to dissociate and the SYBR Green to be released. A single specific amplicon product will dissociate at a given melting temperature, producing a single sharp dissociation curve. Any non-specific products or primer dimers will be detected during this analysis as multiple or broader dissociation peaks.

H.P.S. Makkar and C.S. McSweeney (eds.), Methods in Gut Microbial Ecology for Ruminants, 105–115.
© 2005 *IAEA. Printed in the Netherlands.*

Other detection assay systems have evolved that show greater specificity than the SYBR Green assay by combining a fluorescence reporter dye with a quencher dye. The most common of these, the 5′ Nuclease (Taqman) assay, utilizes target specific amplification primers and a probe that contains both the reporter and quencher chemistry. The probe is designed in such a way, that while intact, the quencher is in close proximity to the fluorescence reporter and quenches any signal. The probe will bind to its target site on the template DNA between the amplification primers. As the *Taq* polymerase extends along the template, the 5′-nuclease activity of the polymerase will degrade the probe, resulting in an increase in detectable fluorescence, as the quencher is separated from the reporter flurophore. This type of detection does not suffer from the presence of non-specific product amplification or primer dimers, as long as the probe is sequence specific.

Regardless of which method is employed, several common requirements should be addressed so as to produce an accurate and robust assay. (1) The amplicon product size should be restricted to a size range of 50–200 bp, as amplification efficiencies of 2 (a doubling of product at each cycle) are more easily obtainable when the amplicon size is kept to a minimum (<100 bp). (2) Optimizing primer location to reduce template secondary structure interference [1] will increase the efficiency of amplification. (3) Assay conditions, such as $MgCl_2$ and primer concentrations, should be optimized [2] prior to undertaking any quantitative assays. The lowest optimal primer concentrations that results in efficient amplification but no primer dimmer are critical when developing an SYBR Green assay. (4) When undertaking the development of a new SYBR Green assay, it is important to initially analyze PCR end products visually, using agarose gel electrophoresis, to ascertain the absence of primer dimers and non-specific amplification products. (5) A serial dilution of template should be performed in order to investigate and calculate the PCR efficiency of the assay.

Two methods are used for quantification of Ct values so as to allow the values to be interpreted with respect to expression levels. Either direct quantification from a generated standard curve of known concentrations of target or by relative expression (ΔCt), a comparison is made between the target gene and a 'house-keeping gene' (reference gene). Theoretically, there is a quantitative relationship between the amount of starting target sample and the amount of amplicon at any given cycle within the exponential stage of amplification [13]. A change of a single Ct value will represent a twofold difference in starting material, whereas a change of 3.331 cycles will represent a 10-fold difference in starting material based on the equation 2^n (where $n = \Delta$Ct) (Fig. 1). The observed Ct values are plotted against the standard curve log concentrations. The slope of the standard curve can be used to determine the exponential amplification and efficiency of the PCR reaction by the following equation [13]:

$$\text{Exponential amplification} = 10^{(-1/\text{slope})}$$

For direct quantification assays, an experimental Ct value is plotted against the standard curve, and the target gene starting concentration can then be extrapolated.

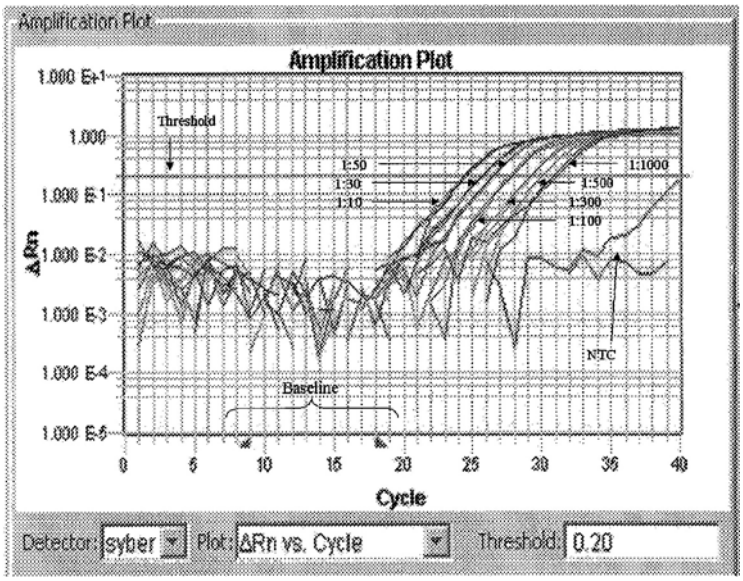


Figure 1. A typical standard curve plot showing the change in Ct values in relation to the amount of template added. Threshold and baseline settings are indicated.

For relative expression, a ΔCt value is calculated as the difference in observed Ct values between the target gene and a reference gene:

$$\Delta\text{Ct} = \text{Ct(target)} - \text{Ct(reference)}.$$

The analysis relies on a consistent expression level being maintained by the reference gene throughout all experiments. The most common genes picked for reference are the ribosomal genes, Glyceraldehyde-3-phosphate dehydrogenase or β-actin [2]. For quantification assays within the field of rumen microbial ecology, the 16S ribosomal gene would be the reference gene of choice. The reference amplicon and the target amplicon should possess similar amplification efficiencies if they are to be considered robust enough for comparative analysis. To calculate this, a plot of the ΔCt values for each target from the same initial template dilution series is created. If the efficiencies of the two amplicons are approximately equal, the plot of log concentrations vs. ΔCt will produce a line approaching the horizontal with a slope of <0.10. This indicates that both PCRs perform with equal efficiency and meaningful data can be calculated from this assay.

$\Delta\Delta$Ct analysis calculates the relative change in expression when comparing the ΔCt value of the target gene to the reference gene under a control condition compared to the ΔCt of the sample condition [12].

$$\text{Comparative expression level} = 2^{-(\Delta\text{Ct sample} - \Delta\text{Ct control})}$$

For example, if ΔCt control = 5 and ΔCt sample = 3, then the comparative expression level = $2^{-(-2)}$resulting in an eightfold increase in gene expression.

If performing relative expression quantification assays with primers that do not exhibit exact amplification efficiencies, then the amplification efficiencies should be considered when calculating the relative expression values. Pfaffl [12] introduced a new mathematical formula that takes into consideration the amplification efficiencies when calculating the relative expression levels. The formula is as follows:

$$\text{Relative expression} = \frac{(E_{\text{target}})^{\Delta\text{Ct target(control} - \text{sample)}}}{(E_{\text{target}})^{\Delta\text{Ct reference(control} - \text{sample)}}}$$

The advantage of comparative expression level quantification methods is that they do not require standard curves to be run with each sample, thus reducing the number of reactions required.

Researches within the rumen microbial ecology field have successfully employed real-time PCR techniques for the enumeration and tracking of *Megasphaera elsdenii* and *Butyrivibrio fibrisolvens* dosed into the cattle rumen ([4, 11], see Chapter 3). Quantification of *M. elsdenii* within the rumen after dosing was achieved by direct comparison to a standard curve of known numbers of *M. elsdenii* cultured *in vitro* [11]. They were also able to demonstrate how a standard curve should be generated, for the analysis of crude rumen fluid samples. The addition of rumen fluid to the *M. elsdenii* DNA, prior to creating the standard curve, was essential for maintaining a more accurate standard curve for extrapolating numbers when handling experimental samples of rumen fluid from cattle. The rumen fluid spiked standard curve was found to produce a curve parallel to that of the pure culture DNA while maintaining a slope approaching 1. If quantification was performed only on the pure DNA standard curve, this would have resulted in a calculation error that would have over-estimated the number of *M. elsdenii* present in the rumen by 10-fold [11].

Tajima and colleagues [15] published the design and evaluation of primers against 12 different rumen bacterial species and clearly demonstrated the importance of primer design when they were able to illustrate large differences in Ct values observed for amplification from the same quantities of starting template [15]. This observation is of importance not just in relation to quantification experiments, but as they discuss, may possibly explain the under-representation of some bacteria such as *Fibrobacter succinogenes* that have been only sparsely identified in 16S clone libraries.

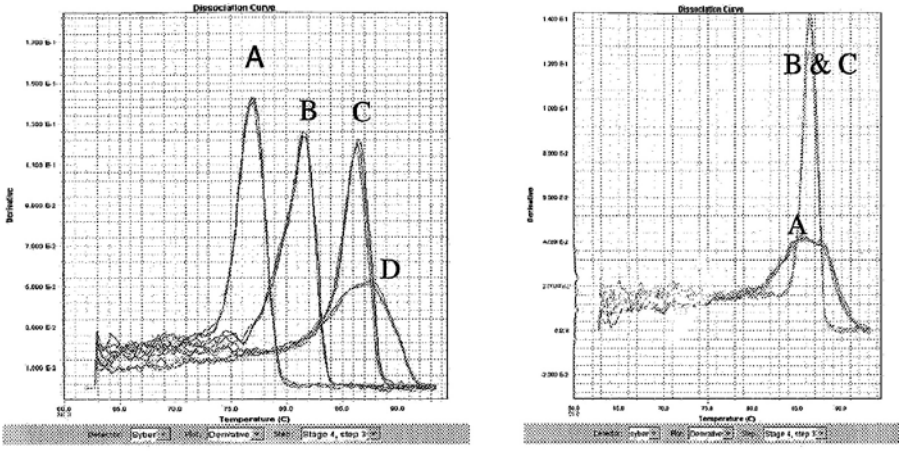
In this chapter, we describe the methods for an SYBR Green qPCR assay, which we have developed for the detection and relative expression calculations of rumen fungi, *F. succinogenes* and *Ruminococcus flavefaciens* populations, compared to total rumen bacterial numbers within the rumen. Newly designed primers were developed to specifically amplify target populations (Table 1). Bacterial primers were targeted against the 16S ribosomal gene while the rumen fungal specific primers were designed to amplify a portion of the internal transcribed spacer 1 (ITS1) region. All primers were designed using primer express software from Applied Biosystems (ABI) and

Table 1. Primers for qPCR

Target species	Forward primer	Reverse primer	Amplicon
General bacterial	CGGCAACGAGCG-CAACCC*	CCATTGTAGCACGT-GTGTAGCC	130 bp
General anaerobic fungi	GAGGAAGTAAAAGTCG-TAACAAGGTTTC	CAAATTCACAAA-GGGTAGGATGATT	120 bp
F. succinogenes	GTTCGGAATTACTGGG-CGTAAA	CGCCTGCCCCTGA-ACTATC	121 bp
R. flavefaciens	CGAACGGAGAT-AATTTGAGTTTACTTAGG	CGGTCTCTGTATGTT-ATGAGGTATTACC	132 bp

*Modified from Ref. [7].

to have a melting temperature (T_m) of ~60°C. It should be noted that a product is expected to amplify in the no template control (NTC) reaction when using recombinant *Taq* polymerase with the broad bacterial primers. This amplification product exhibits a different dissociation curve when compared with rumen microbial sample (Fig. 2B) having a T_m of ~86°C. The use of a native *Taq* polymerase does not produce this amplicon, and it is, therefore, most likely to be an amplification product generated from contaminating *E. coli* DNA during recombinant *Taq* polymerase enzyme production. In addition reactions performed, using the bacterial primer set and *E. coli* DNA as template produced an identical dissociation curve (Fig. 2B).



A. B.

Figure 2. A: Dissociation curves for (A) total fungi (T_m = 77°C), (B) *R. flavefaciens* (T_m = 82°C), (C) *F. succinogenes* (T_m = 86°C) and (D) total bacteria (T_m = 82–89°C). B: Dissociation curves for (A) total bacteria (T_m = 82–89°C), (B) no template control with bacterial primers (T_m = 86°C) and (C) *E. coli* template with bacterial primers (T_m = 86°C).

Standard curve generation for qPCR

Each qPCR reaction is performed in quadruplicate 5 μl reaction volumes if using an ABI PRISM® 7900HT Sequence Detection System 384 well machine. Reaction volumes will need to be increase to 10 μl if using a 96-well plate. Preparation of a primer master mix is crucial to reduce pipetting errors.

Reagents

- Platinum SYBR Green qPCR Supermix UDG (Invitrogen cat # 11733–038).
- Template – Dilutions of DNA extracted from rumen fluid (1 : 10, 1 : 30, 1 : 50, 1 : 100, 1 : 300, 1 : 500, 1 : 1000, NTC). First dilution should be ~10 ng/μl of DNA.
- Primers: specific primers of interest (Table 1).
  - Broad rumen bacterial;
  - Broad rumen fungi;
  - *R. flavefaciens*;
  - *F. succinogenes.*

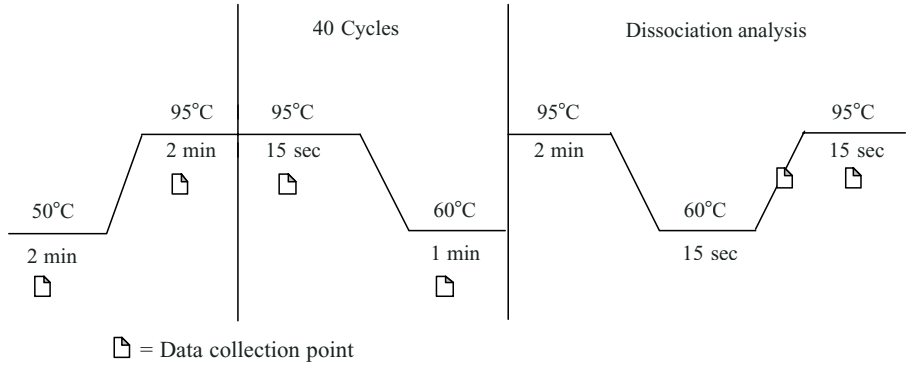
Method

1. Set up a primer master mix for each primer pair with all components except template as specified below:

	Primer master mix (μl)	Final conc.
2× SYBR Master Mix[a]	12.5 × n[b]	1×
50× ROX	0.5 × n	1×
Forward primer (10 μM)	0.75 × n	300 nM
Reverse primer (10 μM)	0.75 × n	300 nM
Template	–	
Distilled H_2O	5.5 × n	
Final volume	25 × n	

[a]Final concentration of Mg^{2+} is 3 mM.
[b]n is the number of reactions + 1.

2. Vortex primer master mix well. Pipette 20 μl of mix into a 96-well PCR plate (or 0.2 ml microcentrifuge tube).
3. Add 5 μl of sample template (i.e. dilutions of rumen samples).
4. Seal and vortex for 5–10 s. Spin for 1 min at 3000 *g* to collect contents to bottom of wells.
5. Transfer each reaction in quadruplicate to a 384-well plate. Seal with optical tape and spin briefly (1 min at 3000 *g*).
6. Place reactions in real-time thermal cycler, programmed as follows:



Data and standard curve analysis

Outliers, baseline and threshold

1. Firstly, exclude outlying wells, including samples with atypical plots or that 'creep', i.e. that do not exhibit bona-fide amplification (with three points in a log phase). This may include NTCs that creep up over the threshold.
2. Set baseline cycles 1–2 cycles before the reporter dye signal begins to increase.
3. Set threshold halfway in the log part of the amplification plot (use semi-log scale).
4. Examine the dissociation curves to ascertain if a single amplicon was generated for each primer pair used.
   - To calculate the amplification efficiency firstly analyse the standard curve, by plotting the log 10 (template dilution) vs. mean Ct of replicate reactions (Fig. 3).
   - Obtain the slope of the line and calculate the R^2 value. R^2 should be approaching a value of 1, thus representing a linear relationship.
   - To calculate the PCR efficiency, use the following equation:

$$\text{Efficiency} = 10^{-1/\text{slope}}$$

   a value approaching 2 = 100% efficiency for amplification.
   - In order to perform ΔCt analysis (relative expression), both primer pairs must have similar amplification efficiencies.

Analysis of rumen samples by qPCR

Each qPCR reaction is performed in quadruplicate 5 μl reaction volumes. Preparation of a primer master mix is crucial to reduce pipetting errors.

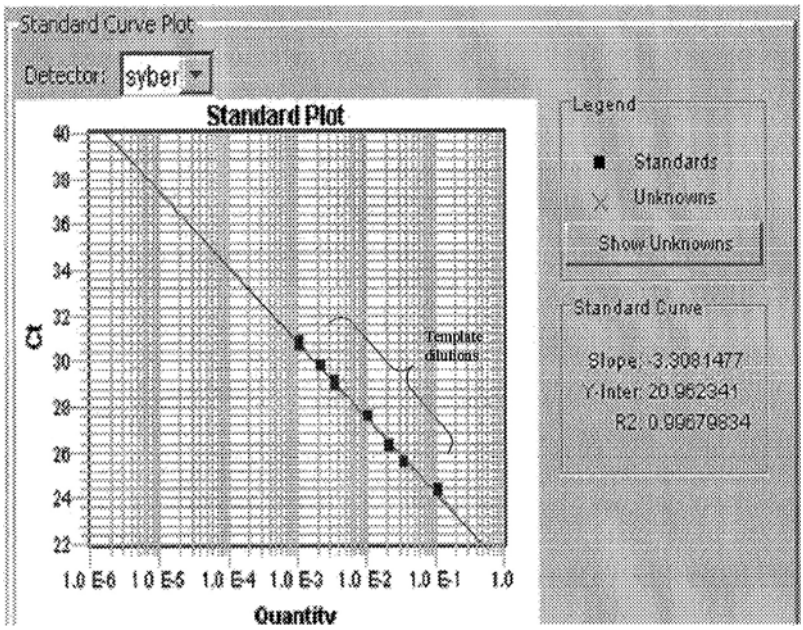


Figure 3. Standard curve plot of log 10 (template dilution) vs. mean Ct value.

Reagents

As per previous setup for standard curve analysis (see above).

Method

1. Set up a primer master mix for each primer pair with all components except template as specified below:

	Reaction mix (μl)
2× SYBR Master Mix[a]	12.5 × n[b]
50× ROX	0.5 × n
Forward primer (10 μM)	0.75 × n
Reverse primer (10 μM)	0.75 × n
Template	–
Distilled H_2O	5.5 × n
Final volume	25 × n

[a]Final concentration of Mg^{2+} is 3 mM.
[b]n is the number of reactions + 1.

2. Vortex primer master mix well. Pipette 20 μl of mix into a 96-well microtitre plate (or 0.2 ml microcentrifuge tube).
3. Add 5 μl of sample template.
4. Seal and vortex well for 5–10 s. Spin to collect contents to bottom of wells.

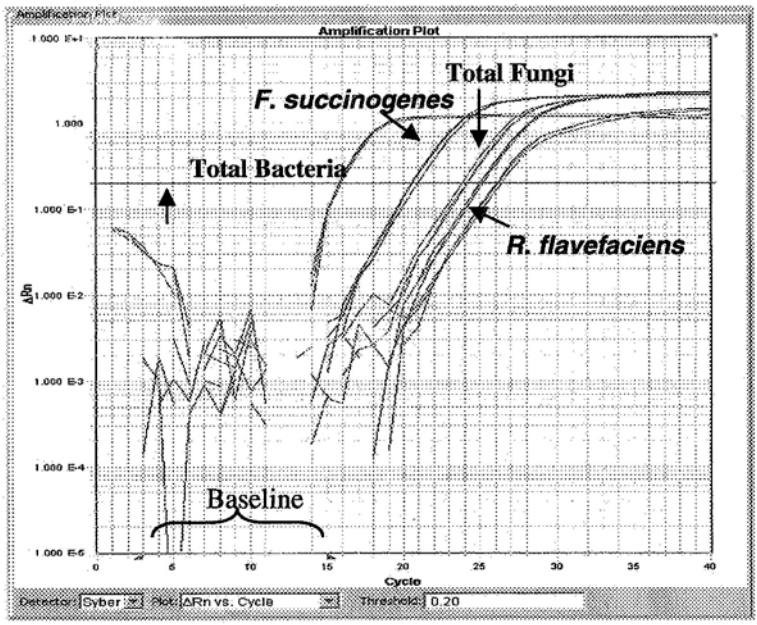


Figure 4. A typical amplification curve plot for the primer sets using microbial rumen DNA as template. Threshold and baseline settings are indicated.

5. Transfer each reaction in quadruplicate to a 384-well plate. Seal with optical tape and spin briefly.
6. Place reactions in real-time thermal cycler programmed as follows:

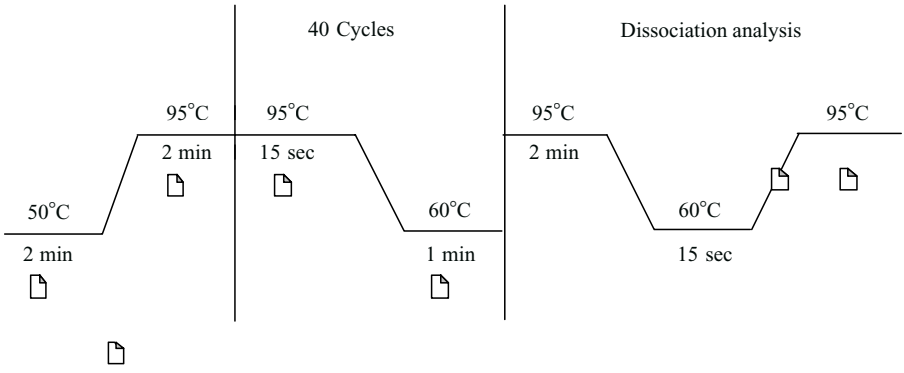


Table 2. Example of data analysis for calculating relative expression to total bacteria

Amplicon	Ct	ΔCt	$2^{-\Delta Ct}$
Total bacteria	16	0	1
Total fungi	24	8	1/256
R. flavefaciens	25	9	1/512
F. succinogenes	21	5	1/32

Table 3. Example of data analysis for calculating relative expression of experimental data to control data

Amplicon	$Ct_{reference}$	Ct_{target}	ΔCt	$\Delta\Delta Ct$	$2^{-\Delta\Delta Ct}$
Control	20	25	5	0	1
Sample 1	21	28	7	2	1/4
Sample 2	20	22	2	−3	8

Data analysis

Process data as before for removal of outliers and the setting of baseline and threshold values (see above) (Fig. 4).

Examine the dissociation curves to ascertain if a single amplicon was generated for each primer pair used (see above Fig. 2).

To convert a ΔCt value to a ratio of target to reference, simply use the following equation (Table 2):

$$2^{-\Delta Ct} = 2^{-(Ct\ target - Ct\ reference)}$$

To calculate $\Delta\Delta Ct$ values and their relative expression values for a target against a reference gene, use the following equation (Table 3):

$$\text{Comparative expression level} = 2^{-(\Delta Ct\ sample - \Delta Ct\ control)}$$

References

1. Boeckman, F., M. Brisson, and L. Tan. 2001. *Real-Time PCR: General Considerations. Bio-Rad Tech Note 2593.* Bio-Rad Laboratories, Inc., Hercules, CA.
2. Bustin, S.A. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J. Mol. Endocrinol.* **25:**169–193.
3. Freeman, W.M., S.J. Walker, and K.E. Vrana. 1999. Quantitative RT–PCR: pitfalls and potential. *Biotechniques* **26:**112–125.
4. Klieve, A.V., D. Hennessy, D. Ouwerkerk, R.J. Forster, R.I. Mackie, and G.T. Attwood. 2003. Establishing populations of *Megasphaera elsdenii* YE 34 and *Butyrivibrio fibrisolvens* YE 44 in the rumen of cattle fed high grain diets. *J. Appl. Microbiol.* **95:**621–630.
5. Koike, S., and Y. Kobayashi. 2001. Development and use of competitive PCR assays for the rumen cellulolytic bacteria: *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*. *FEMS Microbiol. Lett.* **204:**361–366.
6. Krause, D., B. Dalrymple, W. Smith, R. Mackie, and C. McSweeney. 1999. 16S rDNA sequencing of *Ruminococcus albus* and *Ruminococcus flavefaciens*: design of a signature probe and its application in adult sheep. *Microbiology* **145:**1797–1807.
7. Lane, D.J. 1991. 16S/23S rRNA sequencing, pp. 115–175. *In* E. Stackebrandt and M.D. Goodfellow (eds.), *Nucleic Acid Techniques in Bacterial Systematics*. John Wiley and Sons, New York, NY.
8. Lin, C., and D.A. Stahl. 1995. Taxon-specific probes for the cellulolytic genus fibrobacter reveal abundant and novel equine-associated populations. *Appl. Environ. Microbiol.* **61:**1348–1351.
9. McSweeney, C.S., R.I. Mackie, A.A. Odenyo, and D.A. Stahl. 1993. Development of an oligonucleotide probe targeting 16S rRNA and its application for detection and quantitation of the ruminal bacterium *Synergistes jonesii* in a mixed-population chemostat. *Appl. Environ. Microbiol.* **59:**1607–1612.

10. Odenyo, A.A., R.I. Mackie, D.A. Stahl, and B.A. White. 1994. The use of 16S rRNA-targeted oligonucleotide probes to study competition between ruminal fibrolytic bacteria: pure-culture studies with cellulose and alkaline peroxide-treated wheat straw. *Appl. Environ. Microbiol.* **60:**3697–3703.
11. Ouwerkerk, D., A.V. Klieve, and R.J. Forster. 2002. Enumeration of *Megasphaera elsdenii* in rumen contents by real-time *Taq* nuclease assay. *J. Appl. Microbiol.* **92:**753–758.
12. Pfaffl, M.W. 2001. A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Res.* **29:**2002–2007.
13. Rasmussen, R. 2001.Quantification on the LightCycler, pp. 21–34. *In* S. Meuer, C. Wittwer, and K. Nakagawara (eds.), *Rapid Cycle Real-Time PCR*. Methods and Applications Springer Press, Heidelberg.
14. Reilly, K., and G.T. Attwood. 1998. Detection of *Clostridium proteoclasticum* and closely related strains in the rumen by competitive PCR. *Appl. Environ. Microbiol.* **64:**907–913.
15. Tajima, K., R.I. Aminov, T. Nagamine, H. Matsui, M. Nakamura, and Y. Benno. 2001. Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. *Appl. Environ. Microbiol.* **67:**2766–2774.

PART FOUR

Molecular fingerprinting techniques for genotypic analysis of pure cultures and microbial communities

4.1. Denaturing gradient gel electrophoresis

SVETLANA A. KOCHERGINSKAYA, ISAAC K.O. CANN and
RODERICK I. MACKIE
Department of Animal Sciences, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

Introduction

It is worthwhile considering that only some 30 species make up the bulk of the bacterial population in human faeces at any one time based on the classical cultivation-based approach [7, 14]. The situation in the rumen is similar. Thus, it is practical to focus on specific groups of interest within the complex community. These may be the predominant or the most active species, specific physiological groups or readily identifiable (genetic) clusters of phylogenetically related organisms. Several 16S rDNA fingerprinting techniques can be invaluable for selecting and monitoring sequences or phylogenetic groups of interest and are described below.

Over the past few decades, considerable attention was focussed on the identification of pure cultures of microbes on the basis of genetic polymorphisms of DNA encoding rRNA such as ribotyping, amplified fragment length polymorphism and randomly amplified polymorphic DNA [19]. However, many of these methods require prior cultivation and are less suitable for use in analysis of complex mixed populations although important in describing cultivated microbial diversity in molecular terms. Much less attention was given to molecular characterization of complex communities. In particular, research into diversity and community structure over time has been revolutionized by the advent of molecular fingerprinting techniques for complex communities [15]. Denaturing or temperature gradient gel electrophoresis (DGGE/TGGE) methods have been successfully applied to the analysis of human [1, 6, 8, 20, 26–28], pig [22, 23], cattle [9], dog [21] and rodent [5, 25, 12] intestinal populations.

DGGE/TGGE

DGGE is a genetic fingerprinting technique that enables separation of double-stranded DNA fragments up to 500 bp in length utilizing either a denaturing or temperature gradient gel [15, 16]. Separation of similar length PCR amplified fragments is achieved by denaturation within discrete melting domains, which result in characteristic

H.P.S. Makkar and C.S. McSweeney (eds.), Methods in Gut Microbial Ecology for Ruminants, 119–128.
© 2005 *IAEA. Printed in the Netherlands.*

banding patterns from PCR product mixtures. Increased resolution of banding patterns is achieved through addition of a GC clamp during PCR amplification [16]. In principle, DGGE can be used for analysis of PCR amplified ribosomal genes, or functional genes, from mixed microbial communities. For studies on microbial diversity and ecology, or community structure and dynamics, the 16S rDNA is particularly useful due to its mosaic structure comprising highly conserved and hypervariable regions. The application of mixed PCR product to a DGGE gel results in a pattern of bands, which corresponds with the predominant species or assemblages (phylotypes) of the microbial community under study. Individual bands, separated by DGGE, can also be identified by direct cloning and sequencing, or by hybridization with group or genus specific DNA probes. This technique is widely used in molecular microbial ecology and has been successfully applied to analyse intestinal and faecal bacterial banding profiles of pigs, rodents and dogs [5, 21–23]. Improvements from these studies are optimization of DNA extraction from faecal samples, inclusion of standard DNA fragments from known gut bacteria, which allow more precise gel analysis and between gel comparisons, as well as image capture and analysis. These improvements have enabled us to describe temporal and spatial changes in bacterial populations as a result of diet, dosing of exogenous probiotic bacteria and antibiotic therapy. Importantly, this has demonstrated that each individual animal had a unique but stable and repeatable banding pattern over time. This technique is less labour-intensive and biased than traditional cloning and enables rapid estimation of microbial diversity.

Other applications of this technique include identifying 16S rDNA sequence heterogeneity [18]; the study of gene diversity such as tetracycline genes [2, 3]; monitoring specific physiological groups; monitoring enrichment and facilitating isolation [15]. Similarity indices need to be calculated using numerical methods such as the Shannon–Weaver index [18, 27] and other indices [21–23]. These indices result in a more objective approach to analysing the effect of location, diet or supplementation on gut microbial populations.

DGGE protocols

Genomic DNA isolation

There are many different techniques describing isolation of total community genomic DNA from various environments. We use the modified method suggested by Tsai and Olson [24] that is time-consuming, but results in more complete recovery of high quality DNA. Commercially available kits such as UltraClean Soil DNA Isolation kit (Mo Bio Laboratories, Solana Beach, CA, USA) and FastDNA Spin Kit (For Soil) (Q BIOGENE, Carlsbad, CA, USA) can be used for rapid isolation of genomic DNA of suitable quality. If necessary, further purification of extracted DNA to remove inhibitory substances can be achieved by performing spin-column chromatography using PVVP [4], Sepharose 4B or Sephadex 200 [10, 13]. Here, we describe the details of Tsai and Olson [24] protocol.

Reagents
- Lysozyme (Sigma, St. Louis, USA)
- Proteinase K
- Phenol, pH 8.0 (Sigma)
- Phenol : chloroform : isoamyl alcohol, 25 : 24: 1 (Sigma)
- Chloroform : isoamyl alcohol, 24 : 1 (Sigma)
- Tris
- EDTA disodium salt
- SDS
- Isopropanol

Solutions

Phosphate buffered saline (PBS)
- 120 mM Na-phosphate buffer, pH 8.0
- 0.85% NaCl, autoclave

STS solution
- 0.1 M NaCl
- 0.48 M Tris–HCl, pH 8.0, autoclave
- 10% SDS

TE buffer, pH 8.0
- 10 mM Tris–HCl, pH 8.0
- 1 mM EDTA, pH 8.0, autoclave

10.5 M ammonium acetate
- 80.93 g in distilled H_2O, final volume 100 ml, filter sterilize

Lysis solution
- 0.15 M NaCl
- 0.1 M EDTA, pH 8.0, autoclave, then add lysozyme (15 mg/ml)

Proteinase K stock solution
- 5 mg/ml H_2O

Protocol
1. Mix 2–10 g sample with 10 ml PBS, vortex.
2. Centrifuge at 6000 *g*, 4°C, for 10 min.
3. Discard the supernatant.
4. Repeat steps 1–3.
5. Add 2 ml lysis solution per 1 g of the sample.
6. Incubate with gentle shaking for 30 min to 2 h at 37°C.
7. Add an equal volume of STS solution.
8. Incubate for 30 min at 37°C with gentle shaking.
9. Freeze at −20°C, thaw and bring to room temperature, repeat it twice (total three 'freeze–thaw' cycles).

10. Add Proteinase K to a final concentration 50 μg/ml.
11. Incubate for 30 min at 60°C.
12. Centrifuge the lysate at 10 000 *g*, 4°C, for 20 min, transfer the supernatant to a clean tube.
13. Extract the supernatant with an equal volume of phenol, pH 8.0, briefly vortex or mix the phases by gentle shaking.
14. Centrifuge at 10 000 *g*, 4°C, for 20 min, transfer the upper aqueous phase to a clean tube.
15. Extract the aqueous phase with an equal volume of phenol/chloroform/isoamyl alcohol (24 : 24 : 1), briefly vortex or mix the phases by gentle shaking.
16. Centrifuge at 10 000 *g*, 4°C, for 20 min, transfer the upper aqueous phase to a clean tube.
17. Extract the aqueous phase with an equal volume of chloroform/isoamyl alcohol, briefly vortex or mix the phases by gentle shaking.
18. Centrifuge at 10 000 *g*, 4°C, for 20 min, transfer the upper aqueous phase to a clean tube.
19. Add 10.5 M ammonium acetate solution to the aqueous phase from step 18 to a final concentration 2.5 M.
20. Precipitate the DNA with an equal volume of isopropanol. Place the tubes at −80°C for at least 30 min.
21. Centrifuge at 10 000 *g*, 4°C, for 30 min.
22. Discard the supernatant, wash the pellet once with 70% ethanol stored at −20°C. Carefully remove 70% ethanol and dry DNA pellet in the opened tube for 1–2 min (overdried DNA is difficult to dissolve).
23. Resuspend the DNA pellet in 50–200 μl TE buffer, pH 8.0.
24. Determine DNA concentration spectrophotometrically (absorbance at 260 nm) and check the size of DNA molecules using agarose gel electrophoresis.
25. Aliquot the DNA in small volumes and store at −80°C.

PCR

The DNA fragments for DGGE analysis are usually amplified in polymerase chain reaction using the primers towards conserved regions flanking the variable regions of the 16S rRNA genes. To provide more stable melting behaviour of the fragments in DGGE, GC-rich sequence (GC-clamp) is attached to the 5′-end of one of the primers. Most often used primers are the ones for variable region 3 (V3) of 16S rDNA molecule: 341F, 5′-CCTACGGGAGGCAGCAG-3′ ('eubacterial' primer), and 534R, 5′-ATTACCGCGGCTGCTGG-3′ ('universal' primer), the numbering corresponding to *E. coli* 16S rDNA [17]. Primer 341F has a 40-nucleotide GC-clamp at 5′-end: 5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3′; therefore, in combination with 534R primer it amplifies PCR products of approximately 200 bp. Positive and negative control reactions which use as a template individual genomic DNA isolated from any bacterial pure culture and H_2O, respectively, are run in each PCR. Before running in DGGE, the quality and quantity of PCR products (5–10 μl)

are usually verified in 2–4% agarose gel electrophoresis. Single-stranded DNA often remaining in the sample after PCR and presenting a problem for the image analysis can be degraded using mung bean nuclease [22]. Bacterial standard ladder can be created using genomic DNAs extracted from several predominant strains common for certain environments for the amplification of individual V3 fragments with different relative mobility in denaturing gels [23].This ladder is used in all DGGE gels and allows between gel comparison of banding patterns during image analysis.

Generally, PCR program includes the denaturing step (94°C, 5 min), 20 cycles of 'touchdown' (with decreasing annealing temperature from 65°C to 55°C, 1°C every two cycles), 10 cycles with annealing temperature 55°C and additional extension at 72°C for 10 min:

94°C	5 min
20 cycles	
94°C	1 min (30 s)
65–55°C	1 min (30 s)
72°C	3 min (30 s)
10 cycles	
94°C	1 min (30 s)
55°C	1 min (30 s)
72°C	3 min (30 s)
72°C	10 min
4°C	constant

Composition of PCR mixture

–Genomic DNA	125 ng
–Primer 341F	25 pmol
–Primer 534R	25 pmol
–10× ExTaq buffer*	5 μl
–dNTP mixture*	4 μl
–TaKaRa Ex Taq Polymerase*	0.5 μl
–Sterile Milli-Q water	to adjust the volume to 50 μl

*ExTaq PCR kit (PanVera, Madison, WI, USA)

Equipment

–Thermocycler GeneAmp PCR System 2400 or 9600 (Perkin Elmer Biosystems, Foster City, CA, USA)

Denaturing gradient gel electrophoresis

The equipment for DGGE can be purchased from several companies, such as CBS Scientific Co (Del Mar, CA, USA), INGENY (Leiden, the Netherlands) and BioRad (Hercules, CA, USA). A gradient former, a magnetic stirrer and a peristaltic pump are necessary to cast reproducible gradient gels. We describe here how to use BioRad D-Gene System for separation of bacterial fragments in parallel gradient gels containing

a linear ascending gradient of denaturants (urea and formamide) from top of the gel to its bottom.

Gradient choice
To set up a new experiment (new primers, new samples, etc.) initially, a broad gradient of denaturants, such as 15–60%, is chosen. Then a narrower gradient interval can be used, which includes all the highest and lowest bands in different samples. For bacterial samples and primer set 341F/534R, it is typically 35–60% denaturants (a 100% denaturant is a mixture of 7 M urea and 40% deionized formamide). Concentration of acrylamide for both gradients is 8%.

Reagents
- Acrylamide/bis, 40% solution, 37.5 : 1
- APS (ammonium persulphate)
- TEMED (N,N,N',N'-tetramethylethylenediamine) – all from BioRad
- Urea, electrophoresis reagent
- Formamide

Equipment
- Protean II xi Inner Plate, 16 cm
- Protean II xi Outer Plate, 16 cm
- Protean II xi cell spacers, 16 cm × 0.75 mm
- Protean II xi cell comb, 20 well, 0.75 mm
- Sandwich clamps, 16 cm
- Casting stand with sponges
- Electrophoresis/temperature control module
- Electrophoresis tank
- Sandwich core – all included in D-Gene System (BioRad)
- A sheet of GelBond PAG film (corresponding to the size of the outer plate) for each gel (Cambrex, former FMC)
- Double-sided Scotch tape or double-stick tape, 1/2″ wide
- Cello-Seal (Fisher Scientific, USA)
- Gradient former, 20 ml each side

Assembling the plates
1. Clean the glass plate, spacers and combs carefully with detergent and ethanol. Grease from the previous runs should be removed completely.
2. Cut a strip of double-sided Scotch tape approximately two inches longer than the width of the glass plate.
3. Holding the tape tout, align it with the bottom edge of the glass and lay it in place. The tape will attach the film to the glass and prevent acrylamide solution from channelling behind the film during casting.
4. Position the GelBond PAG film (hydrophilic side facing up) so that the bottom edge is aligned with the bottom edge of the glass. Using soft paper tissues, press down firmly to seal the film to the tape.

5. Lift the film by a top corner, put a few drops of water in the centre of the glass, and lay the film down again. Press out the excess fluid, so that a very tight seal is formed between the glass and the film. Avoid air bubbles. Wipe any water from the film.
6. Grease the side spacers with a very thin layer of Cello-Seal or similar grease. Lay the spacers in place, press and wipe off excess grease.
7. Lay the second glass plate down properly and assemble the mould according to the chamber instructions. Tighten all clamp screws only to get a good seal with the greased spacers.
8. Put some grease on the corners of the glass plate, spacer ends and on the rubber gasket of the casting stand to prevent leakage.

Solutions

– 20× TAE buffer (0.8 M Tris base, 0.4 M sodium acetate, 0.02 M EDTA disodium salt, pH 7.4)

For 4 l: 388 g Tris
218 g Na acetate $3H_2O$
30 g Na_2EDTA

Adjust the pH to 7.4 with glacial acetic acid and add deionized water to the final volume of 4 l.

Pouring the gel

Prepare the solutions:

35% denaturing agents (100 ml)
– 20 ml 40% solution acrylamide/bis solution 37.5 : 1 (final concentration 8%)
– 5 ml 20× TAE buffer, pH 7.4 (final concentration 1× TAE)
– 14.7 g urea
– 14 ml formamide
– Adjust the volume up to 100 ml with deionized H_2O

60% denaturing agents (100 ml)
– 20 ml 40% solution acrylamide/bis solution 37.5 : 1 (final concentration 8%)
– 5 ml 20× TAE buffer, pH 7.4 (final concentration 1× TAE)
– 25.2 g urea
– 24 ml formamide
– Adjust the volume up to 100 ml with deionized H_2O

2× gel loading dye (10 ml)
– 2% Bromophenol blue 0.25 ml
– 2% Xylene Cyanol 0.25 ml
– 100 Glycerol 7 ml
– Distilled H_2O 2.5 ml

These solutions can be stored at 4°C for several weeks.
20% APS – prepare fresh for every DGGE run.

Mix the following reagents in 50 ml tubes:
Solution A
– 11.5 ml 35% denaturants solution
– 55 μl 20% APS

Solution B
– 11.5 ml 60% denaturants solution
– 55 μl 20% APS
Attach the exit tubing from the peristaltic pump between the glass plates in the assembled gel sandwich. Add 5.5 μl TEMED to Solution A, mix carefully and pour immediately to the gradient former chamber, not connected to the pump (the valve between the chambers should be closed at this point). Briefly open the valve and let a small portion of Solution A pass to the other outflow chamber of the gradient former. Quickly close the valve, and transfer Solution A from the outflow chamber back. Quickly add 5.5 μl TEMED to Solution B, mix and pour immediately to the outflow chamber. Apply stirrer to the outflow chamber, and turn the magnetic stirrer on. Simultaneously, open the valve between the chambers and turn the peristaltic pump on. When the gel is casted, apply the comb between the glass plates. Let the gel polymerize for at least 2 h.

Running the gel
About 2 h prior to running the gel fill the electrophoresis tank with ~7 l of 1× TAE buffer, pH 7.4, and preheat to 64°C. The buffer will cool to ~60°C during the sample loading. Attach the gel plates to the core apparatus. If running only one gel, make a second 'sandwich' with two glass plates and clamps. This is necessary to complete the upper reservoir of the buffer tank. Add 1× TAE buffer, pH 7.4, to the upper chamber, remove the combs, rinse the wells with the TAE buffer. Add 2× loading buffer to the PCR samples and load them into the wells. Place the core with the gel plates into the electrophoresis tank (64°C), close the buffer chamber, reset the temperature to 60°C, and turn on the recirculation pump and stirrer. Run electrophoresis at low voltage (30–40 V) for the first 5 min, and then at 150 V for 2 h and subsequently at 200 V for 1 h.

Silver Staining [11]
Reagents
– Silver nitrate, $AgNO_3$
– Formaldehyde
– Sodium Borohydride, $NaBH_4$
– NaOH, photographic grade – all from Sigma, St. Louis, MO, USA
After the DGGE run is over remove the gel from the plates and rinse *briefly* in water. Shake the gel gently for at least 2 h – overnight in the *fixing solution I* (10% ethanol, 0.5% acetic acid). Rinse the gel with water and place it in the *staining solution* prepared a few minutes before use (0.2 g $AgNO_3$, 200 ml distilled H_2O). Briefly rinse the gel in water, transfer it to a clean pan and add the *developing solution*, also prepared a few minutes before use (200 ml 1.5% (w/v) NaOH, 0.02 g $NaBH_4$, 0.8 ml formaldehyde).

Gently shake until the desired exposure of the bands is achieved (usually several minutes). Place the gel into water and scan it using GS-710 Calibrating Imaging Densitometer (BioRad) connected to G3 Macintosh computer.

Analysis of gel patterns

Gel images can be captured and analysed using Diversity Database software, part of Discovery Series (BioRad). This software allows band identification and determining the similarities in hundreds of gel images. DGGE profiles can be compared using Dice's similarity coefficient analysis and Ward's algorithm [23].

References

1. Akkermans, A.D.L., E.G. Zoetendal, C.F. Favier, H.G.H. Heilig, W.M. Akkermans-van Vliet, and W.M. de Vos. 2000. Temperature and denaturing gradient gel electrophoresis analysis of 16S rRNA from human fecal samples. *Biosci. Microflora* **19:**93–98.
2. Aminov, R.I., N. Garrigues-Jeanjean, and R.I. Mackie. 2001. Molecular ecology of tetracycline resistance: development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. *Appl. Environ. Microbiol.* **67:**22–32.
3. Aminov, R.I., J.C. Chee-Sanford, N. Garrigues, B. Teferedegne, I.J. Krapac, B.A. White, and R.I. Mackie. 2002. Development, validation, and application of PCR primers for detection of tetracycline efflux genes of gram-negative bacteria. *Appl. Environ. Microbiol.* **68:**1786–1793.
4. Bruns, M.A., and D.H. Buckley. 2001. Isolation and purification of microbial community nucleic acids from environmental samples, pp. 564–572. *In* C.J. Hurst, R.L. Crawford, G.R. Knudsen, M. McInerney, and L. Stetzenbach (eds.), *Manual of Environmental Microbiology*, 2nd ed. American Society for Microbiology, Washington, DC.
5. Deplancke, B., K.R. Hristova, H.A. Oakley, V.J. McCracken, R.I. Aminov, R.I. Mackie, and H.R. Gaskins. 2000. Molecular ecological analysis of dissimilatory sulfidogen succession and diversity in the mouse gastrointestinal tract. *Appl. Environ. Microbiol.* **66:**2166–2174.
6. Favier, C.F., E.E. Vaughan, W.M. de Vos, and A.D.L. Akkermans. 2002. Molecular monitoring of succession of bacterial communities in human neonates. *Appl. Environ. Microbiol.* **68:**219–226.
7. Finegold, S.M., H.R. Attebury, and V.L. Sutter. 1974. Effect of diet on human fecal flora: comparison of Japanese and American diets. *Am. J. Clin. Nutr.* **27:**1456–1469.
8. Heilig, H.G.H.J., E.G. Zoetendal, E.E. Vaughn, P. Marteau, A.D.L. Ackermans, and W.M. de Vos. 2002. Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. *Appl. Environ. Microbiol.* **68:**114–123.
9. Kocherginskaya, S., R.I. Aminov, and B.A. White. 2001. Analysis of the rumen bacterial diversity under two different diet conditions using denaturing gradient gel electrophoresis, random sequencing, and statistical ecology approaches. *Anaerobe* **7:**119–134.
10. LaMontagne, M.G., F.C. Michel Jr., P.A. Holden, and C.A. Reddy. 2002. Evaluation of extraction and purification methods for obtaining PCR-amplifiable DNA from compost for microbial community analysis. *J. Microbiol. Methods* **49:**255–264.
11. Liu, W.-T., and K. Ritalahti. 1996. *Denaturing Gradient Gel Electrophoresis (DGGE) Protocol*. ROME Lab DGGE Workshop. Department of Microbiology and Center for Microbial Ecology, Michigan State University, East Lansing, MI.
12. McCracken, V.J., J.M. Simpson, R.I. Mackie, and H.R. Gaskins. 2001. Molecular ecological analysis of dietary and antibiotic-induced alterations of the mouse intestinal microbiota. *J. Nutr.* **131:**1862–1870.
13. Miller, D.N. 2001. Evaluation of gel filtration resins for the removal of PCR-inhibitory substances from soils and sediments.*J. Microbiol. Methods* **44:**49–58.
14. Moore, W.E.C., and L.V. Holdeman. 1974. Human fecal flora: the normal flora of 20 Japanese-Hawaiians. *Appl. Microbiol.* **27:**961–979.

15. Muyzer, G. 1999. DGGE/TGGE a method for identifying genes from natural ecosystems. *Curr. Opin. Microbiol.* **2:**317–322.
16. Muyzer, G., T. Brinkhoff, U. Nübel, C. Santegoeds, H. Schäfer, and C. Wawer. 1998. Denaturing gradient gel electrophoresis (DGGE) in microbial ecology, pp. 1–27. *In* A. Akkermans, J.D. van Elsas, and F. de Bruijn (eds.), *Molecular Microbial Ecology Manual*, Vol. 3.4.4. Kluwer Academic Publishers, Boston.
17. Muyzer, G., S. Hottentrager, A. Teske, and C. Wawer. 1996. Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA – a new molecular approach to analyse the genetic diversity of mixed microbial communities, pp. 3.4.4.1–3.4.4.22. *In* A.D.L. Akkermans, J.D. Elsas, and F.J. Bruijn (eds.), *Molecular Microbial Ecology Manual*, Kluwer Academic Publishers, Dordrecht, The Netherlands.
18. Nubel, U., F. Garcia-Pichel, M. Kuhl, and G. Muyzer. 1999. Quantifying microbial diversity, morphotypes, 16S rRNA genes, and carotenoids of oxygenic phototrophs in microbial mats. *Appl. Environ. Microbiol.* **65:**422–430.
19. O'Sullivan, D.J. 1999. Methods for the analysis of the intestinal microflora, pp. 23–44. *In* G.W. Tannock (ed.), *Probiotics: A Critical Review*. Horizon Scientific Press, England.
20. Satokari, R., E. Vaughan, A. Akkermans, M. Saarela, and W. de Vos. 2001. Bifidobacterial diversity in human feces detected by genus specific PCR and denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* **67:**504–513.
21. Simpson, J.M., B. Martineau, W.E. Jones, J.M. Ballam, and R.I. Mackie.2002. Characterization of fecal bacterial populations in canines: effects of age, breed and dietary fiber. *Microbiol. Ecol.* **44:**186–197.
22. Simpson, J.M., V.J. McCracken, B.A. White, H.R. Gaskins, and R.I. Mackie. 1999. Application of denaturant gradient gel electrophoresis for the analysis of the porcine gastrointestinal microbiota. *J. Microbiol. Methods* **36:**167–179.
23. Simpson, J.M., V.J. McCracken, H.R. Gaskins, and R.I. Mackie. 2000. Denaturing gradient gel electrophoresis analysis of 16S rDNA amplicons to monitor changes in fecal bacterial populations of weaning pigs following introduction of *Lactobacillus reuteri* strain MM53. *Appl. Environ. Microbiol.* **66:**4705–4714.
24. Tsai, Y.-L., and B. Olson. 1992. Detection of low numbers of bacterial cells in soils and sediments by polymerase chain reaction. *Appl. Environ. Microbiol.* **58:**754–757.
25. Walter, J., G. Tannock, A. Tilsala-Timisjarvi, S. Rodtong, D. Loach, K. Munro, and T. Altossava. 2000. Detection and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primers. *Appl. Environ. Microbiol.* **66:**297–303.
26. Zoetendal, E.G., A.D.L. Akkermans, and W.M. de Vos. 1998. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. *Appl. Environ. Microbiol.* **64:**3854–3859.
27. Zoetendal, E.G., A.D.L. Akkermans, W.M. Akkermans-van Vliet, A.G.M. Visser, and W.M. de Vos. 2001. The host genotype affects the bacterial community in the human gastrointestinal tract. *Microbiol. Ecol. Health Dis.* **13:**129–134.
28. Zoetendal, E.G., A.V. Wright, T. Vilponnen-Salmela, K. Ben-Amor, A.D.L. Akkermans, and W.M. de Vos. 2002. Mucosa-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. *Appl. Environ. Microbiol.* **68:**3401–3407.

4.2. Bacteriophage populations

ATHOL V. KLIEVE[1] and ROSALIND A. GILBERT[2]
[1]*Department of primary industries and fisheries, animal sciences, Yeerongpilly, Queensland 4105, Australia*
[2]*CSIRO livestock industries, St Lucia, Queensland 4072, Australia*

Introduction

Bacteriophages are ubiquitous to the rumen ecosystem; they have a role in nitrogen metabolism through bacterial lysis in the rumen, they may help to regulate bacterial population densities, be an agent for genetic exchange and be of use in biocontrol of bacterial populations through phage therapy [10, 11, 19]. In Chapter 2.1, classical methodologies to enable the isolation, enumeration, storage and morphological characterization of phages were presented.

In addition to these classic procedures, molecular biological techniques have resulted in a range of methodologies to investigate the type, topology and size of phage nucleic acids, to fingerprint individual phage strains and to create a profile of ruminal phage populations [9, 11].

Different phage families possess all the currently identified combinations of double-stranded or single-stranded RNA or DNA [16] and may also possess unusual bases such as 5-hydroxymethylcytosine (found in T-even phage) or 5-hydroxymethyluracil and uracil in place of thymidine [1]. In all morphological groups of phage except the filamentous phages, the nucleic acid is contained within a head or polyhedral structure, predominantly composed of protein. Filamentous phages have their nucleic acid contained inside the helical filament, occupying much of its length [4, 14, 15].

Many of the procedures used with phage nucleic acids and double-stranded (ds) DNA, in particular, are not specific to ruminal phages but are the same as in other areas where nucleic acids are investigated and are covered elsewhere in the literature and this chapter. Most applications with rumen phages are similar to those reported for phages of non-ruminal bacteria and are covered in general texts such as Maniatis et al. [13]. In this chapter, we will concentrate on aspects of methodology as they relate to ruminal phages.

H.P.S. Makkar and C.S. McSweeney (eds.), Methods in Gut Microbial Ecology for Ruminants, 129–137.
© 2005 *IAEA. Printed in the Netherlands.*

Procedures for nucleic acid extraction from samples containing phages

Steps in the extraction of nucleic acids from purified phages

Purified phages are obtained either as high-titre lysates (lytic phages) or from cultures of bacterial lysogens treated to induce temperate phages (integrated into the bacterial chromosome) to excise, enter the lytic phase of growth and be released into the growth medium. Procedures for obtaining both types of lysate and the filtration and storage of these lysates are presented in Chapter 2.2.

1. Bacteriophage particles are concentrated from lysates by centrifugation at 30 000 *g* for 2 h at 4°C.
2. The supernatant is discarded and the pellet is resuspended in a 200 μl volume of phage storage buffer (PSB, see Chapter 2.2).
3. Extraction of nucleic acid follows the method of Maniatis et al. [13]. The basic procedure is
   - Incubate sample with 2 μl of RNase (10 mg/ml) at 37°C for 30 min.
   - Add 2 μl of 10% (w/v) SDS and 2 μl of Proteinase-K (20 mg/ml). Mix well and incubate at 50°C for 1 h.
   - Add an equal volume of phenol (buffered with TE) /chloroform (1 : 1) to the tube and mix by inversion for 5 min.
   - Centrifuge at 10 000–15 000 *g* for 10 min. Remove upper aqueous phase to a fresh microfuge tube and discard lower phase.
   - Add an equal volume of chloroform and mix by inversion for 5 min.
   - Centrifuge as previously retaining the aqueous layer.
   - Add 0.25 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of absolute ethanol (−20°C). Mix thoroughly by inversion and place at −20°C for 1 h.
   - Pellet nucleic acid by centrifugation at 10 000–15 000 *g* for 10 min. Carefully remove and discard the supernatant.
   - The pellet is then dried (vacuum desiccator, freeze drier or simply air dried) resuspended in an appropriate volume of sterile high-purity water and stored at −20°C.

(*Notes: (a)* If the phage could be expected to contain RNA (from morphology), then it is prudent to omit the RNase step. (b) When preparing phage DNA, an additional enzymatic digestion with RNase may be included prior to organic solvent extraction in order to remove large amounts of contaminating RNA. Alternatively, when preparing phage RNA, digestion with DNase may be included.)

When nucleic acid is prepared from phages obtained following host bacterial growth in a rumen fluid (RF) based media, impurities may be retained in the nucleic acid product; therefore, prior to sensitive procedures, such as DNA sequencing, additional purification steps may be required using commercial nucleic acid purification kits for DNA (PCR clean up column, QIAamp, QIAGEN) or RNA (Trizol, RNA clean up column).

Separating the phage fraction from ruminal contents

Although found in very high numbers within the rumen, rumen phage are by far the smallest members of the rumen microbial community; therefore, they can be fractionated on the basis of size using ultrafiltration and differential centrifugation [8, 11].

1. Immediately following collection, crude RF samples should be first heated to 75°C for 20 min in order to eliminate the activity of proteinases and nucleases, which degrade phage particles and nucleic acids. Samples may then be processed immediately, as follows, or stored at −20°C.
2. Centrifuge the RF sample twice at 20 000 *g* for 15 min at 4°C to remove particulate matter, protozoa, bacteria and fungi. The supernatant is retained and the pellet discarded.
3. Filter supernatant using 0.45 μm pore-size low-protein-binding filters using a Sterifil Aseptic Filtering system (Millipore Corp.) connected to a vacuum pump or similar equipment.

   (*Note:* If samples are difficult to filter, this filtration step may be preceded by additional filtration with larger pore-size (e.g. 0.065 μm) low-protein-binding filters (Millipore Corp.))
4. Phage particles will remain in the filtrate and are concentrated by centrifugation at 30 000–50 000 *g* for 2 h at 4°C.
5. The supernatant is discarded and the pellet resuspended overnight in a small volume (100 μl) of PSB.
6. In order to remove impurities, a second wash step may be incorporated where the resuspended phage pellet is diluted with TE (10 mM Tris, 1 mM EDTA, pH 7.6), the volume of which should fill the centrifuge tubes and centrifugation at 30 000–50 000*g*for 2 h at 4°C repeated.
   a. Concentrated phage particles are then resuspended in PSB (e.g. 100 μl PSB per 50 ml volume centrifuge tube) and phage either DNA extracted as for samples of purified phages above, set in soft-agar for analysis by pulsed field gel electrophoresis (PFGE, see below), or stored at −20°C.

Steps in extraction and preparation of phage DNA for PFGE

Following the concentration of phage particles by ultrafiltration and differential centrifugation, phage particles stored in PSB may be prepared for further analysis of their DNA by PFGE. PFGE is used to separate large pieces of DNA, far larger than is possible with conventional agarose gel electrophoresis. However, DNA larger than approximately 50 kbp is subject to physical shearing in aqueous solutions. Many phage genomes are larger than 50 kbp and are typically between 30 and 300 kbp but can be larger still [1]. In order to avoid shearing, the large DNA genomes of phages that may be present in RF samples, intact phage particles must first be embedded in low-melting point agarose.

1. The volume of concentrated phage sample is determined and sufficient powdered low-melting point agarose added to produce a 1.5% (w/v) gel.
2. The mixture is heated to 70°C, mixed thoroughly but gently and allowed to cool in approximately 40 μl aliquots contained in blunt-ended 200 μl micropipette tips or in the plug making apparatus supplied by the PFGE apparatus manufacturer (e.g. BioRad PFGE casting mould).
3. DNA is released from phage particles embedded in low-melting point agarose and prepared for PFGE by the procedures used for viruses of *Chlorella* spp. [17].
   - Low-melting point agarose blocks containing phage particles are incubated overnight at 55°C in a solution containing 25 μl of 20 mg/ml Proteinase-K, 50 μl SDS (20%), 425 μl 0·2M EDTA per sample, to degrade the phage protein coat.
   - The solution is then removed from the agarose blocks and they are washed twice with TE (10 mM Tris, 1 mM EDTA, pH 7.6) buffer, 0·5 ml per sample, for 30 min per wash at room temperature.
   - Agarose plugs are treated with 0.5 ml (per sample) of 1 mM phenylmethylsulfonyl fluoride in TE Buffer (freshly prepared) for 1 h in a 55°C water bath, to remove residual fragments of protein.
   - Agarose plugs are washed twice with TE Buffer (0.5 ml per sample) for 30 min per wash at room temperature.
   - Agarose plugs are washed with 20% *iso*propanol in TE buffer (0.5 ml per sample) for 1 h.
   - *Iso*propanol/TE solution is removed and replaced with TE for storage at 4°C.

   (*Note:* Agarose blocks containing embedded phage DNA may be stored for approximately 6 months at 4°C in TE buffer pH 7.6, as recommended in [13].)

Procedures for defining the genome of individual phages

Characterization of the viral genome in bacteriophages is an important aspect in defining these viruses. However, most methodology for fundamental nucleic acid identification is generally applicable to nucleic acids, independent of their source and widely available, e.g. [1, 2, 13]. We will limit the methodology reported here to the basic determination of nucleic acid type [i.e. DNA or RNA, double or single stranded (ds or ss)], visual evidence of dsDNA topology (cohesive-ended DNA vs. terminally redundant and circularly permuted) and determination of dsDNA genome size.

Key to determining nucleic acid type

Following are a range of simple procedures that rely upon treating the nucleic acid under question in a specific manner and then observing differences between the treated and untreated nucleic acids on electrophoretic gels. Enzymes are used under standard conditions as specified by their manufacturers.

1. Samples of nucleic acid treated with DNaseI for 30 min at 37°C. Nucleic acid no longer present on agarose gel-DNA.

2. If still present after DNase treatment, treat sample with RNase A for 30 min at 37°C. Nucleic acid no longer present on agarose gel-ssRNA. Still present, dsRNA.
3. If dsRNA is suspected, heat sample at 95°C for 5 min to denature dsRNA to single strands, cool on ice slurry, treat again with RNase A. If nucleic acid was dsRNA, then it should have been digested following heat treatment.
4. If ssDNA is likely, heat sample to 95°C for 5 min, immediately place on ice. If identical to unheated sample, then the nucleic acid is most likely ssDNA, dsDNA will denature and migrate differently to the unheated sample.

(*Note:* Once denatured, what was dsDNA and appeared as a single bright band on a gel, usually appears as either two faint bands close together or is partially or totally degraded due to disintegration of the DNA where nicks have been present in the double-stranded structure.)

Visual keys to nucleic acid topology

In addition to genomes being DNA, RNA, double- or single-stranded, they can also be linear or circular and circular genomes can occur relaxed or supercoiled while linear genomes can be packaged into phage heads with cohesive ends [3] or by the 'headful' packaging mechanism [18] which produces a circularly permuted, terminally redundant population of genomes from a given phage. While we cannot delve into the methodologies to unravel these variations in this publication (texts such as Hendrix et al. [6], Lin et al.[12], and Ackermann and DuBow [1] are useful), they can present confusing banding patterns on electrophoretic gels, with or without digestion by restriction endonucleases. As an aid to understanding the banding patterns visualized on gels, those most likely to be encountered are described below. It should be noted that the overwhelming majority, >95% [1], of phages that are likely to be encountered are 'tailed phages' of the families Myoviridae, Siphoviridae and Podoviridae. All of these phages contain genomes with linear dsDNA.

1. Where more than one band appears from undigested nucleic acid from a purified phage.
   - Two bands are present with one fainter than the other but the relative intensities vary from one gel to another. One of two scenarios is common.
     a) Nucleic acid is closed circular and nicked molecules become linear. By electrophoresing the sample on gels of differing agarose concentration (e.g. 0.8%, 1.0%, 1.5% and 2.0%), the linear band will remain aligned with linear size markers, but the circular molecules will vary in relative position. On PFGE, the linear form should not occur and the circular form is unlikely to enter the gel.
     b) dsDNA is linear but has cohesive ends that can temporarily circularize the genome by base pairing of complementary sequences. In this case, the DNA is usually large (above the 23 kbp size marker of the *Hin*dIII digest of phage λ) and one band will migrate slower than that expected for linear DNA of

any length. Heat the DNA to 75°C for 5 min and immediately place on ice prior to electrophoresis; if cohesive ends are present, a single band should be present and the slow migrating band should have disappeared. On PFGE, only the linear form should be present and this will also indicate the length of the linear genome.

- Three bands are present with variable intensities relative to each other between batches of DNA.

a) This pattern is reminiscent of plasmid DNA extracted from bacterial cells and usually for the same reason. Bands are likely to correspond supercoiled nucleic acid, the corresponding closed circular and linear forms. Electrophoresis on gels of differing agarose concentration will confirm whether bands are composed of linear material or material with secondary structure. Incubations of sample with dilute DNase (or RNase for RNA genomes) over a time period should show a gradual change from one form to the another as increased 'nicking' of the genome decreases the amount of the supercoiled form first and then the circular form.

2. Restriction endonuclease digestion of linear dsDNA from tailed phages is commonly used to 'fingerprint' individual phages as digestion patterns are characteristic for a specific phage and are dependent on DNA sequence. The methodology for use of restriction endonucleases is the same as with bacterial DNA (see Chapter 4.3). It is common to define each phage with three or more different restriction enzymes and enzymes with a six-base-pair recognition sequence are used unless the total genome length is very small, i.e. below 20 kbp, and then enzymes with a four-base-recognition may prove more useful. An example of digestion profiles of a typical tailed phage (Siphovirus in this case) from the rumen bacterium *Prevotella ruminicola* are presented in Fig. 1.
3. In addition to 'fingerprinting' individual phages, the total genome length can be determined from endonuclease digests, simply by the addition of the lengths of digestion fragments. However, to ensure accuracy it is best to analyse photographic negatives of the digests with densitometric software (such as Quantity One, Bio-Rad). By calculating band density and dividing by fragment length, the relative number of molecules in each band can be determined. The ratio of molecules in each band should be 1 : 1 or an exact multiple such as 2 : 1 (indicates a doublet, i.e. two different fragments of the same length). The total length from three different digests should be determined and as long as there is good agreement they should be averaged to give the total genome length. Disagreement between digests is often due to the presence of faint and broad diffuse bands that are not in stoichiometric agreement with other bands in the digest (fewer molecules than the 1 : 1 ratio). Such bands (identified by arrows in Fig. 1) are often associated with a genome that is circularly permuted and terminally redundant. Genome size is best estimated by ignoring these bands, but this will introduce some error and slightly underestimate the size. The length of DNA from these phages can be accurately determined by PFGE.

Endonuclease mapping to determine the circular permutation and the degree of redundancy of these genomes is very complex and beyond the scope of this manual.

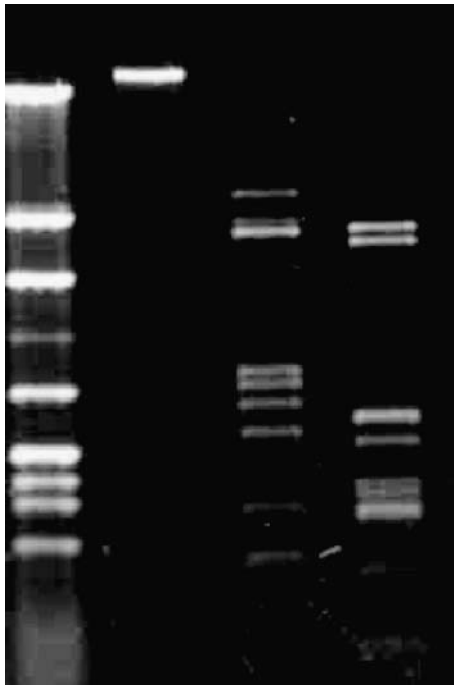


Figure 1. Restriction endonuclease profiles of ϕBrb01 DNA [9]. Lane 1, λ CFK1 DNA size marker. Lane 2, uncut DNA. Lanes 3 and 4, *Eco*RI and *Hin*dIII digests, respectively. Arrows indicate faint and diffuse bands.

To undertake such a task, the reader is referred to Jackson et al. [7], Grund and Hutchinson [5] and Klieve et al. [9].

Procedures for profiling and enumerating phage populations in the rumen ecosystem by PFGE

The development of a method for detecting rumen phages without the use of electron microscopy [11] has allowed further investigation into the nature of the bacteriophage population present in RF samples. This method involves the fractionation of phage from RF samples and the preparation of phage DNA, as detailed previously. PFGE is performed using a commercially available clamped homogenous electric field (CHEF) apparatus and a size marker formulated for PFGE, such as a lambda ladder DNA size marker (Promega), included as a DNA size reference on every gel.

Electrophoresis conditions may be specific to each CHEF apparatus; however, the electrophoresis conditions specified for use with a commercially provided lambda ladder DNA size marker may be used as a programming guide. Examples of PFGE conditions used in our laboratory are detailed below:

1. For the Pulsaphor Plus system (Pharmacia LKB), gels of 1% (w/v) agarose (110 ml volume, pulsed field certified agarose) were prepared with a 1× TBE buffer, as detailed by the manufacturer. Electrophoresis conditions comprised an initial switch time of 50 s for 14 h and then 90 s for 20 h at 150 V. Electrophoresis buffer (1× TBE) was circulated at 9°C.
2. When using the CHEF apparatus supplied by BioRad, 1% agarose, 0.5× TBE gels of 100 ml (10 lane gel) or 150 ml (15 lane gel) volume were prepared, as detailed by the manufacturer. Prior to electrophoresis, agarose plugs containing samples of phage DNA were transferred into the wells of a pre-cast gel and sealed using molten 1% low-melting-point agarose. Electrophoresis conditions involved two sets of parameters. The first set had an initial switch time of 60 s and a final switch time of 90 s for 15 h at 6 V/cm. The second set had an initial switch time of 5 s and ran for 3 h at 6 V/cm. Electrophoresis buffer (0.5× TBE) was circulated at 14°C.

Following electrophoresis, PFGE gels are stained using ethidium bromide and examined with the aid of a medium wavelength (320 nm) UV transilluminator. Due to recent advances in image recording and image analysis software, PFGE gels may be photographed and the image saved in a format allowing further analysis using a software package such as Molecular Analyst or Quantity One (BioRad). In this way, each gel lane, representing the phage population within a RF sample, may be scanned and compared to a DNA size marker standard, in order to obtain a size profile of the phage DNA bands present.

The phage population, as gauged by the appearance of phage DNA on PFGE gels, generally has two major components: (1) a broad region (40–100 kb) comprising DNA from many different phages [11], which would probably encompass the genomes of most of the tailed phages and (2) discrete bands of DNA from one or a few phage genotypes, presumably representing blooms of lytic phage activity [11, 19].

Phage DNA from RF samples separated on PFGE gels may also be further analysed using molecular techniques in order to verify the identity of the phage DNA bands observed. DNA separated by PFGE may be transferred to nitrocellulose or nylon membranes by southern blotting techniques and hybridized using phage sequence-specific DNA probes (standard molecular methods described by Maniatis et al. [13]). Alternatively, for more sensitive detection of specific phage, phage DNA bands within the required size range may be excised from the PFGE gel, the DNA purified from the gel matrix and used in PCR reactions using phage sequence-specific DNA primers.

Quantitation of total phage DNA within an RF sample can be determined using phage DNA embedded in agarose plugs [11].

1. The total DNA content within a volume of agarose plug is determined by electrophoresis of a known quantity of DNA standard, e.g. linear pBR322 plasmid, on a conventional 1% agarose, 1× TBE gel for 30 min at 80 mA.
2. A small volume of phage DNA sample is loaded into the same loading well and electrophoresis recommenced for 1 h at 80 mA.

3. The agarose gel can then be stained and an image saved in a format allowing further analysis using image analysis software. In this way, the image intensity of the two bands within each gel lane, that is, the upper band representing a quantity of phage DNA, and the lower band, representing the DNA quantitation standard, can be compared and the amount of total phage DNA calculated.

References

1. Ackermann, H.W., and M.S. DuBow. 1987. *General Properties of Bacteriophages*. CRC Press, Boca Raton.
2. Ackermann, H.W., and M.S. Dubow. 1987. *Natural Groups of Bacteriophages*. CRC Press, Boca Raton.
3. Arber, W. 1983. A beginner's guide to lambda biology. *In* R.W. Hendrix, J.W. Roberts, F.W. Stahl, and R.A. Weisberg (eds.), *Lambda II.* Cold Spring Harbour Laboratory, New York.
4. Bradley, D.E. 1967. Ultrastructure of bacteriophages and bacteriocins. *Bacteriol. Rev.* **31:**230–314.
5. Grund, A.D., and C.R. Hutchinson. 1987. Bacteriophages of *Saccharopolyspora erythraea*. *J. Bacteriol.* **169:**3013–3022.
6. Hendrix, R.W., J.W. Roberts, F.W. Stahl, and R.A. Weisberg. 1983. *Lambda II.* Cold Spring Harbour Laboratory, New York.
7. Jackson, E.N., D.A. Jackson, and R.J. Deans. 1978. *Eco*R1 analysis of bacteriophage P22 DNA packaging. *J. Mol. Biol.* **118:**365–388.
8. Klieve, A.V., and T. Bauchop. 1988. Morphological diversity or ruminal bacteriophages from sheep and cattle. *Appl. Environ. Microbiol.* **54:**1637–1641.
9. Klieve, A.V., K. Gregg, and T. Bauchop. 1991. Isolation and characteristics of lytic phages from *Bacteroides ruminicola ss brevis*. *Curr. Microbiol.* **23:**183–187.
10. Klieve, A.V., and R. Hegarty. 1999. Opportunities for biological control of ruminal methanogenesis. *Aust. J. Agric. Res.* **50:**1315–1319.
11. Klieve, A.V., and R.A. Swain. 1993. Estimating ruminal bacteriophage numbers using pulsed field gel electrophoresis and laser densitometry. *Appl. Environ. Microbiol.* **59:**2299–2303.
12. Lin, E.C.C., R. Goldstein, and M. Syvanen. 1984. *Bacteria, Plasmids, and Phages: An Introduction to Molecular Biology*. Harvard University Press, Cambridge, Massachussetts.
13. Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, New York.
14. Marvin, D.A., R.D. Hale, C. Nave, and M.H. Citterich. 1994. Molecular models and structural comparisons of native and mutant class I filamentous bacteriophages. *J. Mol. Biol.* **235:**260–286.
15. Model, P., and M. Russell. 1988. Filamentous bacteriophage, pp. 375–456. *In* R. Calendar (ed.), *The Bacteriophages*. Plenum Press, New York.
16. Murphy, F.A., C.M. Fauquet, D.H.L. Bishop, S.A. Ghabrial, A.W. Jarvis, G.P. Martelli, M.A. Mayo, and M.D. Summers. 1995. *Virus Taxonomy, 6th Report of the International Committee on Taxonomy of Viruses*. Springer-Verlag, New York.
17. Rohozinski, J., L.E. Girton, and J.L.V. Etten. 1989. Chlorella viruses contain linear nonpermuted double-stranded DNA genomes with covalently closed hairpin ends. *Virology* **168:**363–369.
18. Streisinger, G., J. Emrich, and M.M. Stahl. 1967. Chromosome structure in phage T4, III. Terminal redundancy and length determination. *Proc. Natl. Acad. Sci. U.S.A.* **57:**292–295.
19. Swain, R.A., J.V. Nolan, and A.V. Klieve. 1996. Natural variability and diurnal fluctuations within the bacteriophage population of the rumen. *Appl. Environ. Microbiol.* **62:**994–997.

4.3. Anaerobic fungal populations

JAYNE L. BROOKMAN[1] and MATTHEW J. NICHOLSON[1,2,*]
[1]*BBSRC Institute for Grasslands and Environmental Research, Department of Plant, Animal and Microbial Science, Plas Gogerddan, Aberystwyth, Ceredigion SY23 3EB, UK.*
[2]*University of Manchester, School of Biological Sciences, 1800 Stopford Building, Oxford Road, Manchester M13 9PT, UK.*
**Current address: AgResearch Grasslands, Private Bag 11008, Palmerston North, New Zealand.*

Introduction

The development of molecular techniques has greatly broadened our view of microbial diversity and enabled a more complete detection and description of microbial communities [2, 13]. The application of these techniques provides a simple means of following community changes, for example, Ishii et al. [6] described transient and more stable inhabitants in another dynamic microbial system, compost.

Our present knowledge of anaerobic gut fungal population diversity within the gastrointestinal tract is based upon isolation, cultivation and observations *in vivo* [4]. It is likely that there are many species yet to be described, some of which may be non-culturable. We have observed a distinct difference in the ease of cultivation between the different genera, for example, *Caecomyes* isolates are especially difficult to isolate and maintain *in vitro*, a feature that is likely to result in the under representation of this genera in culture-based enumerations.

The anaerobic gut fungi are the only known obligately anaerobic fungi. For the majority of their life cycles, they are found tightly associated with solid digesta in the rumen and/or hindgut. They produce potent fibrolytic enzymes and grow invasively on and into the plant material they are digesting making them important contributors to fibre digestion. This close association with intestinal digesta has made it difficult to accurately determine the amount of fungal biomass present in the rumen, with Orpin [11] suggesting 8% contribution to the total microbial biomass, whereas Rezaeian et al. [14] more recently gave a value of approximately 20%.

It is clear that the rumen microbial complement is affected by dietary changes, and that the fungi are more important in digestion in the rumens of animals fed with high-fibre diets [2, 8]. It seems likely that the gut fungi play an important role within the rumen as primary colonizers of plant fibre [1], and so we are particularly interested in being able to measure the appearance and diversity of fungi on the plant tissues within the rumen directly.

To assess the succession and diversity of rumen populations on plant particles within the rumen, we need to be able to detect the rumen microbes in amongst the plant material. We describe below a general approach for DNA extraction from environmental materials, such as plant particles within a feed bolus or faeces. The

H.P.S. Makkar and C.S. McSweeney (eds.), Methods in Gut Microbial Ecology for Ruminants, 139–150.
© 2005 *IAEA. Printed in the Netherlands.*

DNA isolated will contain plant, fungal, bacterial and protozoal material, including DNA from aerobic fungi that are not metabolically active in the rumen but were introduced on the plant material. Other chapters have discussed the methodologies for detection and characterization of bacterial and protozoal components of this mixture; we will consider the gut fungal component only.

Detection of microbes by their DNA requires a sensitive methodology and PCR is commonly used. The ribosomal genes are often chosen as a suitable amplicon for environmental studies as they are multicopy in eukaryotic genomes providing enhanced sensitivity, and can be used for phylogenetic analyses and delimitation of diverse groups of organisms across and between the kingdoms. The rDNA genes are particularly well suited for this purpose as they are present in all forms of life and consist of alternating variable and conserved regions. The small subunit rRNA gene sequences, the 18S gene in eukaryotes, are conventionally used for phylogenetic comparisons. However, as this gene is more than 97% identical between genera of the anaerobic fungi [5] the less conserved internal transcribed spacer (ITS) regions have proven to be more useful [3].

The primer pair described previously for the amplification of the fungal ITS1 sequence from axenic cultures were not suitable for use with more complex samples as they preferentially amplified the aerobic fungal DNA sequences present. We have refined the primer sequences used for environmental samples by analysis of many gut fungal sequences and designed a specific primer set that can be used with environmental samples [10].

Analysis of the population of PCR amplicons produced from a complex sample is required to identify individual sequences and quantify the population diversity for gut fungi present in the environmental sample. A simple shotgun cloning of the PCR fragments necessitates the sequencing of a large number of clones to generate a fair representation of the initial sample. Gel-based profiles provide an alternative and complementary approach enabling many samples to be processed and compared relatively quickly.

One of these gel-based methods, denaturing gradient gel electrophoresis (DGGE) has been used to successfully analyse populations of aerobic fungi in decaying wood [16], and bacterial populations in the rumen [7]. The separation is achieved by fragment size and nucleotide sequence differences. It is a popular methodology as individual bands from within a profile can be picked from the gel and used for reamplification by PCR. The sequence of individual bands and their likely identity can then be determined allowing clear identification of important members of a microbial community. The determination of running conditions for any new group of organisms can be slow, but we have enjoyed success with separation of rumen fungal populations as described below.

The ITS regions from the anaerobic gut fungi show length polymorphisms, and there is an approximate relationship between the length of the ITS1 and genera [3, 9, 15]. The ITS1 fragment length will not absolutely determine the genus of a sequence, but it is sufficiently robust to suggest an alternative method for separation and monitoring of PCR populations. Simple electrophoretic separation of the rDNA ITS1 fragments using high-resolution Spreadex gels that enable separation of closely

sized nucleic acid fragments provides a rapid and reproducible method to monitor fungal populations. By choosing the appropriate grade of polymer to suit the size of fragments to be separated, fragments that differ in length by only 2–4 bp can be resolved. We describe below our use of Spreadex gels for characterization of faecal populations (Nicholson et al., in preparation) and the pattern matching methodology using hidden Markov models [15] developed to rapidly identify the genus of fungal sequences present in these populations.

Preparation of genomic DNA

CTAB method

This method is suitable for extraction of DNA for PCR analysis of environmental samples, such as faeces or for laboratory generated biomass.

1. Grind sample finely in liquid nitrogen using a pestle and mortar. Add to a 1.5 ml Eppendorf tube to the 0.1 ml mark, add 0.8 ml CTAB buffer and vortex thoroughly to mix.
2. Incubate at 70°C for 30 min, vortex samples after 15 min incubation. Add 0.5 ml chloroform, vortex to form a white emulsion. Centrifuge in a microfuge (13 000 *g*, 10 min) to form an aqueous layer above the chloroform.
3. Remove the aqueous layer carefully (~0.5 ml) into a clean microfuge tube. Add 0.3 ml *iso*propanol, invert gently and incubate at room temperature for 10 min. Pellet precipitated DNA in a microfuge (13 000 *g*, 10 min).
4. Remove the supernatant and wash the pellet in 70% (v/v) ethanol, vortex or flick the pellet away from the base of the tube to ensure efficient rinsing. Incubate at 70°C for 10 min; the pellet should become whiter and more clearly visible.
5. Collect pellet by centrifugation as before (13 000 *g*, 10 min) and remove the ethanol. Dry pellet at room temperature and dissolve in 10 mM Tris–HCl (50–100 μl depending on the pellet size/yield of DNA). Warm to 37°C to aid solubilization if necessary.
6. Prepare fivefold serial dilutions of the DNA solution in molecular biology grade water and store at −20°C. Thaw on ice when required.
7. The optimal dilution for PCR should be determined experimentally but is usually 1/25.

Solutions required

CTAB DNA isolation buffer: 100 mM Tris–HCl (pH 8.0); 1.4 M NaCl; 20 mM EDTA (sodium salt); 2% hexadecyltrimethylammonium bromide, filter sterilize the solution.

10 mM Tris–HCl, pH 8.0, filter sterilized.

Fast-Prep method

A method of DNA extraction that we have found to be extremely reproducible for complex substrates, such as feed boli, in environmental programmes is the FastPrep®

approach described below. The FastPrep instrument works by shaking the tube up and down with a slight twisting motion at very high speeds causing cells to lyse and thus removes the need to grind each bolus sample in liquid nitrogen. We have optimized the extraction conditions to provide good quality DNA for PCR rapidly, and the DNA can be used for amplification of fungal, protozoal, bacterial and plant amplicons, if present in the original sample.

The methodology given is for lyophilized bolus samples. Faecal samples can be processed in an identical manner. Samples can also be processed without freeze drying, with approximately 150 mg amounts, although use of a dry sample is more likely to provide a consistent result.

1. Weigh 30 mg lyophilized bolus material into a 2 ml sample tube from the Fast DNA® Kit containing a ceramic sphere and garnet matrix. Add 0.8 ml extraction buffer (CLS-Y).
2. Homogenize samples in the FastPrep instrument at speed 5 for 30 s. Transfer tubes immediately to ice.
3. Centrifuge tubes in a microfuge at full speed (13 000 *g*, 30 min) at 4°C if possible.
4. Remove 0.4 ml supernatant from the tube into a clean 1.5 ml microfuge tube, then add 0.4 ml binding matrix suspension. Mix gently and allow DNA to bind the matrix for 5 min at room temperature.
5. Recover matrix by centrifugation (13 000 *g*, 1 min) in a microfuge.
6. Carefully pour-off supernatant and wash matrix with 0.5 ml wash solution (SEWS-M). Vortex gently to resuspend matrix, recover by centrifugation in a microfuge (13 000 *g*, 1 min).
7. Repeat step 6.
8. Remove supernatant by pouring then spin tube briefly (13 000 *g*, 15 s) to bring all residual SEWS-M solution to bottom of the tube. Carefully remove residual supernatant with a 200 μl pipette.
9. Elute DNA from the binding matrix by resuspending in 75 μl DES solution then incubating for 3 min at room temperature. Spin tube in a microfuge (13 000 *g*, 1 min) to separate matrix from the DNA solution.
10. Remove 55 μl eluate into a clean microfuge tube. Prepare fivefold serial dilutions of the DNA solution in molecular biology grade water and store at –20°C. Thaw on ice when required.
11. The optimal dilution for PCR should be determined experimentally but is usually 1/25.

Equipment and reagents required

– FastPrep® FP120 Cell Disrupter (Qbiogene Inc., Cedex, France).
– Fast DNA® Kit (BIO 101, Carlsbad, California, USA).

Choice of PCR amplicon, primers and PCR conditions

The anaerobic gut fungi are eukaryotic organisms and therefore require a different genetic marker to the rumen bacteria for identification and differentiation. The use of

18S rDNA has not been found to be useful in differentiating between the members of the gut fungal family, *Neocallimastigaceae*, as they are too similar. We have used the more variable, ITS regions 1 and 2 between the structural genes of the ribosomal repeat as sequences with suitable levels of variability for phylogenetic studies. We have also found that in complex environmental samples, such as faeces or bolus material, specific primers are required to avoid amplification of DNA from the many aerobic fungal forms present on plant matter.

Primers used for population analyses

MN100: 5′TCC TAC CCT TTG TGA ATT TG3′ is a forward primer designed from a region of highly conserved sequence towards the 5′ end of the ITS1 sequence.

MNGM2: 5′CTG CGT TCT TCA TCG TTG CG3′ is an improved version of the GM2 reverse primer described in Brookman et al. [3] and is designed from the 5′ end of the 5.8S rRNA gene. For DGGE, this primer was modified to include the 40 bp GC-clamp sequence, giving **MNGM2C:** 5′CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCT GCG TTC TTC ATC GTT GCG3′.

Amplification of DNA with these primers gives an amplicon of approximately 250 bp specifically for the anaerobic gut fungi. In our experience, we have never amplified ITS1 fragments from the aerobic component of the environmental DNA pool using this primer set.

Composition of PCR mixture

Due to the complexity of the environmental samples the selection of a suitable polymerase enzyme system is extremely important. After comparing several we have found the BD Advantage 2 PCR Enzyme System (BD Biosciences, Palo Alto, California) to be extremely reproducible and capable of amplifying many different templates simultaneously from complex environmental samples.

The reaction mix (25 μl) should contain the following:
2.5 μl 10x BD Advantage 2 buffer
10 pmol of MN100
10 pmol of MNGM2 or MNGM2C (for DGGE reactions)
0.2 mM of each dATP, dCTP, dGTP, dTTP
0.5 μl 50$\times$ Advantage 2 polymerase mix
2.5 μl template (see above)
Molecular biology grade water to a final volume of 25 μl.

PCR conditions

A touchdown PCR cycle should be performed to avoid non-specific amplifications; this is especially important when using a primer with the GC-clamp sequence for DGGE:

95°C	5 min	
95°C	30 s	20 cycles
68°C (−0.5°C each cycle)	30 s	
72°C	30 s	
95°C	30 s	10–15 cycles
58°C	30 s	
72°C	30 s	
72°C	6 min	
4°C	hold	

Successful amplification was verified by electrophoresis of an aliquot of the reaction mixture on a 1% (w/v) agarose gel.

Electrophoresis conditions

Preparation and running of DGGE gels

The use of DGGE for characterization of bacterial populations in the rumen has been discussed earlier by Kocherginskaya et al. [7]. A broadly similar approach can be used with the fungal rumen population with some variation to their methods as described below.

Electrophorese amplified anaerobic gut fungal rDNA populations on DGGE gels of 10% acrylamide containing a 15–30% gradient of denaturant at 60°C and 200 V for 2.5 h.

We use an alternative stain for the DGGE gels, SYBR Gold provides a simpler staining method of equivalent sensitivity to silver staining and requires less handling of the fragile polyacrylamide gels. This stain can also be used for the Spreadex gels.

1. Spread 10 ml SYBR Gold solution over the surface of the gel using a disposable plastic spreader.
2. Incubate at room temperature in the dark for 30 min.
3. Rinse twice for at least 15 min with 50 ml Milli-Q water.
4. Carefully remove from the glass plate for photographing using UV transillumination.
5. Bands of interest can be excised from the gels using a clean sharp blade for recovery and reamplification of the DNA. The ITS1 fragments can then be cloned for sequencing to enable identification of community members.

SYBR Gold solution: dilute 1/10 000 (v/v) in Milli-Q water. SYBR Gold is available from Molecular Probes Inc., Oregon, USA.

For the Spreadex gels 10 mM TAE is recommended for dilution of the SYBR Gold solution. (This should be made by dilution from 1.2 M stock solution described below.)

We observed single-stranded profiles in our fungal samples in addition to the usual double-stranded denaturing profiles (Fig. 1). The separation observed with the single-stranded products of the community amplification together with the relative ease of amplification without clamped primers necessary for DGGE, led us to investigate an alternative separation approach to DGGE, as described below.

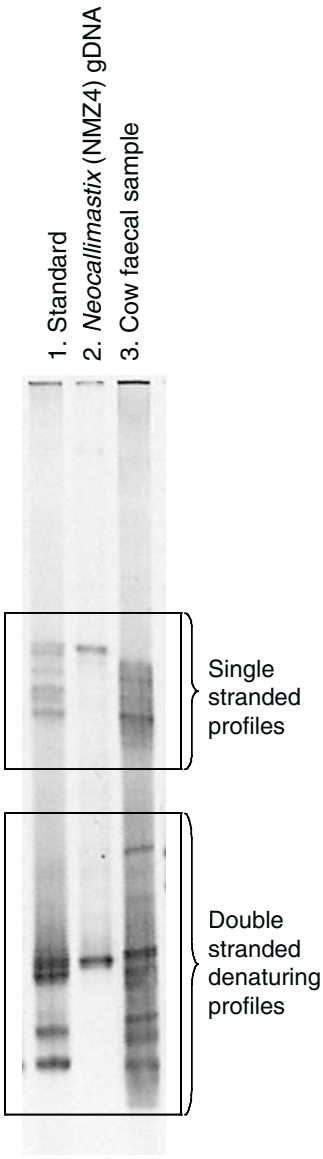


Figure 1. DGGE profiles for ITS1 sequences PCR amplified using anaerobic gut fungal specific primers MN100 and MNGM2C. The standard profile contains sequences amplified from plasmid DNA of ITS1 fragments from eight axenic cultures of gut fungi in our collection, combined into a single sample (Lane 1). Lane 2 was amplified from one of the standard cultures, *Neocallimastix* (NMZ4) genomic DNA. Lane 3 was amplified from total DNA isolated from faecal samples of a domestic cow. In each lane two distinct banding patterns could be seen: the upper patterns were single-stranded PCR products and the lower patterns were the true DGGE profiles.

Preparation and running of Spreadex gels

The size polymorphisms observed in the anaerobic gut fungi make a simpler, size-based separation of ITS1 mixtures extremely effective. We have had considerable success in monitoring population diversity using the Spreadex gel system (Elchrom Scientific, Switzerland). Spreadex gels separate DNA fragments through a non-polyacrylamide matrix by size alone, as incubation at elevated temperatures removes any sequence-dependent mobility differences. We have separated anaerobic gut fungal rDNA amplicons in this way ranging from 206 to 290 bp and bands differing in size by of 1–2 bp can be resolved. The inclusion of a standard profile containing rDNA amplicons of known sizes from a range of anaerobic gut fungal isolates is extremely useful for size estimations of bands in experimental profiles.

1. Fill the electrophoresis tank to the desired level with electrophoresis buffer (approximately 2 l for the SEA 200 and 1 l for the Horizon 11–14) and allow the system to equilibrate to 55°C (approximately 30 min).
2. Prewarm gels by placing on top of the gel tank during the equilibration period.
3. Submerge gels in prewarmed buffer in the tank and rinse the wells with electrophoresis buffer using a hypodermic syringe.
4. Load 5–10 μl PCR product per lane depending on efficiency of PCR amplification as determined by running samples on a simple 1% agarose gel (see above).
5. Electrophorese gels at 10 V/cm (120 V for SEA 2000 or 240 V for Horizon 11–14) for 3 h (mini gels) or 4.5 h (wide mini gels) at 55°C.
6. Remove gels from tank and separate from the backing film using nylon thread.
7. Stain by submerging in a 1/10 000 (v/v) solution of SYBR Gold in 10 mM TAE.
8. Rinse gels twice for at least 30 min in Milli-Q water.
9. Photograph gels as for DGGE gels.
10. Bands of interest can be excised from the gels using a BandPick device (Elchrom Scientific) and included in a 25 μl PCR reaction to reamplify the ITS1 fragment for cloning and sequencing.

(*Note:* We encountered problems with reamplification of the excised bands from the Spreadex gels in many cases. However, the separation obtained and reproducibility of this system recommends its continued use for monitoring the changing diversity in the rumen environment. Where bands will not amplify, we recommend preparing shotgun libraries from an aliquot of the PCR products loaded onto the Spreadex gels. Due to the relatively low level of population complexity ($<$15 bands) in most samples and the high level of accuracy of band size estimations on Spreadex gels (compared with known size standards), shotgun sequences can be matched to corresponding bands in population profiles.)

Reagents and equipment required

SEA 2000® electrophoresis tank (Elchrom Scientific, Switzerland) connected to a recirculating water bath or a standard electrophoresis tank (e.g. Horizon 11–14, Whatman Biometra, Germany) is connected to a recirculating water bath system to maintain temperature control.

EL600 precast gels (Elchrom Scientific), optimum separation range: 150–300 bp.

30 mM TAE diluted from 1.2 M (40×) stock: Add 300 ml Milli-Q water to 72.69 g Tris base, add 40 ml 0.5 M EDTA, pH 8, solution and 17.2 ml glacial acetic acid with mixing. Make up to 500 ml with Milli-Q water.

Shotgun cloning of Spreadex samples

The 15–20 μl PCR mixture remaining after loading a Spreadex gel was used for shotgun cloning of fragments. A QIAquick PCR purification kit (Qiagen) is an effective way to prepare the mixture for ligation. The DNA is eluted into 30 μl volume as per manufacturer's instructions.

The TOPO TA (Invitrogen Ltd., Paisley, UK) cloning vector was used for the shotgun cloning. This vector has a single 3′-thymidine residue at the insertion site of the vector to facilitate cloning of *Taq* DNA polymerase amplified DNA fragments, which have a single deoxyadenosine added, independent of the template, to the 3′ ends of the PCR products. The TOPO vectors are covalently bound to topoisomerase I, which facilitates a more efficient and rapid ligation reaction.

For ligation reactions, 1 μl (50 ng) vector was incubated for 5–30 min with 1 μl of salt solution (1.2 M NaCl; 0.06 M $MgCl_2$) and 50–200 ng of target DNA (usually 2–4 μl from purified PCR mixture) in a final reaction volume of 6 μl according to manufacturer's instructions.

DNA fragments isolated from ligated clones were sequenced, and the ITS sequences are analysed as below to identify the fungal genus. Cloned sequence fragments can also be used as markers for band sizes but as they are excised from the TOPO TA vector by restriction, they will be slightly longer, and hence migrate marginally higher, than the corresponding band from the original PCR mixture.

Sequence analysis

Identification of individual sequences present within a community requires further analysis after visualization by electrophoresis. The gut fungi show a size polymorphism with their ITS1 sequences enabling approximations of the genus composition of a population to be made after separations using Spreadex gels, e.g. Samples 2 and 3 in Figure 2 appear to contain one or more dominant *Piromyces* species and fewer *Neocallimastix* sequences than Sample 4. Unfortunately the ITS1 size does not correspond absolutely to genus as there is some intergeneric overlap. Consequently, analysis of the sequences needs to be performed if community members are to be delineated more definitively.

Phylogenetic analyses such as Maximum Likelihood, Parsimony and Distance Matrix based algorithms have been used to successfully differentiate between the gut fungal genera [3]. These methods, however, are somewhat laborious especially for ongoing studies providing incremental numbers of new sequences, as the analyses will need to be repeated for each new batch of sequence data. More recently, we have used a different approach that allows sequences to be sorted rapidly whether

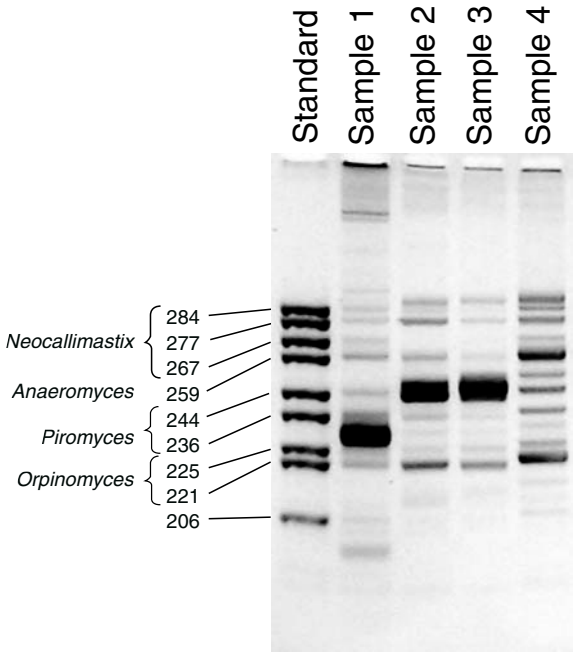


Figure 2. Separation of anaerobic gut fungal ITS1 amplicons according to size using a Spreadex gel. Lane 1, standard profile containing rDNA amplicons from a range of gut fungi, sizes shown in bp. Lanes 2–5, population profiles amplified from environmental (faecal) samples. Samples 2 and 3 were collected from different animals of the same species. All other samples are from animals of different species.

present individually or in large data batches [15]. Ribosomal RNA secondary structure predictions and hidden Markov models have been used to generate sequence fingerprints for the different genera and the several novel sequence groups identified during environmental studies. The fingerprints represent sequence motifs within the variable regions of the ITS1 fragment. The complete ITS1 sequence is composed of four variable regions (Regions I–IV), but the sequences generated using the primers designed to amplify from complex environmental samples contains only three of the four regions (Regions II, III and IV). Matches within any fingerprint to two or three of the three motifs within an environmentally derived ITS1 amplicon enable a likely or confirmed assignment, respectively, to one of the known genera or the novel groups identified to date. Each sequence is given a profile, which identifies the motif present in each of the three variable regions, for example, the sequence from an *Anaeromyces* sp. isolate is Region II Motif 6, Region III Motif 4 and Region IV Motif 4 (II6, III4, IV4). Figure 3 gives an example of this fingerprint technique for sequences AN, OUS1 and TAX1-551. Where two values are given for the same variable region (e.g. sequence AN has two hits for Region II) then the sequence shows similarity to both motifs within that region. The *E* value allows a choice to be made as to which motif is most similar and hence the assigned motif is II6 and not II7 in this case.

AN

HMM II6 Domain start 112 end 159 evalue 1.9e–12

HMM II7 Domain start 112 end 159 evalue 1.7e–05

HMM III4 Domain start 158 end 204 evalue 2.9e–15

HMM IV4 Domain start 203 end 239 evalue 3.4e–11

Anaeromyces - 3/3 fingerprints

OUS1

HMM II8 Domain start 111 end 137 evalue 1.7e–07

HMM III7 Domain start 136 end 183 evalue 3.1e–08

HMM III8 Domain start 136 end 183 evalue 2.2e–14

HMM IV5 Domain start 182 end 203 evalue 2.6e–07

Orpinomyces/PiromycesII - 3/3 fingerprints

TAX1-551

HMM II8 Domain start 112 end 137 evalue 2.3e–07

HMM III6 Domain start 136 end 184 evalue 6.7e–11

HMM IV5 Domain start 183 end 204 evalue 2.6e–07

Orpinomyces/PiromycesII – 3/3 fingerprints

Figure 3. Fingerprints generated for sequences from three fungal isolates: AN, OUS1 and TAX1. The genus assigned from the fingerprints is given in bold together with the number of matched motifs. The matches for the three regions of the ITS1 compared are given for each sample, together with an *E* value as generated by the model.

We reported previously that *Piromyces* is a disparate genus using phylogenetic methodologies and the fingerprinting approach has similar findings. There are three *Piromyces* groups one of which is shared with *Orpinomyces*, e.g. the sequence 551 from a monocentric, *Piromyces*-like isolate TAX1 falls in the *Orpinomyces*/*Piromyces* II group but shows a II8, III6, IV5 fingerprint whereas the *Orpinomyces* strain OUS1 has a II8, III8, IV5 fingerprint.

We have found this approach extremely useful for rapid identification of novel groups within the gut fungi as exemplified by the 206 bp sequence used as the lowest standard marker for the Spreadex gels (Fig. 2, Nicholson et al. in preparation).

References

1. Akin, D.E., G.L.R. Gordon, and J.P. Hogan. 1983. Rumen bacterial and fungal degradation of *Digitaria pentzii* grown with or without sulfur. *Appl. Environ. Microbiol.* **46:**738–748.
2. Bauchop, T. 1979. Rumen anaerobic fungi of cattle and sheep. *Appl. Environ. Microbiol.* **38:**148–158.
3. Brookman, J.L., G. Mennim, A.P.J. Trinci, M.K. Theodorou, and D.S. Tuckwell. 2000. Identification and characterization of anaerobic gut fungi using molecular methodologies based on ribosomal ITS1 and 18S rRNA. *Microbiology* **146:**393–403.
4. Davies, D.R., M.K. Theodorou, M.I. Lawrence, and A.P.J. Trinci. 1993. Distribution of anaerobic fungi in the digestive tract of cattle and their survival in faeces. *J. Gen. Microbiol.* **139:**1395–1400.
5. Doré, J., and D.A. Stahl. 1991. Phylogeny of anaerobic rumen Chytridiomycetes inferred from small subunit ribosomal-RNA sequence comparisons. *Can. J. Bot.* **69:**1964–1971.
6. Ishii, K., M. Fukui, and S. Takii. 2000. Microbial succession during a composting process as evaluated by denaturing gradient gel electrophoresis analysis. *J. Appl. Microbiol.* **89:**768–777.
7. Kocherginskaya, S., R.I. Aminov, and B.A. White. 2001. Analysis of the rumen bacterial diversity under two different diet conditions using denaturing gradient gel electrophoresis, random sequencing, and statistical ecology approaches. *Anaerobe* **7:**119–134.
8. Lee, S.S., J.K. Ha, and K.J. Cheng. 2000. Relative contributions of bacteria, protozoa, and fungi to in vitro degradation of orchard grass cell walls and their interactions. *Appl. Environ. Microbiol.* **66:**3807–3813.
9. Mennim, G. 1997. The application of ribosomal DNA sequence data and other molecular approaches to the study of anaerobic gut fungi. PhD Thesis, Faculty of Science and Engineering, University of Manchester, UK.
10. Nicholson, M.J. 2003. Molecular characterisation of anaerobic gut fungi and their colonisation of plant material in the rumen. PhD Thesis, Faculty of Science and Engineering, University of Manchester, UK.
11. Orpin, C.G. 1984. The role of ciliate protozoa and fungi in the rumen digestion of plant cell walls. *Anim. Feed Sci. Technol.* **10**(2–3)**:**121–143.
12. Pace, N.R. 1997. A molecular view of microbial diversity and the biosphere. *Science* **276:**734–740.
13. Ranjard, L., F. Poly, J.C. Lata, C. Mougel, J. Thioulouse, and S. Nazaret. 2001. Characterization of bacterial and fungal soil communities by automated ribosomal intergenic spacer analysis fingerprints: biological and methodological variability. *Appl. Environ. Microbiol.* **67:**4479–4487.
14. Rezaeian, M., G.W. Beakes, and D.S. Parker. 2002. Distribution of the anaerobic fungi along the digestive tract of sheep. *Reprod. Nutr. Dev.* **42**(Suppl. 1)**:**S84–S85.
15. Tuckwell, D.S., M.J. Nicholson, C.S. McSweeney, M.K. Theodorou, and J.L. Brookman. 2005. The rapid assignment of ruminal fungi to presumpative genera using ITS1 and ITS2 RNA secondary structures to produce group specific fingerprints. *Microbiology* **151:**1557–1567.
16. Vainio, E.J., and J. Hantula. 2000. Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. *Mycol. Res.* **104:**927–936.

4.4. RAPD, RFLP, T-RFLP, AFLP, RISA

STUART E. DENMAN[1], MAKOTO MITSUMORI[2] and CHRISTOPHER S. MCSWEENEY[1]
[1]*CSIRO Livestock Industries, Queensland Bioscience Precinct, 306 Carmody Rd, St Lucia, Queensland 4067, Australia*
[2]*National Institute of Livestock and Grassland Science, 2 Ikenodai, Tsukuba, Ibaraki 305–0901, Japan*

Introduction

Traditional methods of identifying microorganisms through culturing and microscopy techniques can be somewhat tedious and time consuming. A faster and more accurate method for identifying microorganisms is through the sequencing of its ribosomal gene. Classification of microorganisms by ribosomal gene sequencing has become widely accepted within the scientific community. Although this method is quite definitive in its ability to identify the microorganism being studied, it usually involves a pure culture and then the cloning and sequencing of its ribosomal gene. In order to look at complex communities and uncultured microorganisms, many researches have removed the culturing step and moved towards the generation of 16S clone libraries (see Chapter 5.1). Data generated from numerous 16S clone libraries from countless environments have produced databases full of ribosomal sequences that may have never been gathered if culturing of the microorganism had been a prerequisite. Ribosomal clone libraries are still quite time consuming, especially if one is interested in detecting differences between complex community structures under varying conditions, such as the effect, diet can impose on the rumen microbial community. Rapid screening methods that allow for the presentation of phylogenetic ribosomal diversity patterns from complex communities in an easy-to-interpret and reproducible manner have all benefited from the knowledge gained from ribosomal clone libraries. Restriction fragment length polymorphism (RFLP) and terminal restriction fragment length polymorphism (T-RFLP) are two such techniques that will be described in this chapter.

Other methods such as ribosomal intergenic spacer analysis (RISA), which determines diversity through differences, found in the transcribed spacer region between the highly conserved ribosomal genes can also be employed. RISA is a particularly powerful tool for attempting to discriminate between closely related species and strains [9].

Two methods that do not focus on the conserved ribosomal regions for phylogenetic diversity studies are those that amplify up random genomic sequences. The two most common methods are random amplified polymorphic DNA (RAPD) [13] and amplified fragment length polymorphism (AFLP) [11].

H.P.S. Makkar and C.S. McSweeney (eds.), Methods in Gut Microbial Ecology for Ruminants, 151–159.
© 2005 *IAEA. Printed in the Netherlands.*

RFLP, T-RFLP

RFLP is a technique in which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA. Initially, designed methods would involve the extraction of total DNA that was then subjected to restriction digestion with a 4–6 bp cutter and later resolved on an agarose gel. Southern blotting of this gel was then used in conjunction with a gene specific probe [3]; the detection of different size bands would infer polymorphism of that gene. The techniques more commonly employed today to infer phylogenetic diversity within microbial populations focus on the PCR amplification of a portion of the 16S rDNA followed by restriction enzyme digestion. Products are then simply separated using standard gel electrophoresis and visualized for polymorphism [1].

An advancement of this technique has evolved, termed T-RFLP [2, 8], which simply calls for the fluorescent labelling of one or both of the PCR primers. Detection of the terminal restriction fragment fluorochrome is then performed by separating the fragments on an automated sequencer.

RFLP and T-RFLP protocols

These methods can be performed either directly on total community DNA or on 16S rDNA clone libraries generated from total community DNA. In both instances, the initial step involves the amplifying of a fragment of the 16S rDNA with universal primers 27f and 342r [7]. These amplification products are then either cloned and then screened for representatives based on their restriction patterns or immediately digested before separation via gel electrophoresis.

Genomic DNA isolation

Numerous methods are available for genomic DNA extraction; other detailed methods are available in Chapters 3.1, 4.1 and 4.3. We have been employing a bead-beating technique, FastDNA (Q BIOGENE, Carlsbad, CA, USA) following the manufacturer's instructions, which briefly are as follows.

1. Transfer 1.5 ml of sample (rumen fluid or fecal material 200 mg) using a wide bore or cut-off tip into the supplied tube containing the beads.
2. Centrifuge for 5 min at 14 000 *g* in a microcentrifuge.
3. Remove the supernatant and add the following kit reagents 800 μl of lysis buffer CLS-VF, 200 μl of PPS, 200 μl of distilled H_2O and 20 μl of β-mercaptoethanol.
4. Homogenize in FastPrep®instrument for 30 s on a speed setting of 5, place on ice for 2 min before repeating once.
5. Incubate on ice for 5 min and then centrifuge for 15 min at 14 000 *g*at 4°C.
6. Transfer 600 μl to new tube and add 600 μl of binding matrix. Incubate at room temperature for 5 min before centrifuging at 14 000 *g*for 1 min.
7. Remove the supernatant and resuspend the pellet with 500 μl of SEWS-M. Spin for 1 min and remove the supernatant. Spin for 10 s and remove any residual liquid.

8. Elute DNA from matrix by the addition of 100 μl of sterile distilled H_2O and incubate at room temperature for 3 min before centrifuging at 14 000 *g* for 1 min and removing the supernatant to a clean tube.

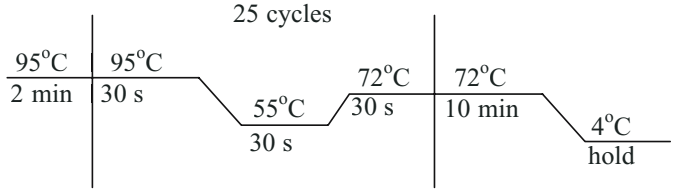
PCR

Amplification of a 16S rDNA fragment is performed on the isolated genomic DNA using primers to bacterial conserved regions at 27f 5′AGAGTTTGATCMTGGCTCAG and 342r 5′CTGCTGCSYCCCGTAG. We have found that Platinum *Taq* from Invitrogen produces the most consistent and sensitive products when using samples from rumen fluid or fecal material.

The PCR is set up as follows:

Components	Volume	Final concentration
10× PCR buffer Mg free	5 μl	1×
10 mM dNTP mixture	1 μl	0.2 mM each
50 mM $MgCl_2$	2.5 μl	2.5 mM
27f primer 10 pmol	1 μl	0.2 pmol
342r primer 10 pmol	1 μl	0.2 pmol
Genomic DNA 100 ng/μl	1 μl	100 ng
Platinum *Taq* (5 U/μl)	0.2 μl	1 U
Sterile distilled water	To 50 μl	

Thermal cycling conditions are as follows:



Cloning of 16S rDNA library

The amplification product generated from total rumen microbial DNA is firstly cloned into a vector system, such as pGEM-T Easy (Promega), before being transformed into *E. coli* competent cells. For a detailed background information and protocols regarding 16S clone libraries, see Chapter 5.1.

Digestion of PCR amplicon

Restriction enzyme digestion of the PCR amplicon will generate products of varying length with respect to sequence diversity. Separation of these products is performed on either 3–4% molecular screening agarose gels or a 15% acrylamide gel. The gels

are then stained and visualized under UV illumination, and the image captured on either polaroid film or a gel documentation system. The data can then be analysed using software such as BIO-RAD's diversity database, where unique fragment patterns can be easily distinguished and phylogenetic trees based on these patterns can be generated. The choice of restriction enzymes to be employed should be based on those that cut frequently (i.e. four base cutters) and produce unique patterns between operational taxonomic units. The choice of enzymes used when screening clone libraries and total community analysis should produce patterns that allow for maximal diversity discrimination. Digestion of the PCR product with two enzymes is sometimes necessary to yield the highest degree of resolution.

The double restriction digestion is set up as follows and incubated at 37°C for 2 h:

Component	Volume
PCR product	20 μl
*Dde*I (10 U/μl)	1 μl
*Alu*I (10 U/μl)	1 μl
10× PCR buffer (Promega buffer C)	2.5 μl

1. After incubation, the digestion products are separated by gel electrophoresis on either 3–4% molecular screening agarose gels or a 15% acrylamide gel (containing 1 ng/ml of ethidium bromide) at 100 V for 2 h.
2. Bands are visualized under UV illumination and the image captured on either polaroid film or a gel documentation system.
3. Analysis of restriction fragments can be performed either visually or by processing image data with band recognition software, such as BIO-RAD's diversity database.

TRFLP analysis

Amplification of a 16S rDNA segment, along with fragmentation through the use of restriction endonucleases, is performed essentially as above for the RFLP protocol. The major modification to the protocol is the use of 5′ fluorescently labelled PCR primer. If both PCR primers are labelled with different fluorophores, then a more complex terminal restriction pattern can be generated. Primers are usually labelled with phosphoramidite dyes, 6-carboxyfluorescein (FAM) or 6-carboxy-2′,4,4′,5′,7,7′-hexachlorofluorescein (HEX). Digestion products are then separated on an automated sequencer, where only the terminal restriction fragments are detected and quantified based on signal intensities and peak area.

Method

1. PCR is performed as above.
2. Prior to the PCR products being analysed, the amplicon product should be run on an agarose gel; if non-specific products are observed, then the desired product should be purified by excising the respective part of the gel using an appropriate gel purification system. Otherwise, the PCR product is purified to remove any

un-incorporated primer using a commercial kit such as QIAquick PCR purification kit from Qiagen.

3. Digestion of PCR product with preferred enzyme should be performed as follows:

Component	Volume
PCR product	200–300 ng
10× buffer	2 μl
Restriction enzyme	2 U
Distilled water	To 20 μl

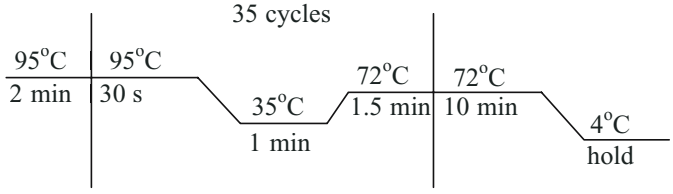
4. Incubate for 2 h at 37°C.
5. To 2 μl of the digestion product, add 2 μl of formamide loading dye [Amersham Biosciences (US79448)] and 0.5 μl of GeneScan 500 ROX internal standard [Applied Biosystems (401734)]. Denature by incubating for 5 min at 95°C and then immediately transferring to ice.
6. Samples are separated on an ABI Prism 377 DNA sequencer using a 36 cm 6% (w/v) polyacrylamide gel containing 8.3 M urea under the following genescan conditions: 2500 V, 40 mA and 30 W for 14 h.
7. The electropherograms are analysed with the GENESCAN analysis software (Applied Biosystems).

RAPD

RAPD was first employed to discriminate between similar genomes in plant species [13]. Initial studies utilizing RAPD analysis within microbiology have focused on clonal and species discrimination [4] rather than total microbial community analysis. Recent studies have used RAPD techniques as a method for addressing microbial community analysis [6, 10, 12]. RAPD analysis differs from ribosomal-based amplification in that no prior genetic knowledge is required. A single small random nucleotide primer of ~10 base pairs is employed under low stringency conditions for amplification. Several primers are usually tested to produce a pattern, which reveals the highest level of community discrimination.

Method

1. Genomic DNA is extracted from environmental samples as mentioned earlier.
2. Amplification reactions are performed essentially as mentioned previously except that only a single arbitrary primer is used as both a forward and reverse primer.
3. Cycling conditions are as below:



4. If analysis is being performed on clonal isolates as a screening method to detect genomic variance, then samples can simply be separated on standard 2% agarose gels, as a relatively simple pattern is expected.
5. For analysis of complex communities, it is suggested that samples be separated on 6% denaturing polyacrylamide gels, for higher resolution.
6. If samples are amplified with fluorescently labelled primers, then they can be detected on an automated sequencing apparatus following similar conditions as described for T-RFLP detection.

AFLP

AFLP developed by Vos et al. [11] involves three steps. Firstly, the genomic DNA is digested, and then adaptors are ligated to the digested fragments. Secondly, amplification is performed by using complementary primers to the adaptor sequence plus an extension of up to three bases at the 3′ end. These extra bases usually allow for the production of a simpler pattern when compared with no extra bases appended. The correct primer combinations must be tested in the first instance for their ability to produce an interpretable pattern. Finally, the gels are analysed for pattern similarities. The use of fluorescently labelled primers allows for analysis to be performed using automated sequencing equipment and GeneScan analysis software from Applied Biosystems. A commercial AFLP microbial fingerprinting kit is available from Applied Biosystems and contains all the required reagents for performing AFLP.

Method

1. Genomic DNA is extracted from environmental samples as mentioned previously.
2. Restriction endonuclease digestion of genomic DNA is performed using *Eco*RI and *Mse*I. A sample aliquot should be tested on an agarose gel to determine if this combination of restriction enzymes is adequate for the genome of interest.
3. Adaptors for ligating to *Eco*RI and *Mse*I digestion ends are as follows:

   *Eco*RI 5′-CTCGTAGACTGCGTACC
   CATCTGACGCATGGTTAA-5′

   *Mse*I 5′-GACGATGAGTCCTGAG
   TACTCAGGACTCAT-5′
4. A ligation reaction is setup as follows and incubated either overnight at room temperature or for 3 h at 37°C.

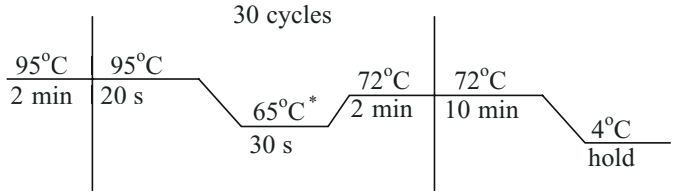
Component	Volume
Digested genomic DNA (100 ng/μl)	1.0 μl
10× T4 DNA Ligase buffer	1.0 μl
0.5 M NaCl	1.0 μl
1.0 mg/ml BSA	0.5 μl
*Mse*I adaptor (20 μM)	1.0 μl
*Eco*RI adaptor (2 μM)	1.0 μl
T4 DNA Ligase	1.0 μl
Water	3.5 μl

5. Dilute by adding 180 μl of TE.
6. An amplification step is performed as set out below using the following primers:
   *Eco*RI 5′-GACTGCGTACCAATTC-NN
   *Mse*I 5′-GATGAGTCCTGAGTAA-NN

Component	Volume
Adaptor ligated DNA (100 ng/μl)	5.0 μl
10× PCR buffer	2.0 μl
*Mse*I primer (5 μM)	0.5 μl
Labelled *Eco*RI primer (1 μM)	0.5 μl
Taq 5 U/ μl	0.2 μl
dNTP mixture (10 mM)	1.0 μl
Distilled water	11.8 μl

(*Note:* N is any nucleotide as an extension base.)

7. Amplification of the fragments is performed under to the following conditions:



*Annealing temperature reduces by 1°C every cycle until it reaches and is maintained at 55°C.

8. To 2 μl of amplification product, add 2 μl of formamide loading dye [Amersham Biosciences (US79448)] and 0.5 μl of GeneScan 500 ROX internal standard [Applied Biosystems (401734)]. Denature by incubating for 5 min at 95°C and then immediately transferring to ice.
9. Samples are separated on 6% denaturing polyacrylamide gel and detected either on ABI 377 or ABI 373 (Applied Biosystems) if using fluorescently labelled primers or in a phosphor imager or X-ray film if radioactive labelled primers were used.

RISA

The ribosomal intergenic spacer region, which lies between the 16S and 23S rRNA genes, can be amplified for determining phylogenetic diversity (RISA). The intergenic transcribed region is not placed under as much mutational constraint as its flanking ribosomal genes and, therefore, contains higher levels of sequence diversity [9]. RISA is a particularly powerful tool for attempting to discriminate between closely related species and strains [9]. Amplification primers target the conserved region at the 3′ end of the 16S gene and the 5′ end of the 23S gene. Products are generated and then

resolved through electrophoresis on either agarose or polyacrylamide gels. The use of fluorescently labelled primers allows for analysis to be performed using automated sequencing equipment and software to analyse the products [5].

Method

1. Genomic DNA is extracted from environmental samples as mentioned previously.
2. Amplification of the ITS region is performed on the isolated genomic DNA using primers to bacterial conserved regions at 1490f 5′GCGGCTGGATCCCCTCCTT and 132r 5′CCGGGTTTCCCCATTCGG.

   The PCR is set up as follows:

Components	Volume	Final concentration
10× PCR buffer Mg free	5 μl	1×
10 mM dNTP mixture	1 μl	0.2 mM each
50 mM $MgCl_2$	2.5 μl	2.5 mM
1490f primer 10 pmol	1 μl	0.2 pmol
132r primer 10 pmol	1 μl	0.2 pmol
Genomic DNA 100 ng/μl	1 μl	100 ng
Platinum *Taq* (5 U/μl)	0.2 μl	1 U
Sterile distilled water	To 50 μl	

3. Samples are resolved on 6% polyacrylamide gel using Tris-borate buffer at 200 V.
4. Gels can be stained with either ethdium bromide or silver stained depending on band intensity expected.
5. Bands are visualized under UV illumination and the image captured on either polaroid film or a gel documentation system.
6. Analysis can be performed either visually or by processing image data with band recognition software, such as BIO-RAD's diversity database.

Silver staining method

1. Wash the gel for 5 min with Buffer A.
2. Discard the buffer and repeat the wash step again.
3. Remove wash buffer and add Buffer B for 10 min.
4. Briefly wash the gel twice with double distilled H_2O.
5. Replace with Buffer C for 10–20 min or until bands are detected.
6. Discard buffer and incubate the gel in Buffer D for 5–10 min.

Solutions required

Buffer A (10% ethanol; 0.5% acetic acid).
Buffer B (0.1% $AgNO_3$).
Buffer C (1.5% NaOH; 0.01% $NaBH_3$; 0.015% formaldehyde) prepared fresh.
Buffer D (0.75% Na_2CO_3).

References

1. Acinas, S.G., F. RodríGuez-Valera, and C. PedróS-Alió. 1997. Spatial and temporal variation in marine bacterioplankton diversity as shown by RFLP fingerprinting of PCR amplified 16s rDNA. *FEMS Microbiol. Ecol.* **24:**27–40.
2. Avaniss-Aghajani, E., K. Jones, D. Chapman, and C. Brunk. 1994. A molecular technique for identification of bacteria using small subunit ribosomal RNA sequences. *Biotechniques* **17:**144–146, 148–149.
3. Busse, H.J., E.B. Denner, and W. Lubitz. 1996. Classification and identification of bacteria: current approaches to an old problem. Overview of methods used in bacterial systematics. *J. Biotechnol.* **47:**3–38.
4. Domig, K.J., H.K. Mayer, and W. Kneifel. 2003. Methods used for the isolation, enumeration, characterisation and identification of Enterococcus spp. 2. Pheno- and genotypic criteria. *Int. J. Food Microbiol.* **88:**165–188.
5. Fisher, M.M., and E.W. Triplett. 1999. Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. *Appl. Environ. Microbiol.* **65:**4630–4636.
6. Harry, M., N. Jusseaume, B. Gambier, and E. Garnier-Sillam. 2001. Use of rapid markers for the study of microbial community similarity from termite mounds and tropical soils. *Soil Biol. Biochem.* **33:**417–427.
7. Lane, D.J. 1991. 16S/23S rRNA sequencing, pp. 115–176. *In* E. Stackebrandt and M. Goodfellow (eds.), *Nucleic Acid Techniques in Bacterial Systematics*. John Wiley & Sons, Inc., New York, NY.
8. Liu, W.T., T.L. Marsh, H. Cheng, and L.J. Forney. 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Appl. Environ. Microbiol.* **63:**4516–4522.
9. Normand, P., C. Ponsonnet, X. Nesme, M. Neyra, and P. Simonet. 1996. ITS analysis of prokaryotes, pp. 1–12. *In* D.L. Akkermans, J.D. van Elsas and E.I. de Bruijn (eds.), *Molecular Microbial Ecology Manual*, Vol. 3.4.5. Kluwer Academic Publishers, Amsterdam, The Netherlands.
10. Vettori, C.D., D. Paffetti, G. Pietramellara, G. Stotzky, and E. Gallori. 1996. Amplification of bacterial DNA bound on clay minerals by the random amplified polymorphic DNA (RAPD) technique. *FEMS Microbiol. Ecol.* **20:**251–260.
11. Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. Van De Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper, and M. Zabeau. 1995. AFLP: a new concept for DNA fingerprinting. *Nucleic Acids Res.* **23:**4407–4414.
12. Wikström, P., L. Häglund, and M. Forsman. 2000. Structure of a natural microbial community in a nitroaromatic contaminated groundwater is altered during biodegradation of extrinsic, but not intrinsic substrates. *Microbiol. Ecol.* **39**:203–210.
13. Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski, and S.V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18:**6531–6535.

PART FIVE

DNA clone libraries of microbial communities

5.1. 16S/18S ribosomal DNA clone library analysis of rumen microbial diversity

ANDRÉ-DENIS G. WRIGHT[1], KIYOSHI TAJIMA[2] and RUSTAM I. AMINOV[3]
[1]*CSIRO Livestock Industries, Centre for Environment and Life Sciences, Wembley, WA 6913, Australia*
[2]*National Institute of Livestock and Grassland Science, National Agricultural Research Organization, Tsukuba, Ibaraki 305-0901, Japan*
and [3]*Rowett Research Institute, Aberdeen AB21 9SB, UK*

Introduction

The rumen contains a complex ecosystem where billions of bacteria, archaea, protozoa and fungi reside. This diverse microbiota is well adapted to live in the rumen and play an important role in the digestion of feed and nutrient supply to the host in the form of microbial protein and volatile fatty acids. It is estimated that the rumen microbial population consists of about 10^6 protozoa/ml, 10^3–10^7 fungi/ml, 10^{10} bacteria/ml, and 10^9 methanogens/ml [11]. To better understand the complex relationships in the rumen, it is necessary to gain an insight into the diversity of the rumen microbes and how the quantity and composition of rumen micro-organisms are altered by a number of different host factors such as age, genetics and diet.

In the past, the diversity of micro-organisms from the digestive tracts of domesticated ruminants has been identified by classical microbiological techniques [43]. However, given the fastidious growth requirements of rumen micro-organisms, it is reasonable to concede that the culture-dependent methods may select against some species, or taxonomic groups, leading researchers to underestimate the microbial diversity that is actually present in the rumen. In fact, it has been speculated that 90% of micro-organisms in nature have escaped traditional cultivation methods [35]. Therefore, a major challenge in microbial ecology has been to assess the diversity and structure of natural microbial communities.

The field of molecular biology has advanced with many innovative technological breakthroughs. The ability to extract and to isolate high-molecular weight DNA from rumen digesta, PCR amplify genes from specific microbial groups and obtain gene sequence data is now a routine event. The small subunit ribosomal RNA (SSU-rRNA) gene, called 16S in prokaryotes and 18S in eukaryotes, is the most widely used molecular marker to presumptively identify morphologically indistinguishable species, to infer their phylogenetic relationships, and to elucidate microbial diversity. Furthermore, the retrieved SSU-rDNA sequence information lays a background for the development of other molecular detection techniques such as FISH, FISH-FC, DNA microarrays and quantitative real-time PCR techniques. The application of molecular techniques has already revealed the enormous wealth of microbial diversity and putative novel species in the rumen [15, 17, 21, 22, 30–32, 34, 39, 40, 45]. In

H.P.S. Makkar and C.S. McSweeney (eds.), Methods in Gut Microbial Ecology for Ruminants, 163–174.
© 2005 *IAEA. Printed in the Netherlands.*

this Chapter, the main steps of SSU-rDNA library generation and analysis will be presented and discussed in detail.

Overview of experimental approach

In general, construction of SSU-rDNA libraries is started from obtaining a fresh sample of the rumen contents from fistulated domestic animals or wild ruminants and fractionation if desirable (rumen fluid, rumen solids and protozoal fractions). At this stage, the sample can be frozen at -80°C for further analysis. Then the total DNA is isolated using either commercially available kits, bead-beating technique, freezing–thawing method or enzymatic lysis. Obviously, this total DNA preparation contains chromosomal DNA of all microbiota, including bacteria, archaea, fungi and protozoa, as well as the host's DNA from sloughed-off epithelial cells. To retrieve specific SSU-rDNA sequences from this mix, PCR amplification with primers that are specific for bacteria, archaea, fungi or protozoa is used. The number of cycles needs to be maintained low (i.e. 10–12 cycles) to avoid preferential amplification of certain templates and/or chimeric molecule formation [3, 42].

Commercially manufactured cloning kits are available to clone the PCR amplified 16S/18S genes from your target group into specially designed plasmids. Positive clones (i.e. white colonies) are randomly selected and checked for the presence of expected size inserts by PCR amplification, this time using the vector primers that surround the insert. These recombinant clones now represent your library and the next step is to analyse them by sequencing.

In a randomly generated library, some clones are closely related and therefore redundant in terms of uncovering the whole diversity. Obviously, an initial selection step before proceeding to the complete sequencing is helpful to reduce the sequencing work while allowing the maximum diversity coverage in a library. This can be done by partial sequencing of PCR templates that were generated in the positive clone screening procedure of the previous step. For this, the PCR products are sequenced with one of the primers used for library production and the sequences are analysed for similarity. Sequences with similarity values $\geq$97% are considered to belong to the same operational taxonomic unit (OTU) and sequencing can be limited to the one representative OTU. This molecular species definition, OTU, is based on correlation between a DNA reassociation value and SSU-rDNA homology. In a polyphasic approach, the 'species' nomination includes bacteria that share a DNA reassociation value of about 70% [37], and this value corresponds to similarities of 97% and higher at the level of SSU-rDNA sequence [28]. For ecological community structure analysis, OTUs are considered as molecular species, while the number of sequences within an OTU represents the population of a given species. Complete sequencing of each representative OTU is done with several sequencing primers complementary to conserved regions of the SSU-rDNA molecule.

Alternatively, the taxonomic groups can be defined on the basis of restriction fragment length polymorphisms (RFLP), also called ribotypes. For this, instead of

sequencing, the PCR fragments are digested with a combination of frequent-cutting restriction enzymes according to the manufacturers specifications. Digested DNA is separated on 3–4% molecular screening agarose gel run at 100 V for at least 2 h. Gels are visualized with UV illumination and documented either by Polaroid photography or by a gel documentation system. The ribotypes are grouped according to their unique pattern and when possible, compared to expected ribotypes for known sequences (of the same length) from the same taxonomic group of interest. Otherwise, 5–10 representatives from each unique ribotype are sequenced for identification or confirmation of presumptive identification based upon expected ribotype pattern.

Further library analysis requires a computer with a set of programs for sequence analysis and Internet access. First sequences from each template are assembled and checked on-line for integrity using the CHIMERA_CHECK program at the Ribosomal Database Project II (http://rdp.cme.msu.edu/cgis/chimera.cgi?su=SSU). This is an important step for cleaning-up a library by eliminating PCR-generated artefacts, such as chimeric sequences. The sequences that passed quality control are aligned together with the type strains and similar sequences from databases. This alignment now can be used for phylogenetic reconstruction using various methods such as neighbour-joining, maximum parsimony or maximum likelihood followed by statistical analysis, such as bootstrapping.

Based on the number of species retrieved (OTUs) and population numbers within the species (number of clones forming an OTU), the ecosystem parameters, such as library coverage, general diversity indices, evenness and species richness, can be elucidated.

Procedures, equipment and reagents

For each animal, fresh rumen content samples should be used and transported on ice in a tightly closed tube to prevent further microbial activity. In a lab, rumen content can be fractionated if desired and immediately frozen and stored at −80°C. Methods to collect, fractionate and extract DNA from rumen digesta and PCR amplification protocols are suggested in Chapter 3.

There are several methods of total DNA isolation, including commercially available kits (e.g. Qiagen QIamp® DNA Stool Mini Kit, Qiagen, Valencia, CA), bead-beating technique, freezing–thawing method or enzymatic lysis. In our experience of bacterial library construction, the bead-beating method, which is also implemented in some kits, results in the maximum recovery of diversity, while the enzymatic lysis method generates libraries biased toward over-representation of Gram-negative bacteria belonging to the CFB phylum (Tajima et al., unpublished observations). Despite the higher probability of chimera formation with template DNA produced by bead-beating, it remains the method of choice for production of PCR-generated SSU-rDNA libraries. This is, in part, because no high-molecular weight DNA is required for this application and the probability of cell disintegration is independent of the cell wall structure.

There are some concerns about library generation by PCR, in that, the number of cycles must be kept as small as possible. It has been shown for the human gut community that the 25-cycle rDNA library displays reduced diversity than the 10-cycle library [3]. The smaller number of cycles imposes strict requirements to DNA quality; it can be additionally purified by gel-filtration, cesium chloride (CsCl) gradient centrifugation or agarose electrophoresis, if PCR-inhibiting substances are suspected in DNA preparations. Another point of the library quality issues is the annealing temperature during PCR. A significant increase in number of OTUs retrieved can be achieved by lowering the annealing temperature [12].

It is also important to mention that most commercial cloning kits use the A-overhang artefact that is generated by *Taq* polymerase and PCR products are ligated into plasmids designed with a T-overhang. Therefore, on the last PCR cycle, the primer extension should be between 7 and 10 min long to ensure completion of the A-overhangs. This will also improve your cloning efficiency. Because there are many commercial cloning kits available, such as the TA cloning kit (Invitrogen), pGEM-T cloning kit (Promega), the Sure Clone Ligation Kit (Pharmacia), etc., it is important to follow the manufacturers instructions. PCR primers used to amplify bacterial, archaeal, protozoal and fungal SSU-rDNA are shown in Table 1.

Cloning

1. After PCR, the mix can be used directly in the cloning reaction with TOPO TA cloning kit (Invitrogen, CA, USA). This kit uses topoisomerase-mediated incorporation of PCR amplicons into the T-overhang vector pCR4 and all cloning procedures can be done within 2 h.
2. To ensure colonies are well spread on the 1.2% LBAXI agar plates (LB agar containing Ampicillin, Xgal and IPTG) (Sigma; Appendix VI), spread three different volumes (120μ, 80μ and 50 μl) using a hockey stick and incubate at 37°C for 14–16 h.

   If using TOP10 cells, no IPTG is required for blue/white screening.

   (*Note*: A bacterium without an insert in the plasmid forms blue colonies (i.e. negative) and a bacterium with an insert in the plasmid forms white colonies in the presence of Xgal (i.e. positive) [25].)
3. Remove the plates with the transformed colonies and store at 4°C for several hours to increase the intensity of blue (i.e. negative) colonies.

Clone confirmation

1. Use the blunt end of an autoclaved toothpick to gently touch the centre of a single white colony and make three streaks 1–2 cm long onto a new 1.2% LBAXI agar plate. Repeat this step using a new toothpick for each white colony, and then incubate at 37°C for 12–16 h.
2. Remove LBAXI agar plates and check triple streaks for blue colouration. Disregard blue steaks and number only those sets of triple streaks that are white.

Table 1. Primers used for library construction and analysis

Primer ID	Primer sequence	References
Archaea		
025eF	5′-CTG GTT GAT CCT GCC AG	[1]
1492R	5′-GGT TAC CTT GTT ACG ACT	[1]
D30	5′-ATT CCG GTT GAT CCT GC	[2]
D33	5′-TCG CGC CTG CGC CCC GT	[2]
Met83F	5′-ACK GCT CAG TAA CAC	[44]
Met86F	5′-GCT CAG TAA CAC GTG G	[44]
Met1340R	5′-CGG TGT GTG CAA GGA G	[44]
Ar1000	5′-AGTCAGGCAACGAGCGAGA	[38]
Ar1500	5′-GGTTACCTTGTTACGACTT	[38]
Bacteria		
27f	5′ AGA GTT TGA TCM TGG CTC AG	[16]
519f	5′ CAG CMG CCG CGG TAA TWC	[16]
519r	5′ GWA TTT TAC CGC GGC KG C TG	[16]
926r	5′ CCG TCA ATT CMT TTR AGT TT	[16]
926f	5′ AAA CTY AAA KGA ATT GAC GG	[16]
1492r	5′ TAC GGY TAC CTT GTT ACG ACT T	[16]
1525r	5′ AAG GAG GTG WTC CAR CC	[16]
Eukarya		
Medlin B	5′-TGA TCC TTC TGC AGG TTC ACC TAC	[19]
Fungi		
nu-SSU-0817f	5′ TTA GCA TGG AAT AAT RRA ATA GGA	[4]
nu-SSU-1196r	5′ TCT GGA CCT GGT GAG TTT CC	[4]
nu-SSU-1536r	5′ ATT GCA ATG CYC TAT CCC CA	[4]
Protozoa		
P-SSU-342F	5′-CTT TCG ATG GTA GTG TAT TGG ACT AC	[13]

3. For each group of white streaks, use the blunt end of an autoclaved toothpick to collect cells from one of the three streaks. Pick one blue streak and use it as your negative control.
4. Stick toothpick and cells into 15 μl of distilled H_2O in a 0.6 ml microfuge tube. Twist toothpick in the distilled H_2O to dislodge the bacterial cells (water should turn cloudy) and discard the toothpick into a biohazardous waste autoclave bag. Do not leave toothpick in tube or it will absorb the solution.
5. Add 15 μl of 2$\times$ plasmid cracking buffer and vortex. Bacterial cells are lysed when the solution turns clear.
6. Add 30 μl of distilled H_2O to the solution and then add 5 μl of 6$\times$ dye.

   To check for plasmids containing the correct size insert, load 30 μl of each tube onto a 1.0% agarose gel and run for 1 h at 100 V. First load a DNA molecular marker and then the sample from the negative control (i.e. blue streak containing plasmid only).

   (*Note*: The DNA will be supercoiled, so it will migrate faster than linear DNA. To determine the approximate size of your positive clone on the gel, take the size of your plasmid and add the size of your PCR product and then look for a band at approximately 60% of your calculation. For example, if your plasmid is 2.9 kb

and your insert is 1.3 kb insert (4.2 kb total), then 60% of 4.2 kb would be the supercoiled size.)

7. Identify and label all samples of the correct molecular weight (i.e. those larger than the negative control).
8. Aseptically remove one of the two remaining triple streaks from each positive clone using an inoculation loop or an autoclaved toothpick and inoculate test tubes containing 10 ml of LB broth and Ampicillin.
9. Agitate tubes overnight on a platform shaker at 37°C.

   (*Note:* The remaining streak can be used as a backup or to make glycerol stocks for storage.)

Alternative rapid protocol for clone confirmation

If you have access to a genomics facility, recombinant colonies are picked up using an automated colony picker into the 96- or 384-well format microtitre plates. The cells are grown overnight in 2× LB or terrific broth and archived with 20% glycerol or 7% DMSO at −80°C. The liquid handling robot is then used for PCR amplification using 1 μl of cell suspension as a template and vector primers (M13 or T3 + T7). One microlitre of the resulting PCR is used for sequencing PCR. If no high-throughput facility is available, follow the protocol given below.

1. Prepare PCR master mix with BIOTAQ Red polymerase (Bioline) and M13 or T3 and T7 primers.
2. Dispense 15 μl aliquots into a 96-well PCR plate on ice using a 12-channel pipette.
3. Use a sterile plastic toothpick to gently touch the centre of a single white colony and dip it into the PCR mix for 1–2 s. Then inoculate the biomass remaining on a toothpick to a corresponding grid on a 96-well microtitre plate with ×2 LB or terrific broth. Repeat the step using a new toothpick for each white colony.
4. Pick a blue colony as a negative control.
5. Seal the plate and run PCR with initial DNA denaturation at 95°C for 10 min, followed by 35 cycles of 95°C for 10 s, 55°C for 10 s and 72°C for 30 s, then the final elongation step at 72°C for 10 min and hold at 4°C.
6. In the meantime, prepare a 1% agarose gel in TBE with a GelStar fluorescent dye (BMC).
7. Load 5 μl aliquots into the gel directly from the PCR plate using a 12-channel pipette. No need to add loading buffer. Run phoresis at 100 V for 1 h.
8. After electrophoresis, document the gel with the GelDoc system. ‘Empty’ clones will produce a small PCR product, 150–190 bp of length, migrating together with the negative control, while recombinants will produce larger amplicons (ca. 1.5 kb if bacterial libraries were produced with 27f and 1525r or 27f and 1492r primer pairs).
9. The remaining PCR product (step 7) can be stored at 4°C for sequencing with a single primer (e.g. with 27f primer in a bacterial library that was produced with 27f and 1492r primers) to define the number of OTUs in your library.

10. Incubate the plate from step 3 at 37°C for 12–16 h with gentle agitation and archive it at −80°C after adding glycerol (20% final concentration) or DMSO (7%).

Plasmid miniprep

Plasmids are harvested and purified from the overnight cultures using plasmid minipreps such as the Flexi-Prep 100 Kit (Qiagen) and GenElute™ Plasmid Miniprep kit (Sigma). The plasmid insert is then re-amplified by PCR using the vector-specific primer, usually M13 or T3 and T7.

Determination of OTUs by ribotyping

1. Frequent-cutting restriction endonucleases are used, in accordance with manufacturer's specifications, to digest the amplified SSU-rDNA sequence.
2. Digested fragments with a 100 bp DNA molecular weight marker (to determine the size of the bands) are then separated by gel electrophoresis performed at 100 V for 2 h on high-resolution 3–4% molecular screening agarose gel.
3. Gels are visualized with UV illumination and ribotypes documented either by Polaroid photography or by a gel documentation system.
4. Ribotypes are grouped according to their unique pattern.
5. At least 5–10 representatives from each unique ribotype are sequenced for identification, ideally in both directions using a set of primers covering the whole amplicon.

Determination of OTUs by sequencing

1. Perform sequencing PCR with a 1 μl template from a positive PCR reaction (Chapter 3) and an internal sequencing primer (e.g. 338F for bacterial libraries, or Met448F for methanogen libraries [44]).

   (*Note:* If PCR was performed with a large excess of primers and dNTPs, they may interfere with the subsequent sequencing PCR and need to be removed by ExoSAP-IT (Amersham Biosciences). For this, add 1 μl of the ExoSAP-IT enzyme mix to 5 μl of PCR product and incubate at 37°C for 15 min, followed by enzymes inactivation at 80°C for 15 min. Use 1 μl of it in cycle sequencing (e.g. with BigDye v.3.1, Applied Biosystems, or other chemistries.)
2. Clean up the sequences and generate a DNA similarity matrix. Reads with more than 97% similarity represent a single OTU and 1–2 clones from each OTU are submitted to complete sequencing by a combination of primers covering the whole amplicon on both strands (e.g. in the case of 27r and 1492r-generated bacterial libraries, these are 27f, 338f, 338r, 907r, 926f and 1492r; or in the case of Met83F or Met86F and Met1340R-generated methanogen libraries these are Met86F, Met448F, Met448R, Met1027F, Met1027R and Met1340R [44]).

Bioinformatics

Phylogenetic classification is an evolutionary hypothesis of a historical sequence of speciation events that suggests that species or groups of species classified together are more closely related genealogically to each other than they are to any other taxa [10, 41]. This system is based on the principle of homology and uses *a priori* arguments based on comparisons to an outgroup. Theoretically, any taxonomic unit not in the ingroup can be used as the outgroup. However, ideally you should use the sister group for comparisons [18, 27, 29, 36].

1. Assemble several sequencing reads of each OTU using Sequencher or AutoAssembler (PE-Applied Biosystem). Clean up from vector sequences and edit the sequence if necessary.
2. Check the integrity of your sequence using the CHIMERA_CHECK program at the Ribosomal Database Project II (http://rdp.cme.msu.edu/cgis/chimera.cgi?su = SSU).
3. Align new sequences against existing sequences for your taxonomic group using sequence alignment software such as Dedicated Comparative Sequence Editor (DCSE) [23] or CLUSTALX [33] (available at http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html). For phylogenetically coherent sequences, remember to use sequences from closely related species for your outgroup. If the library is generated with primers targeting the larger order taxa such as the *Bacteria* domain, use the deep-branching sequences of *Thermotoga* and *Aquifex*.
4. Check the alignment quality and edit if necessary (now the alignment is ready for phylogenetic analysis). A phylogenetic inference package such as PHYLIP (v. 3.51C) [6] can be used to calculate the sequence similarity and evolutionary distances between pairs of nucleotide sequences using a distance model such as the Kimura two-parameter model [14].
5. Construct a distance-matrix tree using either the Fitch and Margoliash least squares method [7] or the neighbour-joining method [24].
6. To determine the stability of particular groups of taxa, bootstrap resamples [5] the data 1000 times. When phylogenetic trees are constructed by distance-matrix methods, the horizontal length of the branches produced from the analysis indicates the amount of genetic difference between the organisms, in relation to each other on the tree.
7. Visualize and edit the tree using TreeView, NJ plot, or MacClade program.

The Shannon index values

The Shannon index illustrates the difference in diversity between SSU-rDNA clone libraries. The Shannon index (H) of general diversity can be calculated for each clone library using the following equation [20].

$$H = -\sum \left(\frac{ni}{N}\right) \times \log \left(\frac{ni}{N}\right)$$

where ni = importance value for each species
N = total of importance values

The Shannon index is calculated as follows: the total importance value is the total number of clones identified in that sample. Thus, the importance value for the individual clone was the number of times it was identified in the sample. The SSU-rDNA clone library with the greatest diversity has the highest Shannon index value.

Rarefaction analysis

The diversity of your clone libraries can be estimated by rarefaction analysis [9] using the Analytic Rarefaction v.1.3 program (S.M. Holland, University of Georgia), which is freely available on the Web (www.uga.edu/strata/software).

Library coverage

The library coverage value is calculated according to Good [8] using the formula $[1 - (n/N)]$, where n is the number of OTUs represented only by a single clone and N is the total number of clones in the library. The more unique OTUs in the library, the lower the diversity coverage. This approach also helps to test whether two libraries (e.g. from different diets) are significantly different and can be calculated with the LIBSHUFF program [26] available on the Web (www.arches.uga.edu/∼whitman/libshuff.html).

Solutions

1.0 M Tris–HCl, pH 8.0
0.5 M Na-EDTA, pH 8.0
0.5 M Na-EDTA, pH 7.5
5 M NaCl

Xgal/IPTG (5 : 1) solution
- 5-Bromo-4-chloro-3-indolyl galactopyranoside (Xgal) 100 mg
- Isopropyl thio-β-D-galactoside (IPTG) 200 mg
- Dimethylformamide 5 ml
- Autoclaved distilled H_2O 1 ml

Dissolve 100 mg of Xgal in 5 ml of dimethylformamide (20 mg/ml).
Dissolve 200 mg of IPTG in 1.0 ml of autoclaved distilled H_2O (200 mg/ml)
Mix 5 ml of Xgal solution with 1 ml of IPTG and store at −20°C.

Luria Bertini (LB) broth
- NaCl 5.0 g
- Yeast extract 2.5 g
- Bacto tryptone 5.0 g

Mix ingredients and bring to 500 ml with distilled H_2O, then aliquot 10 ml into 40 test tubes. Use 100 ml for 100× Ampicillin solution (see below). Autoclave test tubes, and when cool using sterile techniques add 100 μl of the 100× Ampicillin solution to each tube. Store tubes at 4°C.

100× Ampicillin solution

- LB broth 100 ml
- Ampicillin 1.0 g

Dissolve 1.0 g of Ampicillin into 100 ml of LB broth. No need to autoclave as this is 100× recommended strength. Keep frozen at −20°C.

1.2% LB–Ampicillin–Xgal–IPTG (LBAXI) agar

- NaCl 10.0 g
- Yeast extract 5.0 g
- Bacto tryptone 10.0 g
- Agar 12.0 g
- Xgal : IPTG (5 : 1) solution 1.0 ml
- 100× Ampicillin solution 10.0 ml (or 100 mg Ampicillin)

Mix all dry ingredients and bring to 1 l with distilled H_2O and autoclave. When solution is cool enough to touch against wrist, add Ampicillin and 1 ml of Xgal : IPTG (5 : 1) solution. Swirl the contents and pour into (30–40) Petri dishes.

2× Plasmid Cracking Buffer

- 10 M NaOH 0.5 ml
- 0.5 M Na-EDTA pH 8.0 1.0 ml
- 20% (v/v) SDS 2.5 ml
- Glycerol 5.0 ml
- Distilled H_2O 41.0 ml

Mix solution and store at room temperature. No need to autoclave.

6× Dye

- Bromophenol blue (final concentration 0.3%) 0.3 g
- Xylene Cyanol FF (final concentration 0.3%) 0.3 g
- 0.5 M Na-EDTA (pH 7.5) (final concentration 10.0 mM) 2.0 ml
- 40% (w/v) Glucose solution 98.0 ml

Mix solution and store at −20°C. No need to autoclave.

Other reagents and equipment

DNA isolation kits, PCR kits, primers, pipettes and tips, electrophoresis unit, thermocycler, UV transilluminator, spectrophotometer (A260 nm capable), restriction enzymes, DNA size markers, agarose (multipurpose and molecular screening), centrifuge, incubator, water bath, capillary sequencer, computer and software for sequence alignment and phylogenetic analysis.

References

1. Achenbach, L., and C. Woese. 1995. 16S and 23S rRNA-like primers, pp. 521–523. *In* K.R. Sower and H.J. Schreier (eds.), *Archaea: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
2. Arahal, D.R., F.E. Dewhirst, B.J. Paster, B.E. Volcani, and A. Ventosa. 1996. Phylogenetic analyses of some extremely halophilic archaea isolated from Dead Sea water, determined on the basis of their 16S rRNA sequences. *Appl. Environ. Microbiol.* **62:**3779–3786.
3. Bonnet, R., A. Suau, J. Dore, G.R. Gibson, and M.D. Collins. 2002. Differences in rDNA libraries of faecal bacteria derived from 10- and 25-cycle PCRs. *Int. J. Syst. Evol. Microbiol.* **52:**757–763.
4. Borneman, J., and R.J. Hartin. 2000. PCR primers that amplify fungal rRNA genes from environmental samples. *Appl. Environ. Microbiol.* **66:**4356–4360.
5. Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39:**783–791.
6. Felsenstein, J. 1993. PHYLIP (Phylogeny Inference Package) documentation files. Version 3.51 c. Distributed by the author, Department of Genetics, University of Washington, Seattle, WA.
7. Fitch, W.K., and E. Margoliash. 1967. Construction of phylogenetic trees. *Science* **155:**279–284.
8. Good, I.J. 1953. The population frequencies of species and the estimation of population parameters. *Biometrika* **40:**237–262.
9. Heck, K.L., G. Van Belle Jr., and D. Simberloff. 1975. Explicit calculation of the rarefaction diversity measurement and determination of sufficient sample size. *Ecology* **56:**1459–1461.
10. Hennig, W. 1966. *Phylogenetic Systematics*, 264 pp. University of Illinois Press, Urbana, IL.
11. Hespell, R.B., D.E. Akin, and B.A. Dehority. 1997. Bacteria, fungi, and protozoa of the rumen, pp. 59–141. *In* R.I. Mackie, B.A. White, and R.E. Isaacson (eds.), *Gastrointestinal Microbiology*, Vol. 2. Chapman & Hall, New York.
12. Hongoh, Y., H. Yuzawa, M. Ohkuma, and T. Kudo. 2003. Evaluation of primers and PCR conditions for the analysis of 16S rRNA genes from a natural environment. *FEMS Microbiol. Lett.* **221:**299–304.
13. Karnati, S.K., Z. Yu, J.T. Sylvester, B.A. Dehority, M. Morrison, and J.L. Firkins. 2003. Specific PCR amplification of protozoal 18S rDNA sequences from DNA extracted from ruminal samples of cows. *J. Anim. Sci.* **81:**812–815.
14. Kimura, M. 1980. A simple method of estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16:**111–120.
15. Kocherginskaya, S.A., R.I. Aminov, and B.A. White. 2001. Analysis of the rumen bacterial diversity under two different diet conditions using denaturing gradient gel electrophoresis, random sequencing, and statistical ecology approaches. *Anaerobe* **7:**119–134.
16. Lane, D.J. 1991. 16S/23S rRNA sequencing, pp. 115–175. *In* E. Stackebrandt and M. Goodfellow (eds.), *Nucleic Acid Techniques in Bacterial Systematics*. Wiley and Sons, New York.
17. Lin, C., L. Raskin, and D.A. Stahl. 1997. Microbial community structure in gastrointestinal tracts of domestic animals: comparative analysis using rRNA-targeted oligonucleotide probes. *FEMS Microbiol. Ecol.* **22:**281–294.
18. Maddison, W.P., M.J. Donoghue, and D.R. Maddison. 1984. Outgroup analysis and parsimony. *Syst. Zool.* **33:**83–103.
19. Medlin, L., H.J. Elwood, S. Stickel, and M.L. Sogin. 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene* **71:**491–499.
20. Odum, P.E. 1971. Principles and concepts pertaining to organization at the community level, pp. 140–161. *In Fundamentals of Ecology*. W.B. Saunders Company, Philadelphia.
21. Ramsak, A., M. Peterka, K. Tajima, J.C. Martin, J. Wood, M.E. Johnston, R.I. Aminov, H.J. Flint, and G. Avgustin. 2000. Unravelling the genetic diversity of ruminal bacteria belonging to the CFB phylum. *FEMS Microbiol. Ecol.* **33:**69–79.
22. Rieu-Lesme, F., J.J. Godon, and G. Fonty. 2000. Remarkable archaeal diversity detected in the rumen of a cow. *Reprod. Nutr. Dev.* **40:**179–180.
23. Rijk, P. de, and R. de Wachter. 1993. DCSE, an interactive tool for sequence alignment and secondary structure research. *Comput. Appl. Biosci.* **9:**735–740.

24. Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4:**406–425.
25. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed., Vols. 1–3. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
26. Singleton, D.R., M.A. Furlong, S.L. Rathbun, and W.B. Whitman. 2001. Quantitative comparisons of 16S rRNA gene sequence libraries from environmental samples. *Appl. Environ. Microbiol.* **67:**4374–4376.
27. Smith, A.B. 1994. Rooting molecular trees: problems and strategies. *Biol. J. Linn. Soc.* **51:**279–292.
28. Stackebrandt, E., and B.M. Goebel. 1994. A place for DNA-DNA reassociation and 16S ribosomal-RNA sequence-analysis in the present species definition in bacteriology. *Int. J. Syst. Bacteriol*. **44:**846–849.
29. Stackebrandt, E., and W. Ludwig. 1994. The importance of using outgroup reference organisms in phylogenetic studies: the *Atopobium* case. *Syst. Appl. Microbiol.* **17:**39–43.
30. Tajima, K., R.I. Aminov, T. Nagamine, K. Ogata, M. Nakamura, H. Matsui, and Y. Benno. 1999. Rumen bacterial diversity as determined by sequence analysis of 16S rDNA libraries. *FEMS Microbiol. Ecol.* **29:**159–169.
31. Tajima, K., S. Arai, K. Ogata, T. Nagamine, H. Matsui, M. Nakamura, R.I. Aminov, and Y. Benno. 2000. Rumen bacterial community transition during adaptation to high-grain diet. *Anaerobe* **6:**273–284.
32. Tajima, K., T. Nagamine, H. Matsui, M. Nakamura, I. Rustam, and R.I. Aminov. 2001. Phylogenetic analysis of archaeal 16S rRNA libraries from the rumen suggests the existence of a novel group of archaea not associated with known methanogens. *FEMS Microbiol. Lett.* **200:**67–72.
33. Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin, and D.G. Higgins. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **24:**4876–4882.
34. Tokura, M., I. Chagan, K. Ushida, and Y. Kojima. 1999. Phylogenetic study of methanogens associated with rumen ciliates. *Curr. Microbiol.* **39:**123–128.
35. Ward, D.M., M.M. Bateson, Z.R. Weller, and A.L. Ruff-Roberts. 1992. Ribosomal rRNA analysis of microorganisms as they occur in nature. *Adv. Microbiol. Ecol.* **12:**219–286.
36. Watrous, L.E., and Q.D. Wheeler. 1981. The out-group comparison method of character analysis. *Syst. Zool.* **30:**1–11.
37. Wayne, L.G., D.J. Brenner, R.R. Colwell, P.A.D. Grimont, O. Kandler, M.I. Krichevsky, L.H. Moore, W.E.C. Moore, R.G.E. Murray, E. Stackebrandt, M.P. Starr, and H.G. Truper. 1987. International committee on systematic bacteriology. Report of the adhoc committee on reconciliation of approaches to bacterial systematics. *Int. J. Syst. Bacteriol.* **37:**463–464.
38. Weisburg, W.G., S.M. Barns, D.A. Pelletier, and D.J. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **173:**697–703.
39. Whitford, M.F., R.J. Forster, C.E. Beard, J. Gong, and R.M. Teather. 1998. Phylogenetic analysis of rumen bacteria by comparative sequence analysis of cloned 16S rRNA genes. *Anaerobe* **4:**153–163.
40. Whitford, M.F., R.M. Teather, and R.J. Forster. 2001. Phylogenetic analysis of methanogens from the bovine rumen. *BMC Microbiol.* **1:**5.
41. Wiley, E.O. 1981. *Phylogenetics; The Principles and Practice of Phylogenetic Systematics,* 439 pp. John Wiley and Sons, New York.
42. Wintzingerode, F. von, U.B. Gobel, and E. Stackebrandt. 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiol. Rev.* **21:**213–229.
43. Wolin, M.J, T.L. Miller, and C.S. Stewart. 1997. Microbe–microbe interactions. *In* P.N. Hobson and C.S. Stewart (eds.), *The Rumen Microbial Ecosystem*. Blackie Academic and Professional, New York.
44. Wright, A.-D.G., and C. Pimm. 2003. Improved strategy for presumptive identification of methanogens using 16S riboprinting. *J. Microbiol. Methods* **55:**337–349.
45. Yanagita, K., Y. Kamagata, M. Kawaharasaki, T. Suzuki, Y. Nakamura, and H. Minato. Phylogenetic analysis of methanogens in sheep rumen ecosystem and detection *Methanomicrobium mobile* by fluorescence *in situ* hybridization. *Biosci. Biotechnol. Biochem.* **64:**1737–1742.

PART SIX

Use of small subunit ribosomal RNA directed oligonucleotide probes for microbial population studies

6.1. Northern blot analysis to investigate the abundance of microorganisms

DENIS O. KRAUSE
Department of Animal Science and Department of Medical Microbiology and Infectious Disease, University of Manitoba, Winnipeg, MB, Canada

Introduction

Modern molecular microbial ecology has its origins in the analysis of informative macromolecules [5]. Zuckerkandl and Pauling [23] proposed that certain macromolecules are relatively free from evolutionary pressure and may be considered a molecular document of the evolutionary history of the organism that carries the molecule. In their paper, they proposed that the sequence difference of a molecule is proportional to the evolutionary distance between the organisms; the greater the sequence differences the greater the evolutionary distance.

A significant breakthrough with this approach in microbial systematics resulted from the work of Woese and Fox [21] who used oligonucleotide cataloguing of 16S-rRNA to delineate the phylogenetic relationships between microorganisms. By using this approach, it was possible to demonstrate that all life on earth could be divided into three kingdoms: eukarya, procarya and archaea [20]. The unique findings of this research was that the archaea, made up of many methanogenic and thermophilic microorganisms, were probably the most ancient life forms on earth and were not bacteria at all.

One of the first applications of rRNA genes was the recovery of unique 5S-rRNA sequences from the Yellowstone hot spring [18]. Even though the statistical utility of the short 5S sequences was limited, it demonstrated that there was a great deal of uncultured diversity within the ecosystem. This uncultured diversity was demonstrated to be highly significant when clone libraries were constructed from the Yellowstone hot spring [3]. Universal PCR primers were used to amplify 16S-rDNA from the microbial community, and these mixed amplicons were cloned into a vector. Each insert, potentially representing a different species, was sequenced giving a snapshot of microbial diversity in the sample.

A unique feature of the rRNAs is that they are hierarchical molecules [6, 14]. This means that there are regions where the molecules is highly conserved, others where the sequence is variable, and even areas known as hyper-variable regions which have a high degree of sequence variation. As a result of this structure, it is possible to design signature oligonucleotide probes varying in length from about 15 to 30 nucleotides that are diagnostic of microorganisms at the kingdom, domain, genus and even species

H.P.S. Makkar and C.S. McSweeney (eds.), Methods in Gut Microbial Ecology for Ruminants, 177–190.
© 2005 *IAEA. Printed in the Netherlands.*

level [20]. These signature sequences can be used in a variety of applications such as PCR analysis, construction of clone libraries or direct probing of bulk rRNA.

Some of the first applications of this methodology were in fact in the rumen [17]. Ribosomal RNA was extracted from cattle that were fed the ionophore antibiotic monensin. A variety of microbial species were assessed with specific 16S-rRNA directed oligonucleotide probes. This basic technique has been applied in a number of rumen experimental protocols [7, 8, 13] and is still one of the most robust molecular methods for assessing microbial species in the rumen. Some of the reasons for this included:

1. rRNA is more sensitive than DNA because for every one copy of DNA in the cell, there can be as many as 10 000 copies of rRNA [1, 5, 20].
2. rRNA is indicative of function because only growing cells produce RNA [1, 15, 20].
3. Contaminants that inhibit PCR are not a problem because there are no enzymatic steps in northern blot analysis [4, 10].

Ribosomal sequences are stored in large databases, one of the best being The Ribosomal Database Project (RDP) [12]. Sequences are extracted from other database such as NCBI and EMBL, assessed for their integrity, and then placed within a phylogenetic framework that is searchable. Assessment for integrity of the sequence is important because the integrity of the database is founded on the quality of data. Computational checks such as searches for chimeric sequences and secondary structure motifs are done by database staff before the sequences are included in the phylogenetic trees.

Databases are extremely important for use in oligonucleotide design. The principle of oligonucleotide design will not be discussed here, and the reader should refer to other publications for this very important topic [1]. Recent publication provides a series of probes that can be used in rumen microbiology, and readers should refer to Chapter 3 and these manuscripts [16, 19]. There are also software programs available such as PRIMROSE which can import aligned sequence files directly from the RDP and has easy to use facilities that aid the user to design oligonucleotide probes [2].

In this chapter, I provide detailed protocols for the analysis of extracted rRNA and give detailed procedures that must be followed to do northern blot analysis of bulk RNA extracted from the rumen.

Methodology

Background

This protocol is divided into two sections: (a) analysis of rRNA extracts using electrophoresis under denaturing conditions in an agarose–formaldehyde gel and (b) slot-blot hybridization for quantitative analysis of the relative abundance of targets in the rumen. See Chapter 3 for RNA extraction methods. Extracted rRNA is run on a formaldehyde gel to determine the concentration and quality of the 16S band in the case of bacteria and archea, and the 18S in the case of fungi. These molecules have

extensive secondary structure and need to be denatured when run on a gel. By not denaturing the ribosome, the migration patterns of each subunit of the rRNA can vary significantly.

Materials required

- 10× and 1× MOPS running buffer (see recipe for 10× buffer).
- 12.3 M (37%) formaldehyde, pH >4.0.
- RNA sample.
- Formamide.
- Formaldehyde loading buffer.
- 0.5 M ammonium acetate and 0.5 μg/ml ethidium bromide in 0.5 M ammonium acetate or 10 mM sodium phosphate (pH 7.0; see recipe)/1.1 M formaldehyde with or without 10 μg/ml acridine orange.
- 0.05 M NaOH/1.5 M NaCl (optional).
- 0.5 M Tris·Cl (pH 7.4, 1.5 M NaCl (optional).
- 20×, 2× and 6× SSC.
- 0.03% (w/v) methylene blue in 0.3 M sodium acetate, pH 5.2 (optional).
- SSU-based oligonuctleotides suitable for use as probes.
- Formamide prehybridization/hybridization solution.
- 2× SSC/0.1% (w/v) SDS.
- 0.2× SSC/0.1% (w/v) SDS, room temperature and 42°C.
- 0.1× SSC/0.1% (w/v) SDS.
- 55°C, 60°C, 68°C and 100°C water baths.
- RNase-free glass dishes.
- Whatman 3 mm filter paper sheets.
- UV-transparent plastic wrap.
- Nylon membrane.
- Glass plate of appropriate size.
- Oven.
- Hybridization oven and tubes.

(*Note:* Preparation of RNAase-free glassware and solutions with DPEC is essential. Please see protocol at the end of the chapter.)

Agarose/formaldehyde gel electrophoresis

Gel preparation

1. Melt 1.0 g agarose in 72 ml water and cool to 60°C. The ideal temperature is when you can comfortable pick up the bottle without burning yourself. If the agarose is too hot, it will crack the gel tray.

   (*Note:* This step will result in a 1.0% gel, which is suitable for rRNA molecules that would typically be recovered from the rumen. The recipe may be scaled up or down depending on the size of gel; the gel should be 2–6 mm thick after it is poured and the wells large enough to hold 60 μl of sample. The thinner the gel the higher the resolution of the bands.)

2. After cooling, place the flask in a fume hood and add 10 ml of 10× MOPS running buffer and 18 ml of 12.3 M formaldehyde. Using this recipe, the formaldehyde concentration in the gel should be 2.2 M. Lower concentrations can be used, but as the concentration declines, so does the ability to denature the RNA.

   (*Note:* Formaldehyde is toxic through skin contact and inhalation of fumes. All handling involving formaldehyde should be carried out in a fume hood.)
3. Pour the gel and allow it to set. Remove the comb, place the gel in the gel tank and add sufficient 1 × MOPS running buffer to cover to a depth of ~1 mm.

   Prepare sample and run gel.
4. The volume of each sample is adjusted to 11 μl with water, then add:
   - 5 μl 10× MOPS running buffer;
   - 9 μl 12.3 M formaldehyde;
   - 25 μl formamide.

   This solution can be made as a cocktail immediately prior to addition to the sample but should not be stored as such. Mix by vortexing, microcentrifuge briefly (5–10 s) to collect the liquid in the bottom of the tube and incubate for 15 min at 55°C in a water bath.

   (*Note:* Formamide is a teratogen and should be handled with care.)
5. Add 10 μl formaldehyde loading buffer, vortex and microcentrifuge to collect liquid, and load onto gel. About 0.5–10 μg of RNA should be loaded per lane. If too much is loaded, you will get a big 'blob' of RNA on the gel and it will be difficult to distinguish the different bands. If this happens, dilute the RNA and rerun the gel. Duplicate samples should be loaded on one side of the gel for ethidium bromide or acridine orange staining.
6. Run the gel at 5 V/cm until the bromphenol blue dye has migrated one-half to two-thirds the length of the gel. This usually takes ~3 h. Lengthy electrophoresis (>5 h) is not recommended for northern transfers as this necessitates more formaldehyde in the gel. The other problem is that RNA degrades very easily; so, if the gel run is too long, the RNA can degrade to such an extent that you will not see any RNA on the gel.

Stain and photograph gel

7a. Remove the gel and cut off the lanes that are to be stained. Place this portion of the gel in an RNase-free glass dish, add sufficient 0.5 M ammonium acetate to cover the gel and soak for 20 min. Change the solution and incubate for an additional 20 min to ensure all the formaldehyde is removed. Replace solution with 0.5 μg/ml ethidium bromide in 0.5 M ammonium acetate, and stain for 40 min. If background fluorescence makes it difficult to visualize RNA fragments, destain in 0.5 M ammonium acetate for up to 1 h. This process can be sped up by using higher concentration of reagents and might be preferable if you are having a problem with RNA degradation.

7b. Alternatively, remove gel, cut off lanes, and stain 2 min in 1.1 M formaldehyde/10 mM sodium phosphate containing 10 μg/ml acridine orange. If necessary, destain 20 min in the same buffer without acridine orange.

8. Examine gel on a UV transilluminator to visualize the RNA and photograph.

9. Molecular weight markers should be run with the RNA. The purpose of this step is to ensure that (a) all the DNA has been digested and (b) to quantify the rRNA band of interest. For example, if doing bacterial work, the target band would typically be the 16S. Molecular weight markers with know molecular weights of the bands in the marker are required. It is useful if one can obtain a marker with bands in the range of 2 kb–500 bp. Image analysis software is used to obtain an accurate estimate of the density of the target band by referencing against the markers.

   (*Note:* If the quality of the sample is not adequate (DNA is present or the target rRNA is insufficient in concentration), the sample must be re-extracted. Obtaining high quality RNA is absolutely critical and is the most difficult step in the analysis.)

Northern blot analysis of extracted rumen rRNA using a slot-blot apparatus

RNA dot blots can be prepared by hand, but slot blots constructed using a manifold are preferable because the slots make it easier to compare hybridization signals by densitometry scanning.

Materials required

- 0.1 M NaOH.
- 10× SSC.
- 20× SSC, room temperature and ice cold.
- Denaturing solution.
- 100 mM sodium phosphate, pH 7.0.
- Dimethyl sulfoxide (DMSO).
- 6 M (40%) glyoxal, deionized immediately before use.
- Manifold apparatus with a filtration template for slot blots (e.g. Bio-Rad Bio-Dot SF, Schleicher and Schuell Minifold II) 50°C and 60°C water baths.

(*Note:* All solutions should be prepared with sterile deionized water that has been treated with DEPC as described at the end of the chapter.)

Preparation of membrane for transfer

1. Clean the slot-blot apparatus with 0.1 M NaOH and rinse with distilled water. This is an important step because nucleic acid sticks to the apparatus and can cause contamination of new membranes.
2. Cut a piece of nylon membrane to the size of the manifold. Pour 10× SSC (for nylon membrane) into a glass dish. Place the membrane on top of liquid and allow submerging. Leave for 10 min.

   (*Note:* Do not handle nylon membranes with hands but use clean blunt-ended forceps instead.)
3. Secure the membrane in the manifold. Assemble the manifold according to manufacturer's instructions and fill each slot with 10× SSC. Ensure there are no air leaks in the assembly.

Denaturing RNA samples

4a. Add 3 vol. denaturing solution to RNA sample. Incubate 15 min at 65°C, and then place on ice.

(*Note:* The amount of RNA added to the membrane will depend on the probe and target. The critical parameters are that the signal intensity when plotted against the reference rRNA should be in the linear range. As a rule of thumb, concentrations of between 500 ng and 1 μg are sufficient.)

4b. Alternatively, mix:
- 11 μl RNA sample;
- 4.5 μl 100 mM sodium phosphate, pH 7.0;
- 22.5 μl DMSO;
- 6.6 μl 6 M glyoxal.

Mix by vortexing, spin briefly in a microcentrifuge to collect liquid and incubate 1 h at 50°C.

5. Add 2 vol. ice-cold 20× SSC to each sample.

Pass samples through manifold

6. Switch on the suction to the manifold device and allow the 10× SSC added in step 3 to filter through. Leave the suction on.
7. Load each sample to the slots and allow it to filter through, being careful not to touch the membrane with the pipette tip.
8. Add 1 ml of 10× SSC to each slot and allow to filter through. Repeat.
9. Dismantle the apparatus, place the membrane on a sheet of Whatman 3 mm paper, and allow it to dry.
10. Immobilize RNA and carry out hybridisation.
11. Bake in an oven for 2 h at 80°C.

(*Note:* Membranes can be stored dry between sheets of Whatman 3 mm filter paper for several months at room temperature. For long-term storage, they should be placed in a desiccator at room temperature or 4°C.)

Hybridization analysis

After rRNA has been immobilized on nylon membranes, the abundance of specific targets can be assessed with oligonucleotide probes to small subunit rRNA. For this to become a reality, the oligonucleotide must be labelled with a molecule that is complementary to a detection system. In this protocol, I will only discuss labelling and detection of oligonucleotides labelled with digoxigenin, but there are several other detections systems in use.

1. Prepare DNA probe with digoxigenin.
2. Wet the membrane carrying the immobilized RNA in 6× SSC.
3. Place the membrane RNA-side-up in a hybridization tube and add ~1 ml formamide prehybridization/hybridization solution per 10 cm^2 of membrane.

(Note: Prehybridization and hybridization are usually carried out in glass tubes in a commercial hybridization oven. Alternatively, a heat-sealable

polyethylene bag and heat-sealing apparatus can be used. The membrane should be placed in the bag, all edges sealed and a corner cut off. Hybridization solution can then be pipetted into the bag through the cut corner and the bag resealed.)

4. Place the tube in the hybridization oven and incubate with rotation for at least 2 h at 42°C. The hybridization temperature should ideally be 10°C below the T_m of the probe. If using a nylon membrane, it is possible to reduce the prehybridization period to 15 min, but the longer period is preferred.
5. Pipette the desired volume of probe into the hybridization tube and continue to incubate with rotation overnight (16 h) at 42°C. The amount of probe added depends on the detection system used, and investigators should refer to the manufacturers instructions. If digoxygenin-labelled probes are used, 10 ng of probe per millilitre of hybridization solution is usually sufficient.

Wash membrane and perform autoradiography

6. Pour off the hybridization solution and add 2× SSC/0.1% SDS. Incubate with rotation for 15 min at room temperature, change the wash solution, and repeat. The volume of wash solution should be at least 10× the volume of the hybridization solution.
7. Replace wash solution with an equal volume of 1× SSC/0.1% SDS and incubate for at least 20 min at the desired wash temperature for the probe.

   (*Note:* The stringency conditions for 16S-rRNA targeted probes are often developed using 1× SSC, and for the purposes of this protocol, assume that all probes were optimized with 1× SSC. Investigators should, however, ensure that the temperature of the wash solution and the salt conditions are the same as those recommended for the specific probe being used.)
8. If desired, carry out two further washes with 1× SSC/0.1% SDS.

Detection with digoxigenin system

The digoxigenin-based detection system is a non-isotopic labelling method offered by Roche. Detection is achieved by incubation with antidigoxigenin antibodies coupled directly to one of several fluorochromes or enzymes. The availability of uncoupled antibodies also permits signal-amplification protocols to be employed. Biotin- and digoxigenin-labelled probes can be visualized simultaneously using a different fluorochrome for each probe.

Digoxigenin-11-dUTP can be incorporated into DNA by either of the nick translation or random oligonucleotide-primed synthesis protocols. There is also an oligotailing reaction that is ideal for oligonucleotide probes. Roche advises that nick translation incorporation is not as efficient as random priming; however, nick translation affords greater control over the final probe size. The labelling techniques used depend largely on the experimental question being asked.

9. Incubate nylon membrane for 30 min in Buffer 2.
10. Dilute anti-DIG-AP conjugate (1 : 20 000) in Buffer 2.
11. Incubate membrane for 30 min in antibody solution.
12. Wash 2 × 15 min in washing buffer.

13. Equilibrate for 2–5 min in Buffer 3.
14. Roche produces several alkaline phosphatase reagents that can be used at this point, e.g. CDP-Star or CSPD. Refer to manufactures instructions for this step.
15. Perform autoradiography.

    (*Note:* If the membrane is to be reprobed, the probe can be stripped from the hybridized membrane without removing the bound RNA using stripping solution. Do not add NaOH. The membrane must not be allowed to dry out between hybridization and stripping, as this may cause the probe to bind to the matrix.)

Labelling with dig oligonucleotide tailing reaction

There are several labelling options available, but only the oligonucleotide tailing reaction is described here.

1. Mix the following on ice:
   - 4 μl reaction buffer;
   - 4 μl $CoCl_{2;}$
   - No more than 100 pmol of probe;
   - 1 μl of DIG-dUTP solution;
   - 1 μl dATP;
   - 1 μl terminal transferase;
   - distilled H_2O to 20 μl.

   Incubate at 37°C for 15 min.
2. Stop reaction. Add 2 μl of a glycogen stop solution and 200 μl EDTA
3. Precipitate the oligonucleotide. With 2.5 μl of LiCl and 75 μl of cold EtOH.

Statistical considerations

The purpose of statistics is to assess the variation (variance) of means and then to determine whether this variation is such that confident conclusions can be drawn about the means. Usually, the classical procedures used for this are ANOVA or analysis of variance. It is not usually appreciated by non-statisticians that it is analysis of the data variation, not of the means that constitute the basis of an ANOVA.

It therefore follows that in the experimental process, the sources of variation need to be controlled, as the final mean, or variance, is a composite of all the sources of variation in the experiment. In the case of northern analysis of rRNA, the sources of variation are animal [8], rRNA extraction, denaturation, prehybridization, hybridization and post-hybridization steps (washing, densitometry) [16]. The idea of good experimental design is to quantify the sources of variation and then try and reduce them as far as possible. Where the sources of variation are large, replication is required to control and quantify the variation. Quantification of variation has found that animal variation [8] and the variation due to the post-hybridization steps are the greatest [16].

If animals are pen fed and automated feeders are available, it is possible to produce rumen data with very little variation [9, 22]. Rumen populations typically undergo diurnal variation associated with feed intake events [11]. By feeding animals on 12-times per day feeders, this diurnal variation can be almost entirely eliminated [9, 22]. In situations in which automated feeders are not available, animals can be sampled on at least four consecutive days at approximately the same time after feeding. The statistical basis of this process has been established [8].

The following protocol is used to control for the sources of variation discussed:

1. Rumen samples at least five times, samples pooled.
2. rRNA extracted three times from each pooled sample.
3. Extracted and quantified rRNA blotted onto three separate membranes in triplicate. Thus for one animal sample, there will be nine slots (3×3).
4. On each membrane, the target species (e.g. *R. albus*) is blotted in a serial dilution.
5. The total amount of rRNA at each slot is quantified by hybridizing with a universal probe.
6. The specific target (e.g. *R. albus*) is determined with a specific probe using the same dilution series (e.g. *R. albus*).
7. Relative abundance is the proportion of specific to universal.
8. Variation between the membranes must be <5%. If not, repeat the experiment.

(*Note:* The same dilution series must be used for the universal and the specific probe. This makes six membranes for both the universal and the specific probe.)

Buffers and stock solutions

Ammonium acetate, 10 M
Dissolve 385.4 g ammonium acetate in 150 ml distilled H_2O.
Add distilled H_2O to 500 ml.

Blocking reagent for rRNA
10% (w/v) in maleic acid buffer (5 g maleic acid in 50 ml of Buffer 1).
Dissolve blocking reagent by constantly stirring on a heating block (65°C).
Store at −20°C.

Bromophenol blue (1%)
10 μg in 1 ml of water.

Buffer 1: Maleic acid buffer
0.15 g NaCl.
23.2 g maleic acid.
17.6 g NaCl.
Make up to 2 l.
Adjust pH to 7.5.

Buffer 2: blocking solution of rRNA
1 : 10 dilution in Buffer 1.

Buffer 3: Detection buffer
0.1 M Tris–HCl.
0.1 M NaCl.
Adjust to pH 9.5.

Denaturing solution
500 μl formamide.
162 μl 12.3 M (37%) formaldehyde.
100 μl MOPS buffer.
Make fresh from stock solutions immediately before use.

EDTA (ethylenediamine tetra-acetic acid), 0.5 M (pH 8.0)
Dissolve 186.1 g $Na_2EDTA.2H_2O$ in 700 ml distilled H_2O.
Adjust pH to 8.0 with 10 M NaOH ($\sim$50 ml).
Add distilled H_2O to 1 l.

Ethidium bromide, 10 mg/ml
Dissolve 0.2 g ethidium bromide in 20 ml distilled H_2O.
Mix well and store at 4°C in dark.

Formaldehyde loading buffer
1 mM EDTA, pH 8.0.
0.25% (w/v) bromphenol blue.
0.25% (w/v) xylene cyanol.
50% (v/v) glycerol.
Store up to 3 months at room temperature.

Glyoxal, 6 M, deionized
Immediately before use, deionize glyoxal by passing through a small column of mixed-bed ion-exchange resin [e.g. Bio-Rad AG 501-X8 or X8(D) resins] until the pH is $>$5.0.

Glyoxal loading buffer
10 mM sodium phosphate, pH 7.0.
0.25% (w/v) bromphenol blue.
0.25% (w/v) xylene cyanol.
50% (v/v) glycerol.
Store up to 3 months at room temperature.

Diethylpyrocarbonate (DEPC) treatment of solutions
Add 0.2 ml DEPC to 100 ml of the solution to be treated. Shake vigorously to get the DEPC into solution. Autoclave the solution to inactivate the remaining DEPC. Many investigators keep the solutions they use for RNA work separate to ensure that ‘dirty’ pipettes do not go into them.

Hybridization buffer
To make 500 ml add the following:
125 ml formamide (25%).
125 ml SSC (20×).
25 ml Na_2HPO_4 (1 M stock).
100 ml Blocking reagent (10%).
50 ml SDS (20%).
5 ml *N*-laurolsarcosine (10%).

$MgCl_2$, 1 M
20.3 g $MgCl_2.6H_2O$.
Distilled H_2O to 100 ml.

NaCl, 5 M
292 g NaCl.
Distilled H_2O to 1 l.

NaOH, 10 M
Dissolve 400 g NaOH in 450 ml H_2O.
Add distilled H_2O to 1 l.

MOPS buffer
0.2 M MOPS [3-(*N*-morpholino)-propanesulfonic acid], pH 7.0.
0.5 M sodium acetate.
0.01 M EDTA.
Store up to 3 months at 4°C.
Store in the dark and discard if it turns yellow.

MOPS running buffer, 10×
0.4 M MOPS, pH 7.0.
0.1 M sodium acetate.
0.01 M EDTA.
Store up to 3 months at 4°C.

Sodium phosphate, pH 7.0, 100 mM and 10 mM
100 mM stock solution:
5.77 ml 1 M Na_2HPO_4.
4.23 ml 1 M NaH_2PO_4.
Distilled H_2O to 100 ml.
Store up to 3 months at room temperature.

10 mM solution:
Dilute 100 mM stock 1/10 with distilled H_2O.
Store up to 3 months at room temperature.

Stripping solution
1% (w/v) SDS.
0.1× SSC.
40 mM Tris Cl, pH 7.5–7.8.
Store up to 1 year at room temperature.
Where formamide stripping is desired, prepare the above solution and add an equal volume of formamide just before use.

Sodium acetate, 3 M
Dissolve 408 g sodium acetate·$3H_2O$ in 800 ml distilled H_2O.
Add distilled H_2O to 1 l.
Adjust pH to 4.8 or 5.2 (as desired) with 3 M acetic acid.

SSC (sodium chloride/sodium citrate), 20×
3 M NaCl (175 g/l).
0.3 M Na_3citrate.$2H_2O$ (88 g/l).
Adjust pH to 7.0 with 1 M HCl.

TAE (Tris/acetate/EDTA) electrophoresis buffer
50× stock solution:
242 g Tris base.
57.1 ml glacial acetic acid.
37.2 g Na_2EDTA.$2H_2O$.
distilled H_2O to 1 l.
Working solution, pH ~8.5:
40 mM Tris·acetate.
2 mM Na_2EDTA·$2H_2O$.

TBE (Tris/borate/EDTA) electrophoresis buffer
10× stock solution, 1 l.
108 g Tris base (890 mM).
55 g boric acid (890 mM).
40 ml 0.5 M EDTA, pH 8.0.

TE (Tris/EDTA) buffer
10 mM Tris·Cl, pH 7.4, 7.5 or 8.0.
1 mM EDTA, pH 8.0.

Tris·Cl [tris(hydroxymethyl)aminomethane], 1 M
Dissolve 121 g Tris base in 800 ml distilled H_2O.

Adjust to desired pH with concentrated HCl.
Mix and add distilled H_2O to 1 l.
Approximately 70 ml of HCl is needed to achieve a pH 7.4 solution, and approximately 42 ml for a solution that is pH 8.0.

(*Note:* The pH of Tris buffers changes significantly with temperature, decreasing approximately 0.028 pH units per 1°C. Tris-buffered solutions should be adjusted to the desired pH at the temperature at which they will be used. Tris should not be used as a buffer below pH ~7.2 or above pH ~9.0 since the pK_a of Tris is 8.08.)

References

1. Amann, R., and W. Ludwig. 2000. Ribosomal RNA-targeted nucleic acid probes for studies in microbial ecology. *FEMS Microbiol. Rev*. **24:**555–565.
2. Ashelford, K.E., A.J. Weightman, and J.C. Fry. 2002. PRIMROSE: a computer program for generating and estimating the phylogenetic range of 16S rRNA oligonucleotide probes and primers in conjunction with the RDP-II database. *Nucleic Acids Res.* **30:**3481–3489.
3. Barnes, S.M., R.E. Fundyga, M.W. Jeffries, and N.R. Pace. 1994. Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. *Proc. Natl. Acad. Sci*. **91:**1609–1613.
4. Chandler, D.P., C.A. Wagnon, and J.H.B. Bolton. 1998. Reverse transcriptase (RT) inhibition of PCR at low concentrations of template and its implications for quantitative RT–PCR. *Appl. Environ. Microbiol*. **64:**669–677.
5. Harris, J.K., S.T. Kelley, G.B. Spiegelman, and N.R. Pace. 2003. The genetic core of the universal ancestor. *Genome Res*. **13:**407–412.
6. Hugenholtz, P., B.M. Goebel, and N.R. Pace. 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J. Bacteriol.* **180:**4765–4774.
7. Krause, D.O., R.J. Bunch, L.L. Conlan, P.M. Kennedy, W.J. Smith, R.I. Mackie, and C.S. McSweeney. 2001. Repeated ruminal dosing of *Ruminococcus* spp. does not result in persistence, but changes in other microbial populations occur that can be measured with quantitative 16S-rRNA-based probes. *Microbiol*. **147:**1719–1729.
8. Krause, D.O., B.P. Dalrymple, W.J. Smith, R.I. Mackie, and C.S. McSweeney. 1999. 16S rDNA sequencing of *Ruminococcus albus* and *Ruminococcus flavefaciens*: design of a signature probe and its application in adult sheep. *Microbiol.* **145:**1797–1807.
9. Krause, D.O., and J.B. Russell. 1996. An rRNA approach for assessing the role of obligate amino acid-fermenting bacteria in ruminal amino acid deamination. *Appl. Environ. Microbiol*. **62:**815–821.
10. Krause, D.O., W.J. Smith, and C.S. McSweeney. 2001. Extraction of microbial DNA from rumen contents containing plant tannins. *Biotechniques* **31:**294–298.
11. Leedle, J.A.Z., M.P. Bryant, and R.B. Hespell. 1982. Diurnal variations in bacterial numbers and fluid parameters in ruminal contents of animals fed low- or high-forage diets. *Appl. Environ. Microbiol.* **44:**402–412.
12. Maidak, B.L., J.R. Cole, T.G. Lilburn, C.T. Parker Jr., P.R. Saxman, R.J. Farris, G.M. Garrity, G.J. Olsen, T.M. Schmidt, and J.M. Tiedje. 2001. The RDP-II (Ribosomal Database Project). *Nucleic Acids Res.* **29:**173–174.
13. McSweeney, C.S., B. Palmer, R. Bunch, and D.O. Krause. 2001. Effect of the tropical forage calliandra on microbial protein synthesis and ecology in the rumen. *J. Appl. Microbiol.* **90:**78–88.
14. Pace, N.R. 1997. A molecular view of microbial diversity and the biosphere. Science **276:**734–740.
15. Pace, N.R., D.A. Stahl, D.J. Lane, and G.J. Olsen. 1985. Analyzing natural microbial populations by rRNA sequences. *ASM News* **51:**4–12.
16. Raskin, L., W.C. Capman, R. Sharp, L.K. Poulsen, and D.A. Stahl. 1997. Molecular Ecology of Gastrointestinal Ecosystems, pp. 243–298. *In* R.I. Mackie, R.E. White, and R.E. Isaacson (eds.), Chapman & Hall, Melbourne.

17. Stahl, D.A., B. Flesher, H.R. Mansfield, and L. Montgomery. 1988. Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. *Appl. Environ. Microbiol.* **54:**1079–1084.
18. Stahl, D.A., D.L. Lane, G.J. Olsen, and N.R. Pace. 1985. Characterization of a Yellowstone hot spring microbial community by 5S rRNA sequences. *Appl. Environ. Microbiol.* **49:**1379–1384.
19. Teather, R.M., M.A. Hefford, and R.J. Forster. 1997. Genetics of rumen bacteria, pp. 427–466. *In* Anonymous. The Rumen Microbial Ecosystem. Blackie Academic & Professional, Melbourne.
20. Woese, C. R. 1987. Bacterial evolution. *Microbiol. Rev.* **51:**221–271.
21. Woese, C.R., and G.E. Fox. 1977. Phylogenetic structure of the prokaryotic domain: the primary kingdoms. *Proc. Natl. Acad. Sci. U.S.A.* **74:**5088–5090.
22. Yang, C.-M.J., and J.B. Russell. 1993. The effect of monensin supplementation on ruminal ammonia accumulation in vivo and numbers of amino acid fermenting bacteria. *J. Anim. Sci.* **71:**3470–3476.
23. Zuckerkandl, E., and L. Pauling. 1965. Molecules as documents of evolutionary history. *J. Theor. Biol.* **8:**357–366.

6.2. Whole cell probing with fluorescently labelled probes for *in situ* analysis of microbial populations

LINDA L. BLACKALL
Advanced Waste Water Management Centre, The University of Queensland, St Lucia, Queensland 4072, Australia

Introduction

Until 1965, microbiologists struggled with simplicity of bacterial morphology and phenotypic characters in an attempt to construct a phylogenetic division for the prokaryotes. Then, it was found that molecular sequences were the source of much evolutionary information. Consequently, the way from phenotypic to genotypic characteristics for evolutionary inference was clear. Ribosomes within biological cells are the sites of protein synthesis. They are composed of a mixture of nucleic acids [ribosomal RiboNucleic Acids (rRNA)] and proteins and have an average size of 70S in bacteria. Because of their role in cell survival, maintenance and reproduction, rRNAs and their genes are described as being evolutionally conserved. Other genes can also be used to infer evolutionary relationships, and phylogenies inferred from all these molecules tend to concur.

Comparative analyses of small subunit rRNA gene sequences were used in the 1980s to create a phylogeny or natural division for life on earth. It is composed of three domains – Bacteria, Archaea and Eucarya [9]. The database of small subunit rRNA sequences is very large and allowed this broad comparative analysis to be done. In addition, the databases of these gene sequences are cumulative and constitute a growing resource available by modern communication channels to all researchers. The phylogenetic information has been used to clarify classification and taxonomic anomalies in the Bacteria and Archaea. Within the Bacteria, the small subunit rRNA is the 16S rRNA and the genes that code for this molecule are 16S rDNAs. In most cases, the 16S rDNA is exactly transcribed to form the 16S rRNA – i.e. the primary nucleic acid sequences of these two molecules are the same. Additionally, ribosomes of Bacteria contain the larger 23S rRNA (genes = 23R rDNAs), and sequence information from 23S rDNAs is also used to address evolutionary relationships between different Bacteria.

Fluorescence *in situ* hybridization or whole cell probing

Oligonucleotides (short strands of nucleic acids – usually 15–30 nucleotides in length), complementary to 16S rRNA sequence regions with an intermediate

H.P.S. Makkar and C.S. McSweeney (eds.), Methods in Gut Microbial Ecology for Ruminants, 191–199.
© 2005 *IAEA. Printed in the Netherlands.*

degree of conservation and characteristic for phylogenetic entities like genera, families, subclasses, have been used successfully for rapid identification of bacteria. The oligonucleotides are able to enter fixed bacterial cells and once inside the cells, they may form stable associations (hybrids via hydrogen bonding between complementary nucleotides) with the 16S rRNA in the ribosomes. If the complementary sequence for the oligonucleotide is not present in the 16S rRNA in the ribosome, stable hybridization does not occur and the oligonucleotide is washed from the bacterial cell. Thus, the 'targets' for the oligonucleotides are the ribosomes of which there are up to 10^4 per actively growing bacterial cell. In order to observe when hybridization occurs, the oligonucleotides also contain a 'reporter' molecule, and in this description of the technique, the reporter is a fluorochrome. Cells in which the fluorescently labelled oligonucleotide has hybridized with the 16S rRNA in the ribosome can be directly visualized by epifluorescence microscopy. The technique is called fluorescence *in situ* hybridization (FISH) or whole cell probing [2].

Whole cell probing with fluorescently labelled probes

Special notes

- Gloves should be used for all FISH procedures and the gloves used must not have too much powder on them, as this is highly autofluorescent.
- Some dangerous chemicals (e.g. formamide) are used in FISH. Their material safety data sheets should be consulted.

Sample fixation

Method for fixation of liquid samples for whole cell or *in situ* hybridization:

For Gram negatives (paraformaldehyde)
Add 3 volumes of paraformaldehyde (PFA) fixative to 1 volume of sample and hold at 4°C for 1–3 h. Pellet the cells by centrifugation (5000 *g*) and remove fixative. Wash the cells in 1× phosphate buffered saline (PBS) and resuspend in 1× PBS to give 10^8–10^9 cells/ml. Add 1 volume of ice-cold ethanol and mix. Fixed cells can be spotted onto glass slides for FISH. They can be stored at −20°C for several months. Since the suspension of cells is in 50% ethanol, it does not freeze at −20°C.

Paraformaldehyde. Heat 65 ml of high purity water to 60°C. Add 4 g PFA. Add a drop of 2 M NaOH solution and stir rapidly until the solution has nearly clarified (ca. 1–2 min). Remove from the heat source and add 33 ml of 3× PBS. Adjust pH to 7.2 with HCl. Remove any remaining crystals by sterile filtration (0.2 μm). Quickly cool to 4°C and store at this temperature.

PBS. 130 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2 (see details below).

PBS (for pH 7.2, the ratios of disodium : sodium phosphates must be 7.2 : 2.8)

	M (×100*)	g (×100*) for 1 l	M (×30*)	g (×30*) for 1 l	g (×30*) for 500 ml
$Na_2HPO_4 \cdot 12H_2O$ (MW = 358.14)	0.72	257.9	0.216	77.37	38.7
$NaH_2PO_4 \cdot 2H_2O$ (MW = 156.01)	0.28	42.7	0.084	12.1	6.6
NaCl (MW = 58)	13	754	2.9	226.2	112.1

* This is for a final buffer concentration of 10 mM phosphate and 130 mM NaCl.

Note: To prepare a 3× solution of PBS, dilute 1 in 10 (1 + 9); to prepare a 1× solution of PBS, dilute 1 in 30 (1 + 29).

For Gram positives (ethanol)

Add 1 volume of 98% ethanol fixative to 1 volume of sample and hold at 4°C for 4–16 h. Pellet fixed cells by centrifugation (5000 *g*) and remove fixative. Wash the cells in 1× PBS and resuspend in 1× PBS to give 10^8–10^9 cells/ml. Add 1 volume of ice-cold ethanol and mix. Fixed cells can be spotted onto glass slides for FISH. They can be stored at −20°C for several months. Since the suspension of cells is in 50% ethanol, it does not freeze at −20°C.

[*Note:* The fixed cell suspension can be transported and can travel at room temperature for a short period in the order, a few days before being restored at −20°C. Alternately, the fixed cell suspensions can be spotted onto glass slides, the material dehydrated in the ethanol series (see the section on 'Treatment of Teflon-coated microscope slides – optional' below) and then either stored at −20°C or transported.]

Requirements

- PFA solution (PFA, NaOH, HCl, 0.2 μm membrane filters).
- 1× and 3× phosphate buffered saline (1× – 130 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2).
- Ethanol.

Treatment of Teflon-coated microscope slides – optional

The Teflon-coated slides, we typically use, have 'spots' of ca. 5–8 mm diameter on them. A supplier in Australia is Biofusion Pty. Ltd., and the cost of slides is ca. $A135 for 144 slides – making them ca. $A1.00 each.

Cleaned slides (warm detergent for 1 h, thorough wash, air dry), coated with gelatin by dipping into warm (70°C) solution of 0.1% gelatin, 0.01% chromium potassium sulphate, then air dry.

Requirements
- Detergent.
- gelatin.
- chromium potassium sulphate.

Application of samples to slides and dehydration

A volume of between 3 and 30 μl of fixed cell suspension (need to apply ca. 10^5–10^6 cells) is applied to the glass slide, air dried and dehydrated in an ethanol series (3 min in each) 50%, 80% and 98% ethanol. After this procedure, the dehydrated cell suspensions on the slides can be stored indefinitely, but it is wise to store them in the dry, the dark and at -20°C.

Requirements
- Solutions of ethanol – 50%, 80% and 98% in Coplan jars or 50 ml Falcon tubes.

Probe hybridization

Requirements
- 50 ml polypropylene screw top tubes (Falcon tubes) – one slide per tube for hybridisation.
- tissues.
- hybridization buffer (final concentrations – 0.9 M NaCl, 0.01% sodium dodecyl sulphate (SDS), 20 mM Tris–HCl, pH 7.2).
- 2 ml microcentrifuge tubes (sterile).
- formamide (aliquoted and frozen).
- (optional) mutanolysin (Fluka) and sodium phosphate buffer for some cells like '*Microthrix parvicella*,' which require permeabilization.
- hybridization oven at 46°C.
- probes.

(*Note:* The hybridization buffer is prepared in 2 ml microcentrifuge tubes at the time of use. Two millilitres prepared – this allows 8 μl to each well on the slide and the remainder in the hybridization tube for keeping the chamber in the tube moist. Formamide – frozen at -20°C in 2 ml aliquots – this will depend on the number of hybridizations that are done. This should have no colour. When thawed, restore at 4°C and use within 1 week. A range of formamide concentrations is needed for different probes. SDS is added last (in the lid of the tube, therefore, added when lid closed and mixed) because it can interact with the concentrated NaCl and precipitate.)

In a 2 ml microcentrifuge tube add the following – the final volume is 2 ml:
360 μl of 5 M NaCl (autoclaved).
40 μl of 1 M Tris–HCl (autoclaved).
2 μl of 10% SDS – not autoclaved – placed in the lid of the centrifuge tube.
x μl of formamide (see table in what follows).
y μl of autoclaved MilliQ water (depends on amount of formamide – see table)

Amount of formamide (μl) = x	x = %formamide on the well	Amount of MilliQ H_2O (μl) = y
0	0	1598
100	5	1498
200	10	1398
300	15	1298
400	20	1198
500	25	1098
600	30	998
700	35	898
800	40	798
900	45	698
1000	50	598

Hybridization buffer (8 μl) is added to each well on the slide, the remainder is used to moisten a tissue paper in the 50 ml tube. Then, add 1 μl of probe to a final concentration of 25 ng/μl, mix carefully. There is no need to warm the tube or the hybridization buffer. Place slide in the 50 ml tube containing the moistened tissue. Close and put into hybridization oven at 46°C for 1–2 h.

Washing

After hybridization, the slides are carefully removed from the tube and rinsed immediately with wash buffer by pipetting a small amount of the wash buffer gently over the slide.

Preparation of wash buffer (total volume = 50 ml)

NaCl	(5 M – autoclaved)	x μl (see wash buffer table)
Tris–HCl	(1 M – autoclaved)	1 ml
SDS	(10% not autoclaved – added last)	50 μl
Total volume		to 50 ml with MilliQ water

Wash buffer

Hybridization at 46°C % formamide	NaCl (M)	2 μl 5 M NaCl	μl 0.5 M EDTA for 20% formamide and above
0	0.900	9000	–
5	0.636	6300	–
10	0.450	4500	–
15	0.318	3180	–
20	0.225	2150	500
25	0.159	1490	500
30	0.112	1020	500
35	0.080	700	500
40	0.056	460	500
45	0.040	300	500
50	0.028	180	500

$$[\mathrm{NaCl}][\mathrm{mM}]\{0.5 \times (\%\mathrm{FA}) = -16.6\log[\mathrm{Na}^+]\}$$

Take a new 50 ml tube, add the NaCl and then the Tris–HCl, then fill to the 50 ml mark with MilliQ water, mix and then add the SDS. Warm the wash buffer in the bath at 48°C during the hybridization. New tubes are used for washing, then rinsed and used for hybridization in the next round. After this, the tubes are incinerated – formamide is toxic and discard tubes must have the lids tightly screwed to prevent toxic vapours escaping. Washing is done at 48°C.

After hybridization, slides are carefully removed from their tube (then the tube is incinerated), and a small amount of warm wash buffer is pipetted over the slide with excess going into a beaker. The slide is then placed into the washing buffer tube and into the water bath at 48°C for 10–15 min. Rapid transfer of slides prevents cooling which could lead to non-specific probe binding.

After the wash, the slide is removed, wash buffer tipped down the sink and the tube rinsed for reuse. The slide is gently rinsed in MilliQ water from a wash bottle. Water is directed above wells and allowed to flood over them. Both sides of the slide are washed to remove all salts which are highly autofluorescent. After the washing step, it is ensured that all droplets of water are removed from the wells – the probe can dissociate and leave the cells due to osmotic pressure. Compressed air directed at the side of the slide ensures that all water droplets are removed. The slides are now dried in a vertical position.

Mounting slides

When air dry, the slides are mounted in Citifluor – this is toxic and must be used in a fume hood. A very thin film of Citifluor is used. The coverslip is applied and pressure applied to force the Citifluor to completely cover the wells. In the viewing of the slides, the Citifluor must not contact the immersion oil from the top.

Viewing slides

Viewing and photographing should be on the day done. Viewing the next day is acceptable if the slides are stored in the fridge or freezer with Citifluor removed by rinsing with water. As both formamide and Citifluor are toxic, slides must be discarded in the fume hood.

Oligonucleotide probes used in FISH

Some commonly used oligonucleotides probes used in FISH are presented in the table in what follows, and additional probes are given in Chapter 3.1.

List of general oligonucleotide probes used in FISH

Probe	Probe sequence (5′–3′)	rRNA target site[a]	Specificity	Formamide (%)	Reference
EUB338[b]	GCTGCCTCCCGTAGGAGT	16S, 338–355	Many but not all *Bacteria*	0–70	[1]
EUB33811[b]	GCAGCCACCCGTAGGTGT	16S, 338–355	*Planctomycetales*	0–50	[4]
EUBA338-III[b]	GCTGCCACCCGTAGGTGT	16S, 338–355	*Verrucomicrobiales*	0–50	[4]
ALF1b	CGTTCG(C/T)TCTGAGCCAG	16s, 19–35	*Alphaproteobacteria*	20	[6]
ALF969	TGGTAAGGTTCTGCGCGT	16S, 969–986	*Alphaproteobacteria*	20	Modified [7]
BET42a	GCCTTCCCACTTCGTTT	23S, 1027–1043	*Betaproteobacteria*	35	[6]
GAM42a	GCCTTCCCACATCGTTTA	23s, 1027–1043	*Gammaproteobacteria*	35	[6]
HGC69a	TATAGTTACCACCGCCGT	23S, 1901–1918	Actinobacteria	25	[8]
CF319a	TGGTCCGTGTCTCAGTAC	16S, 319–336	*Cytophaga-Flavobacterium* of *Bacteroidetes*	35	[5]

[a] rRNA, *E. coli* numbering [3].
[b] EUB338, EUB338-II and EUB338-III are used in a mixture called EUBMIX.

Making FISH reagents

Phosphate buffered saline, pH 7.2

30× PBS: Add 38.7 g ($Na_2HPO_4 \cdot 12H_2O$), 6.6 g ($Na_2HPO_4 \cdot 2H_2O$), and 113.1 g (NaCl) to 500 ml of MilliQ. Autoclave and store as stock.
Dilute 1:10 for 3× PBS (for making PFA) and autoclave.
Dilute 1:30 (for other uses) and autoclave.

4% PFA

ALWAYS use nitrile gloves and do everything in the fume hood.
1. Set up the balance and a heated stirrer in the fume hood.
2. Warm 65 ml of purified water to 60°C (use the microwave to get it close).
3. Weigh out 4 g of PFA powder (DO NOT inhale).
4. Add the PFA to the water. It should be cloudy.
5. Add 2 drops of 2 M NaOH, and the PFA should be dissolved in 1–2 min.
6. Cool to room temperature and add 33 ml of 3× PBS (safe to remove from the hood now).
7. Adjust the pH to 7.2 with 1 M HCl.
8. Filter through 0.2 μm filter to remove any undissolved crystals.
9. Aliquot to applicable volumes and freeze.

5 M NaCl

Add 58 g of NaCl to 200 ml of MilliQ water. This is a lot of salt – be patient – it will dissolve. Autoclave.

1 M Tris–HCl

Add 31.5 g of Tris–HCl to 150 ml of MilliQ water. Dissolve and adjust pH to 7.2 with 2 M NaOH. Make up to 200 ml with MilliQ water. Autoclave.

DO NOT use Tris. Use Tris–HCl.

(*Note:* Formamide is TOXIC – work in the fumehood with this compound. Aliquot in 2 ml volume and freeze.)

10% SDS

Work in the fume hood. SDS is an irritant.
Dissolve 10 g of SDS in 100 ml of MilliQ water. It takes 20 min or so to dissolve.

0.5M EDTA

1. Add 18.6 g of EDTA disodium hydrate to 75 ml of MilliQ water.
2. Adjust to pH 7.2 with NaOH pellets (you will need a lot).

3. Make up to 100 ml with MilliQ water.
4. Autoclave.

References

1. Amann, R.I., B.J. Binder, R.J. Olson, S.W. Chisholm, R. Devereux, and D.A. Stahl. 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl. Environ. Microbiol.* **56:**1919–1925.
2. Amann, R.I., W. Ludwig, and K.H. Schleifer. 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59:**143–169.
3. Brosius, J., T.L. Dull, D.D. Steeter, and H.F. Noller. 1981. Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli. J. Mol. Biol.* **148:**107–127.
4. Daims, H., A. Bruhl, R. Amann, K.H. Schleifer, and M. Wagner. 1999. The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. *Syst. Appl. Microbiol.* **22:**434–444.
5. Manz, W., R. Amann, W. Ludwig, M. Vancanneyt, and K.H. Schleifer. 1996. Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment. *Microbiology* **142:**1097–1106.
6. Manz, W., R. Amann, W. Ludwig, M. Wagner, and K.-H. Schleifer. 1992. Phylogenetic oligonucleotide probes for the major subclasses of proteobacteria: problems and solutions. *Syst. Appl. Microbiol.* **15:** 593–600.
7. Neef, A., R. Witzenberger, and P. Kampfer. 1999. Detection of sphingomonads and *in situ* identification in activated sludge using 16S rRNA-targeted oligonucleotide probes. *J. Ind. Microbiol. Biotechnol.* **23:**261–267.
8. Roller, C., M. Wagner, R. Amann, W. Ludwig, and K.-H. Schleifer. 1994. *In situ* probing of Gram-positive bacteria with high DNA G+C content using 23S rRNA-targeted oligonucleotides. *Microbiology* **140:**2849–2858.
9. Woese, C.R., O. Kandler, and M.L. Wheelis. 1990. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. *Proc. Natl. Acad. Sci. USA* **87:**4576–4579.

6.3. Combined fluorescence *in situ* hybridization and microautoradiography (FISH–MAR)

MANEESHA P. GINIGE
Advanced Wastewater Management Centre, The University of Queensland, St. Lucia 4072, Queensland, Australia

Introduction

Cultivation independent studies have revealed, that due to the complexity of natural ecosystems such as activated sludge, rhizosphere, rumen etc., the pure cultivation of all related micro-organisms in these diverse ecosystems is quite unsuccessful [2]. Accordingly, the small subunit ribosomal RNA (SSU rRNA, i.e. 16S and 18S rRNA) or genes, obtained from these ecosystems without cultivation has become a widely accepted approach to describe the phylogenetic diversity of microbial communities present in these ecosystems [1, 6]. The rapid growth of the rRNA gene (rDNA) sequence data bank, accessible via the Internet (http://www.ncbi.nlm.nih.gov/BLAST/) has enabled us to compare microbial diversities across the globe without cultivation. However, these rRNA gene sequences provide very few direct clues regarding the interactions and the metabolic capabilities of the identified micro-organisms [9]. Accordingly, the knowledge available on the *in situ* physiology of the inhabitants of most microbial ecosystems is quite remote and the availability of such knowledge will enable our ability to manipulate ecosystems (e.g. activated sludge) to achieve better process performances with the aid of improved mathematical models.

In both natural and engineered systems, there are a diverse group of micro-organisms cohabiting within matrix-encloses such as bio-films or flocs rather than single planktonic cells [5]. In order to manipulate these systems to obtain higher productivity, it is important to understand population structure, dynamics, functionality and also special distributions in the matrix-encloses. It is an accepted fact that pure cultivation of micro-organisms in these complex ecosystems does not permit to resolve any of the above mentioned issues since (1) rigorous biomass disaggregation which is a common practice in cultivation-based approaches results in a loss of special information; (2) cultivation approaches also introduces significant biases resulting in pronounced population shifts resulting in an inaccurate evaluation of the natural population dynamics; and (3) the influence caused by artificial environments where cultivation studies are performed also influences gene expression of related organisms. Hence, an accurate functional analysis of the active communities in these complex communities is not feasible through pure cultivation studies.

H.P.S. Makkar and C.S. McSweeney (eds.), Methods in Gut Microbial Ecology for Ruminants, 201–206.
© 2005 *IAEA. Printed in the Netherlands.*

Until late 1990s, fluorescent *in situ* hybridization (FISH) and confocal laser scanning microscopy enabled culture-independent quantitative examination of structure (shapes of individual species and special arrangements within bacterial habitats) and dynamics of complex microbial communities [2, 8, 10]. However, it was only during early 1999 that researchers succeeded in linking the identity of these complex micro-organisms with their function while they were in their natural habitat. The approach was a combined use of FISH and microautoradiography (MAR). MAR, which was first reported in 1968, allows the analysis of *in situ* metabolic activities of micro-organisms with the aid of radiolabelled substrates while they are in their natural habitat [4]. The main limitation of MAR alone was its inability to correlate the activity detected with the identity of the responsible organism. The direct combined use of FISH and MAR has enabled simultaneous *in situ* analysis of the identity, functionality and activity of micro-organisms found in complex ecosystems without the need for pure cultivation [7].

Experimental approach

The FISH–MAR protocol comprises of four steps. They are: (1) incubation of sample with radioactive carbon source; (2) cell fixation, sectioning and FISH hybridization; (3) nuclear emulsion coating, exposure and development; and (4) visualization. The methods described below for each step are slight modifications of Lee et al. (7).

1. Sample incubation with radioactive carbon sources

Primarily, it is important to have a deeper understanding about the overall metabolic capabilities of the biotic sample on which FISH–MAR would be carried out. An understanding such as on the physiological and biochemical properties of the biomass would enable to carry out the incubation step of the experiment in an environment that would be close to its natural environment.

The incubation procedure explained below is based on a denitrifying bio-reactor sludge, which is experimented to elucidate its capability to utilize a given source of carbon under strict anoxic (nitrate present in an anaerobic environment) conditions.

For this FISH—MAR incubation enriched denitrifying sludge from a stable bio-reactor showing good denitrification is used. The bio-reactor is fed with a specific carbon source for denitrification and the enriched populations are presumed to be capable of utilizing the carbon source provided or, metabolic by-products of other micro-organisms in the reactor. The bio-reactor is operated such that no carbon (electron donor), nitrate or nitrite (electron acceptor) is remaining at the end of a reactor cycle.

A sludge sample obtained from the end of a reactor cycle is diluted using reactor effluent to achieve a mixed liquor volatile suspended solids (MLVSS) value of 1–2 g/l. To demonstrate active carbon substrate uptake, a control experiment is also performed with some pasteurized biomass. Pasteurization is performed by placing a 15 ml Falcon 2059 polypropylene tube containing sludge in an 80°C water bath

for 15 min. For each carbon source evaluated, three sets of 9 ml vials are used. One set contains 4 ml of pasteurized sludge while the other two sets contained 4 ml of non-pasteurized sludge each. The first set of vials with pasteurized sludge is used as a control to demonstrate no uptake of carbon by inactive cells. A volume of 500 μl of reactor feed which includes the electron acceptor (nitrite or nitrate) but not the donor (carbon source) is added into vials of sets one and two to mimic the exact conditions that prevailed in the reactor. To the third set of vials containing non-pasteurized sludge, 500 μl of sterile reactor feed containing neither the electron acceptor (nitrate) nor the donor (carbon source) is added to demonstrate whether the target cells could carry out any anaerobic uptake of the carbon source. The glass vials are then sealed using butyl rubber stoppers and flushed using oxygen-free nitrogen for 5–10 min. To further remove any traces of oxygen from within these vials, they are placed in a rotary shaker at 200 rpm for 30 min. Finally, 500 μl of non-radioactive carbon together with 20 μCi of ^{14}C-labelled radioactive carbon is introduced into each vial to provide electron donor concentrations equivalent to that of the reactor. Strict anaerobic techniques are used on these anoxic samples at all times, and the incubation of the samples is performed at room temperature (20°C) on a rotary shaker (200 rpm). The duration of incubation is dependent on the rate of substrate utilization by the biomass. With respect to this experiment, under the assumption that there is a rapid utilization of the carbon source, the duration of incubation on multiple non-pasteurized samples (in individual 9 ml vials) range from 1 to 4 h where some vials are incubated for just 1 h and some for up to 4 h. A time series is necessary to determine the minimum duration of incubation, since excessive incubation could result in false interpretation of FISH–MAR images due to a possible uptake of radioactive by-products by non-target organisms. The control vials are incubated for the entire 4 h duration.

2. Cell fixation, sectioning and FISH hybridization

Following incubation, further uptake of the carbon source is stopped by fixing the samples (see Chapter 6.2 – FISH protocol). The cell fixation procedure also facilitates the removal of excess radioactive substrate reducing background and interpretation difficulties on FISH–MAR images.

In FISH—MAR, the objective is to elucidate whether individual cells from certain populations have produced a MAR signal or not. To achieve this, it is important to perform FISH hybridization and film-coating over a single individual layer of cells. Obtaining 2 μm sections of the fixed sludge using a Kryo 1720 cryostat (Leica Microsystems Pty Ltd, Australia) makes it possible to generate a single cell layer on a microscopic slide. Sample preparation for sectioning is done by first pelleting the fixed cells in a 2 ml microcentrifuge tube. The PBS–ethanol is then removed from the fixed cells leaving the minimum and 0.5 ml of Tissue-Tek® OCT compound is introduced over the pellet and the microcentrifuge tube is frozen at −20°C overnight. The frozen OCT with the trapped pelleted cells within its matrix is scooped out of the tube using a pointed needle. It is then mounted and sectioned maintaining a chamber temperature of −20°C and a sample holding temperature of −30°C. The 2 μm

sections are positioned over one single end of clean cover slips (22 mm × 75 mm). The cover slips with the sections are then stored at −20°C until further use.

Standard FISH (see Chapter 6.2) is carried out on the sections using a specific FISH probe labelled in Cy3 and either group probes or EUBMix probe labelled in FITC to target most bacteria in the sample. Since the use of epifluorescence microscopy together with light microscopy is critical for the initial interpretation of FISH–MAR results, FITC labelled group or EUBMix probes rather than Cy5 labelled probes is employed. Once FISH is carried out, the cover slips are stored in the dark at −20°C.

3. Nuclear emulsion coating, exposure and development

The nuclear emulsion Hypercoat LM-1 (Code No. RPN40, Amersham Biosciences Pty Ltd, Australia) is used to coat the samples. The sample coating protocol described is a slight modification of the manufacture's coating protocol and is performed in a dark room fitted with a dark red lamp (PF712B, 15W, Philips, Australia). Initially, the hypercoat LM-1 emulsion is melted in the dark at 43°C for 15 min and the melted emulsion is gently poured into a dipping chamber (Code No. RPN39, Amersham Biosciences Pty Ltd, Australia). A dry cover slip with previously FISH-probed sample is dipped for 10 s vertically into the emulsion. Thereafter, the cover slip is gently removed draining excess emulsion off by brushing the rear of the cover slip against the edge of the chamber and by holding the cover slip vertically over a paper tissue for 5 s. Subsequently, any emulsion remaining on the rear of the cover slip is removed using a paper tissue and the cover slip is placed for 10 min over a cool flat metal surface for rapid solidification of the emulsion. The coated cover slip is placed horizontally in a dry, lightproof box at 28°C for 3–4 h to dry, and then is transferred vertically into a slide rack containing a small amount of anhydrous silica gel and is sealed in light, tight. The coated cover slips are then stored at 4°C for the required duration of exposure.

To determine the optimal exposure time for the cover slips, the number of cells covered by the silver grains is monitored by developing cover slips weekly over a duration of 3 weeks using standard photographic developing procedures. The slides are first placed in a common slide rack normally used for staining purposes, which allows the processing of batches of cover slips. The developer is Kodak D19 (40 g/l of Milli-Q water), the stop solution is Milli-Q water and the fixation solution is 30% (w/v) sodium thiosulphate in Milli-Q water [3]. The slide rack is placed in the developer for 3 min, transferred to the stop solution for 1 min and finally placed in the fixation solution for 4 min. The cover slips are finally rinsed by dipping gently in fresh Milli-Q water twice, for 3 min. The slides are then air dried and microscopically examined.

4. Visualization

A Nikon Eclipse TE300 inverted microscope fitted with Nikon Plan APO 60X oil (NA 1.4) and Plan APO 100X oil (NA 1.4) lenses could be used to inspect the samples. The 100 W high-pressure mercury bulb and filter sets (Nikon B-2A for FITC and

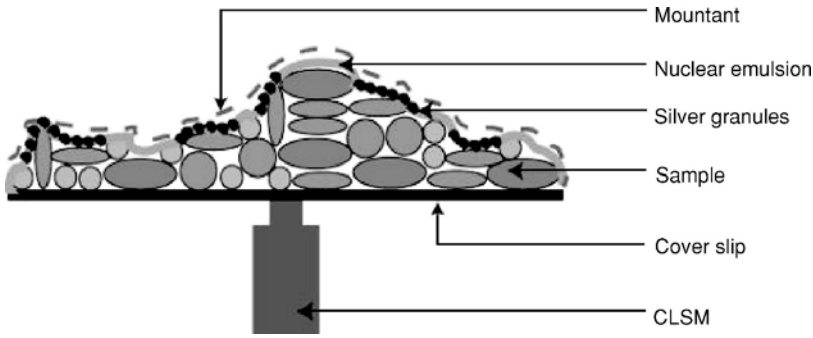


Figure 1. Schematic diagram of the inverse confocal laser scanning microscopic observation (adapted from Lee et al. [7]).

G-2A for Cy3) of the microscope are used for epifluorescence microscopic observations of the probed labelled cells, and bright field microscopy is used for observing the silver grains (MAR signal) on the cover slip. The positive uptake of radiolabelled substrate by bacterial cells is clearly seen as a dense cluster of silver grains on the top of the cells. The confocal laser scanning microscope is used for combined confocal, epifluorescence and bright field (to capture the MAR signal) image acquisition. Fig. 1 illustrates the inverse confocal laser scanning microscopic observation technique.

Accordingly, FISH provides *in situ* evidence on the occurrence of specific microbial populations in mixed culture and MAR enables us to elucidate whether the FISH probe targeted micro-organisms could metabolize substrates under certain environmental conditions.

References

1. Amann, R., and W. Ludwig. 2000. Ribosomal RNA-targeted nucleic acid probes for studies in microbial ecology. *FEMS Microbiol. Rev.* **24:**555–565.
2. Amann, R.I., W. Ludwig, and K.H. Schleifer. 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59:**143–169.
3. Andreasen, K., and P.H. Nielsen. 1997. Application of microautoradiography to the study of substrate uptake by filamentous microorganisms in activated sludge. *Appl. Environ. Microbiol.* **63:**3662–3668.
4. Brock, T.D., and M.L. Brock. 1968. Autoradiography as a tool in microbial ecology. *Nature* **209:**734–736.
5. Costerton, J.W., Z. Lewandowski, D.E. Caldwell, D.R. Korber, and H.M. Lappinscott. 1995. Microbial biofilms. *Annu. Rev. Microbiol.* **49:**711–745.
6. Dahllof, I. 2002. Molecular community analysis of microbial diversity. *Curr. Opin. Biotechnol.* **13:** 213–217.
7. Lee, N., P.H. Nielsen, K.H. Andreasen, S. Juretschko, J.L. Nielsen, K.H. Schleifer, and M. Wagner. 1999. Combination of fluorescent *in situ* hybridization and microautoradiography – a new tool for structure-function analyses in microbial ecology. *Appl. Environ. Microbiol.* **65:**1289–1297.
8. Moller, S., A.R. Pedersen, L.K. Poulsen, E. Arvin, and S. Molin. 1996. Activity and three-dimensional distribution of toluene-degrading *Pseudomonas putida* in a multispecies biofilm assessed by

quantitative *in situ* hybridization and scanning confocal laser microscopy. *Appl. Environ. Microbiol.* **62:**4632–4640.

9. Radajewski, S., I.R. McDonald, and J.C. Murrell. 2003. Stable-isotope probing of nucleic acids: a window to the function of uncultured microorganisms. *Curr. Opin. Biotechnol.* **14:**296–302.
10. Schramm, A., L.H. Larsen, N.P. Revsbech, N.B. Ramsing, R. Amann, and K.H. Schleifer. 1996. Structure and function of a nitrifying biofilm as determined by *in situ* hybridization and the use of microelectrodes. *Appl. Environ. Microbiol.* **62:**4641–4647.

PART SEVEN

Genomic analysis of microbial ecosystems

7.1. Metagenomic analysis of the microbiomes in ruminants and other herbivores

MARK MORRISON[1,4], SARAH E. ADAMS[1,5], KAREN E. NELSON[2,4] and GRAEME T. ATTWOOD[3]
[1]*Department of Animal Sciences, The Ohio State University, Columbus, OH, USA*
[2]*The Institute for Genomic Research, Rockville, MD, USA*
[3]*AgResearch, Grasslands Research Centre, Palmerston North, New Zealand*
[4]*The North American Consortium for Genomics of Fibrolytic Ruminal Bacteria*
[5]*The IAMS Company, Dayton, OH, USA*

Introduction

Many conceptual breakthroughs in the life sciences would not have been possible without first developing techniques and instrumentation to investigate biological processes and molecules. In 1995, The Institute for Genomic Research (TIGR) completely sequenced, assembled and published the first genome of a free-living organism, that of *Haemophilus influenzae* Rd [4]. This milestone in scientific achievement has allowed microbiologists to progress from a reductionist approach of studying one gene at a time to the examination of microbial biology from an organismal perspective, using a combination of existing and newly developed (bio)chemical and computational (*in silico*) approaches. These fields of investigation are often defined with an 'omics' suffix. Hence, genomics refers to the holistic examination of the genetic blueprint that a microbe has acquired, at that point in evolutionary time, to support its lifestyle. Transcriptomics, proteomics and metabolomics refer to a similar level of analysis at the RNA, protein and metabolite levels, respectively.

Furthermore, the latest advances in sequencing technologies and cloning vectors better enable a detailed examination of the structure and function of microbial communities, including those organisms that cannot readily be cultured, and we refer to the integrative use of the following methods as the basis of an emerging scientific discipline referred to as metagenomics:

1. *Bacterial artificial chromosome and fosmid cloning technologies:* Community genomic DNA is cloned in large fragments (>50–150 kilobases [kb]) to create libraries of bacterial artificial chromosomes (BACs), or smaller fragments (~40 kb) are cloned into fosmid vectors. These libraries can then be screened by DNA- and activity-based screens for genes encoding any number of particular functions including hydrolytic and other enzymes central to schemes of carbon sequestration.
2. *High throughput DNA sequencing and bioinformatics:* Both BAC and fosmid libraries and whole genomes of select bacteria can be sequenced, and function inferred, in relatively short periods of time by using high throughput sequencing systems and bioinformatics.

H.P.S. Makkar and C.S. McSweeney (eds.), Methods in Gut Microbial Ecology for Ruminants, 209–220.
© 2005 *IAEA. Printed in the Netherlands.*

3. *Use of small subunit ribosomal RNA as a measure of biodiversity:* An extensive database of RNA sequences supports both PCR- and hybridization-based methods of assessing microbial diversity and population dynamics. Through clonal frequencies, the relative population sizes can be estimated.

Although some readers and users of this manual are unlikely to be directly involved with microbial genome sequencing projects, this does not exclude the reader from contributing to, or capitalizing on, the information and resources made available via such projects. In this context, we will review some of the databases and technologies that are finding application in the study of genome composition of individual strains or species of ruminal bacteria, as well as the metagenomic analysis of gastrointestinal microbiomes. For more extensive reviews of genome sequencing and functional microbial genomics, the reader is encouraged to obtain publications edited by Fraser et al. [5] and Wren and Dorrell [20]. Because the current and emerging protocols applied to ribosomal RNA and gene-based biodiversity studies are covered extensively in other chapters of this manual, they will only be tangentially addressed here and only in relation to the construction of specific metagenomic libraries.

Genome-sequence databases

Whole genome sequencing serves as the foundation for a multitude of comparative and functional lines of investigation that seek to understand, at an organismal level, the molecular biology underpinning the lifestyle of specific microbe. Table 1 lists the ruminal bacteria that, at the time of writing, are the subject of genome sequencing projects, as well as the sequenced genomes that are publicly available for phylogenetically related species and strains, and the websites from which to access this information. Although the total number of sequenced rumen microbial genomes is comparatively low, there is sequence data available for phylogenetically related species and strains. A compilation of all ongoing and completed genome-sequencing projects is maintained at the genomes online database (GOLD, see http://wit.integratedgenomics.com/GOLD/). The comprehensive microbial resource (CMR) curated by TIGR (http://www.tigr.org) also contains the completely sequenced genomes of more than 150 microbes, representing virtually all branches in the three domains of life. In North America, the Joint Genome Institute (JGI) sponsored by the Department of Energy (DOE, http://www.jgi.doe.gov/JGI_microbial/html/index.html) has also played a dominant role in microbial genomics, largely by the provision of 'draft' sequences (where gaps remain in the sequence data, precluding the opportunity to precisely assemble the genome into its cognate molecule[s]) for many more microbes, especially those of environmental and(or) industrial relevance. Another valuable resource is the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.ad.jp/kegg/kegg2.html). All these resources provide access to genome sequence data in tabular and graphical formats and support the users' ability to acquire the available information about a specific gene, entire pathways coordinating physiological processes or a functional category of gene products.

Table 1. Sequencing projects for prokaryotes either present or phylogentically related to species found in rumen and gastrointestinal microbiomes. The institutions coordinating the project and websites for access to the data are also shown.

Microbes of ruminal origin	Strain and coverage	Institution	Website
Prevotella ruminicola	strain 23, closed	TIGR	www.tigr.org
Prevotella bryantii	strain B_14, draft (8x)	TIGR	www.tigr.org
Fibrobacter succinogenes	strain S85, closed	TIGR	www.tigr.org
Wolinella succinogenes	DSMZ 1740, closed	Max-Planck Inst. Tuebingen	www.wolinella.mpg.de
Ruminococcus albus	strain 8, closed	TIGR	www.tigr.org
Ruminococcus flavefaciens	strain FD-1, draft (2x)	Univ. of Illinois	www.biotech.uiuc.edu
Clostridium proteoclasticum	B316, draft (9x)	AgResearch, New Zealand	
Methanobrevibacter ruminantium	DSM 1093, draft (8x)	Pastoral Greenhouse Gas Research Consortium, New Zealand	
Relevant, non-ruminal prokaryotes:			
CFB phylum			
Bacteroides thetaiotaomicron	VPI 5482, closed	Washington Univ., St Louis	genome.wustl.edu
Bacteroides fragilis	NCT 9343, 638R closed	Sanger Inst., Cambridge, U.K.	www.sanger.ac.uk
Bacteroides fragilis	YCH46, closed	Tokishima and Kitasato Univ.	genome.ls.kitasato-u.ac.jp
Bacteroides vulgatus	ATCC8482, draft	Washington Univ.	genome.wustl.edu
Bacteroides distasonis	ATCC8503, draft	Washington Univ.	genome.wustl.edu
Cytophaga hutchinsonii	ATCC 33406, draft	DOE-JGI	www.jgi.doe.gov
Prevotella intermedia	17, closed	TIGR	www.tigr.org
Spirochetes			
Treponema pallidum	Nichols, closed	TIGR	www.tigr.org
Treponema denticola	ATCC 35405, closed	TIGR	www.tigr.org
Clostridial lineages			
Clostridium thermocellum	ATCC 27405 draft	JGI-DOE	www.jgi.doe.gov
Moorella thermoaceticum	ATCC 39073 draft	JGI-DOE	www.jgi.doe.gov
Enterococci			
Enterococcus faecalis	Closed	Genome Therapeutics	www.oscient.com
Enterococus faecium	Closed	Genome Therapeutics	www.oscient.com
Enterococcus faecium	DO, draft	JGI-DOE, BCM-HGSC	www.jgi.doe.gov
Enterococcus faecium	ATCC 35667, closed	Integrated Genomics Inc.	www.integratedgenomics.com
Lactobacilli			
Lactobacillus acidophilus	ATCC 700396, closed	Cal. Poly. Env. Biotech. Inst.	www.calpoly.edu/~rcano/
Lactobacillus brevis	ATCC 367, draft	JGI-DOE	www.jgi.doe.gov
Lactobacillus casei	ATCC 334, draft	JGI-DOE	www.jgi.doe.gov
L. delbrueckii subsp. bulgaricus	ATCC BAA-365, draft	JGI-DOE	www.jgi.doe.gov
Lactobacillus gasseri	ATCC 33323, draft	JGI-DOE	www.jgi.doe.gov
Lactobacillus lactis	IL1403, closed	INRA, Genoscope	www.genoscope.cns.fr
Lactococcus lactis subsp. cremoris	SK11, draft	JGI-DOE	www.jgi.doe.gov
Fusobacterium			
F. nucleatum subsp. polymorphum	ATCC 10953	BCM-HGSC, UCLA	
F. nucleatum subsp. vincentii	ATCC 49256	Integrated Genomics Inc.	www.integratedgenomics.com
Streptococci			
Steptococcus agalactiae	A909, closed	TIGR	www.tigr.org
Streptococcus equi	Closed	Sanger Institute	www.sanger.ac.uk
Syntrophic bacteria			
Syntrophobacter fumaroxidans	MPOB, draft	JGI-DOE	www.jgi.doe.gov
Syntrophomonas wolfei	DSM 2245B, draft	JGI-DOE	www.jgi.doe.gov
Archaebacteria			
Methanosarcina mazei	Go1 (DSMZ 3647)	Gottingen Genomics Lab.	www.g2l.bio.uni-goettingen.de
Methanosarcina barkeri	Fusaro, draft	JGI-DOE	www.jgi.doe.gov
Methanothermobacter thermoautotrophicus	Delta H, closed	Ohio State Univ.	www.biosci.ohio-state.edu
Methanosarcina acetivorans	C2A, closed	Whitehead Institute	www.broad.mit.edu/annotation
Methanospiril lum hungatei	JF1, draft	JGI-DOE	www.jgi.doe.gov

[a]For brevity, *Escherichia coli* strains and *Salmonella* spp. are not included. A complete and updated list of completed and ongoing genome sequencing projects can be found at http://www.genomesonline.org

The FibRumBa database created by the North American Consortium for Genomics of Fibrolytic Ruminal Bacteria is another emerging resource that should be valuable to those interested in herbivore microbiology (www.tigr.org/tdb/rumenomics). The genome module of this database will contain not only the genomes sequenced via the consortium, but also the sequence data produced for other bacteria, including *Bacteroides thetaiotaomicron*, *B. fragilis*, *Clostridium thermocellum*, *Cytophaga hutchinsonii*, *Enterococcus fecalis*, *Thermobifida fusca* and *Wolinella succinogenes.* The genome sequence data for all these microorganisms will be formatted to facilitate the use of many of the analytical features available through the CMR (which are primarily restricted for use with genomes sequenced to closure). The subtractive hybridization module will contain the information derived from suppressive subtractive hybridisation (SSH) and representational difference analysis (RDA) studies undertaken by consortium members (see the section on 'comparative genomics' in what follows). The annotation data will be presented in a tabular format, and the sequences (both nucleotide and peptide) will be retrievable by links associated with each annotation entry. Additionally, the clones will also be presented in a graphical format to illustrate the orientation and length of sequence information for each clone (and any overlaps among multiple clones) and the region of homology with their 'best hit'. Although still in its infancy, the FibRumBa database will provide a comprehensive compilation of raw data, reference information and analytical tools, which should prove useful for anyone with interests in microbial biology, polysaccharide degradation and(or) rumen/gastrointestinal microbiology. For instance, are there 'conserved hypothetical' genes in ruminal bacteria that are central to the colonization and persistence of these microbes in rumen microbiomes? Are there genes unique to specific ruminal bacteria, and(or) do genes of ruminal microbes encode module(s) unique to these bacteria and what are the roles of these genes in colonization and persistence? Such databases support the examination of genome composition and organization, and the opportunity to extend our understanding of gastrointestinal microbiomes beyond the degradative and metabolic characteristics predictably relevant to host animal health and nutrition.

Comparative genomics

The bacterial genomes we have sequenced do not account for the full genetic potential resident within these species. Despite the expediency and cost savings associated with new sequencing technologies, it is still cost prohibitive in many instances to have multiple genomes sequenced for a selected group of related bacteria. Comparative genome hybridizations or 'genomotyping' use whole-genome microarrays to visualize the extent of genomic similarity among related strains and species of bacteria. Genomic DNA from the 'tester' and the reference (sequenced) strains is fractionated and differentially labelled, then hybridized to the microarray. The signal ratio can then be used to establish whether a particular gene is present or divergent in the genome of the tester strain [8]. While such methods are rapid and effective

in providing information of genomic similarity and divergence among strains, the techniques are also 'one-sided': no information is generated concerning what genomic content is present in the tester strain, but absent from the reference strain. As such, SSH [1] and RDA [3] offer rewarding alternatives to whole genome sequencing of related strains. Both methods depend upon the hybridization of DNA fragments with common genomic sequence from the two closely related strains, followed by the isolation of those genomic fragments unique to one of the bacteria. The SSH method is the most widely used approach with both genomic DNA (the PCR-Select Bacterial Genome Subtraction Kit) and cDNA (the PCR-Select cDNA Subtraction Kit). All reagents and controls are contained in these kits, and the researcher supplies genomic DNA (or cDNA) from the strain of interest (tester) and the strain with which to subtract (driver). Essentially, digested genomic DNA fragments from the prokaryotic strain of interest (the tester strain) are modified by the ligation of different adaptor sequences. The DNA fragments are then mixed with an excess of digested genomic DNA fragments from the reference strain (the driver strain). After heat denaturation, the genomic fragments with a high degree of sequence identity in both the tester and driver genomes anneal with each other. The population of 'unique' double-stranded tester DNA fragments can then be selectively amplified by PCR, using primers designed to amplify from the adaptor sequences. The resulting PCR products are cloned and propagated in *Escherichia coli.* After screening for insert by PCR and for specificity by Southern blot, the clones representing genomic differences are sequenced and annotated.

Subtractive hybridization approaches offer the opportunity to examine the genetic potential of a much broader range of ruminal bacteria isolated from different host animals and(or) geographical locations. Furthermore, the sequenced bacterial strains have been maintained in laboratory culture collections for many years; so, it is possible that some genes critical to colonization and persistence in the ruminal environment have been lost. For these reasons, we believe that some studies should be undertaken with freshly isolated strains of ruminal bacteria, which have been screened and characterized with respect to their fibre-degrading potential (kinetics of cellulose solubilization and growth) or other phenotypic characteristics, with the intention of producing new insights into the genetic potential affiliated with these bacterial groupings.

However, the reader should also appreciate that there are possible limitations associated with these studies. Using glycoside hydrolases as an example, the possibility exists that the genomes of the sequenced and freshly isolated strains both contain the same glycoside hydrolase gene(s), but the gene(s) are no longer effectively expressed in the laboratory strains. This would result in the subtraction of the gene(s) by SSH and RDA procedures and concealing these 'genomic differences' between the lab strains and freshly isolated strains. Such a scenario emphasizes the importance of conducting both microarray and proteomic analyses in conjunction with the RDA and SSH analyses, to establish whether the phenotypic differences are attributable to a difference at the DNA, RNA or protein level.

Metagenomic analysis

Metagenomic research is an emerging line of investigation that offers the opportunity to understand and access the genetic potential present in microbial communities, regardless of whether we currently have the means to culture all those microorganisms. Exciting findings have been produced from soil [13], marine microbial communities [2], enrichment cultures [9], and most recently from oceans and acid mine drainage systems [18, 19]. Several groups are currently producing metagenomic libraries of rumen microbiomes, and it is anticipated that these libraries too will reveal significant findings relevant to our understanding of microbial biology and fibre degradation.

Metagenomic libraries can be constructed in a variety of vectors, the choice of which is largely predicated by the nature of the analyses to be conducted and the integrity of the metagenomic DNA that can be recovered from the sample to be analysed. The two principal vectors used, at present, are BAC and fosmid-based, which are typically low copy number vectors that support the cloning of large (50–150 kb) and intermediate-sized (45 kb) fragments, respectively. The BAC vectors afford the opportunity of recovering large biosynthetic or metabolic operons in their entirety and also support a more detailed assessment of (meta)genome organization, due to the larger amount of information archived in each clone [2], although the BAC libraries can also be subjected to functional screens that are dependent on heterologous gene expression, most often in *E. coli* [13]. The most widely used DNA extraction method for BAC library construction employs an in-gel lysis of bacterial cells, followed by partial restriction endonuclease digestion of the DNA and pulse field gel electrophoresis, to recover DNA fragments of the desired size. The set of methods presented in Table 2 were derived from protocols described by Beja et al. [2] and Rondon et al. [13, 14] for the recovery of high molecular weight DNA for BAC library construction using a tube-gel format. At the Ohio State University, we have further modified these methods to include an additional enzyme (mutanolysin) for cell wall lysis and also employed the use of PFGE gel plug moulds to embed cells prior to lysis and partial DNA digestion (methods described in Table 3). As shown in Fig. 1A, the DNA released from cells embedded in PFGE plug moulds appears to be less fragmented prior to restriction endonuclease digestion, but the yield is substantially lower than that recovered from tube-gel slices. When these different DNA preparations are used with either denaturing gradient gel electrophoresis or RIS-specific DNA primers, the diversity profiles are very similar to that obtained from community DNA extracted using the RBB + C method described by Yu and Morrison [22, see Chapter 3.1], which involves bead beating and shearing of DNA (Fig. 1B). The in-gel lysis methods, therefore, produce a good representation of community diversity, and the rate-limiting step to library construction will most likely be the amount of DNA recovered of the desired size range from cells present in the liquid-based fractions of digesta.

Although fosmid clones are inherently smaller, due to the biology underpinning library construction, the cloning efficiency of metagenomic DNA tends to be

Table 2. Procedures for cell suspension in agarose tube gels, in-gel cell lysis and partial digestion of released DNA with *Hin*dIII, developed from protocols described by Beja et al. [2], Rondon et al. [14], Zimmer and Verrinder-Gibbins [23] and Stein et al. [16]

Preparing tube gels

Centrifuge cells at 4°C for 10 min at maximum speed and remove supernatant Add 0.5 ml of buffer A to the cell pellet and resuspend. Make a direct count of the cells and dispense $\sim 10^{10}$ cells per aliquot in 1.5 ml tubes

Centrifuge aliquots and remove the supernatant. Resuspend the cell pellet in 0.25 ml buffer A, then add 0.25 ml of 1% (w/v) SeaPlaque GTG agarose solution preheated to 65°C

Immediately draw the mixture into 1 ml syringes. Let gel solidify at 4°C for at least 30 min

In-gel cell lysis

Remove the tube gel from the syringe mould by cutting off the lure hub of the syringe and use the plunger to extrude the gel into a 15 ml Falcon tube. Add 10 ml of cell-lysis buffer B and 50 mg lysozyme. Incubate at 37°C for 5 h with end-over-end mixing every 30 min

Decant the lysis buffer; add 10 ml of fresh lysis buffer B and 50 mg of lysozyme. Lie the tube horizontally and incubate at 37°C overnight

Replace the above buffer with 10 ml of buffer C containing 1% (w/v) Sarkosyl and 1 mg/ml proteinase K. Incubate at 55°C for 12 h, then replace the solution and repeat Transfer the tube gels into a 50 ml Falcon tube and add 40 ml of TE buffer (10 mM Tris–HCl and 5 mM EDTA [pH 8.0]). Change the buffer four times. Store the tube gels at 4°C or proceed with partial digestion with *Hin*dIII

Partial HindIII digestion

Transfer tube gel slices into microcentrifuge tubes and add 1× React 2 (*Hin*dIII) buffer containing 0.1 mg/ml BSA and 4 mM spermidine to cover the gel slices (~1.5 ml). Incubate on ice for 20 min

Add 5 U *Hin*dIII per 100 μl buffer for each tube gel, except for the control tube gel (no DNA digestion). Incubate on ice for another 20 min

Incubate the mixture at 37°C for 15 min. Add 0.1 vol. of 500 mM EDTA (pH 8) to stop the digestion and then place tubes on ice or store at 4°C

Buffers used

Buffer A: 50 mM Tris–HCl (pH 8.0) containing 1 M NaCl, sterilized by autoclaving

Buffer B: 50 mM Tris–HCl (pH 8.0) containing 100 mM NaCl, 100 mM EDTA, 0.5% (w/v) *S*-laurylsarcosine, 0.2% (w/v) sodium deoxycholate and 0.5% (w/v) Brij-58. Sterilize and add 5 mg/ml lysozyme just before use

Buffer C: 50 mM Tris–HCl (pH 8.0) containing 100 mM NaCl and 500 mM EDTA (pH 8.0), sterilized by autoclaving. Add 1.0% (w/v) *S*-laurylsarcosine and 1.0 mg/ml proteinase K just before use

substantially higher with this type of vector. Fosmid vectors also afford the use of more robust DNA extraction methods, which expedites the processing of larger amounts of digesta samples and the recovery of greater amounts of metagenomic DNA. Additionally, these more robust methods of DNA extraction can be more readily applied to biofilms adherent to particulates and other surfaces, such as plant matter and mucosal scrapings. At TIGR, the guanidine isothiocyanate method developed by Parrish and Greenberg [12] has been used to isolate high-molecular weight genomic DNA for the construction of fosmid libraries. Briefly, the sample is subjected to freeze thawing, and then lysozyme and mutanolysin are used to hydrolyze microbial cell walls. The nucleic acids are extracted with a guanidine isothiocyanate-containing buffer, then adsorbed to diatomaceous earth, washed to remove impurities and eluted in aqueous buffer. This method has been shown to effectively extract and recover DNA from

Table 3. Procedures for cell suspension in PFGE gel moulds and a modified in-gel cell lysis procedure, followed by partial *Hind*III digestion of DNA, developed from protocols described by Youssef et al. [21], Beja et al. [2], Krause et al. [10], Rondon et al. [14], Zimmer and Verrinder-Gibbins [23] and Stein et al. [16]

Preparing PFGE gel plugs
Count cells and dispense $\sim 10^{10}$ cells per aliquot in 2 ml microcentrifuge tubes
Centrifuge tubes at maximum speed for 1 min and remove supernatant. Resuspend each pellet in 250 μl cell wash buffer
Centrifuge at maximum speed for 30 s and remove supernatant
Resuspend pellet in 200 μl Lysis buffer without added lysozyme and mutanolysin
Incubate at 75°C for 10 min to inactivate nucleases, and then place in a 50°C water bath for 15 min to cool mixture to 50°C
Add 0.2 mg of lysozyme and 10 U of mutanolysin to each tube and mix gently
Add 200 μl of 2% (w/v) clean-cut agarose solution to each tube, kept molten at 50°C
Pipet 50 μl aliquots of the agarose-cell mix into PFGE plug moulds
Cool plugs at 4°C for at least 20 min, prior to use
In-gel cell lysis
Extrude the gel plugs into 2 ml microfuge tubes
Make fresh, sterile lysis buffer and add 1 mg/ml lysozyme and 20 U/ml mutanolysin
Add 1 ml of the above buffer to each tube and incubate the gel plugs at 37°C overnight
The next day, remove the lysis buffer and add 1 ml fresh Proteinase K buffer to each tube and incubate the gel plugs at 50°C for at least 12 h
Wash gel plugs overnight with 2 ml of TE, shaking the tubes gently. Alternatively, use five volume changes of TE and shake gently for 1 h between each washing step
Store the washed gel plugs at 4°C or proceed with partial digestion with *Hin*dIII
Partial HindIII digestion
Transfer gel plugs to fresh tubes and add 300 μl of 1× React 2 (*Hin*dIII) buffer containing 0.1 mg/ml BSA and 4 mM spermidine to each tube and place on ice for 20 min
Add 15 U *Hin*dIII to each tube, except the control (no DNA digestion). Leave on ice for 20 min
Incubate at 37°C for 15 min, add 0.1 vol. of 500 mM EDTA (pH 8.0) and then place tubes on ice or store at 4°C
Buffers used
Cell wash buffer: 10 mM Tris–HCl (pH 8.0), 1 M NaCl, sterilized by autoclaving
Cell lysis buffer: 6 mM Tris–HCl (pH 8.0), 1 M NaCl, 100 mM EDTA, sterilized by autoclaving. When required, add 1 mg/ml lysozyme and 20 U/ml mutanolysin
Proteinase K buffer: 250 mM EDTA (pH 8.0), add 1.0% (w/v) *S*-laurylsarcosine and 100 μg/ml proteinase K just prior to use

multiple Gram-positive, Gram-negative and spirochete bacteria, and the DNA prepared in this manner is typically ~40–60 kb, needing no additional shearing prior to fosmid library construction and can be easily end repaired prior to size fractionation by agarose gel electrophoresis. For these reasons, fosmid vectors and methods such as that described by Parrish and Greenberg [12] and the RBB + C method described by Yu and Morrison [22, see Chapter 3.1. in this issue] may be the preferred methods for

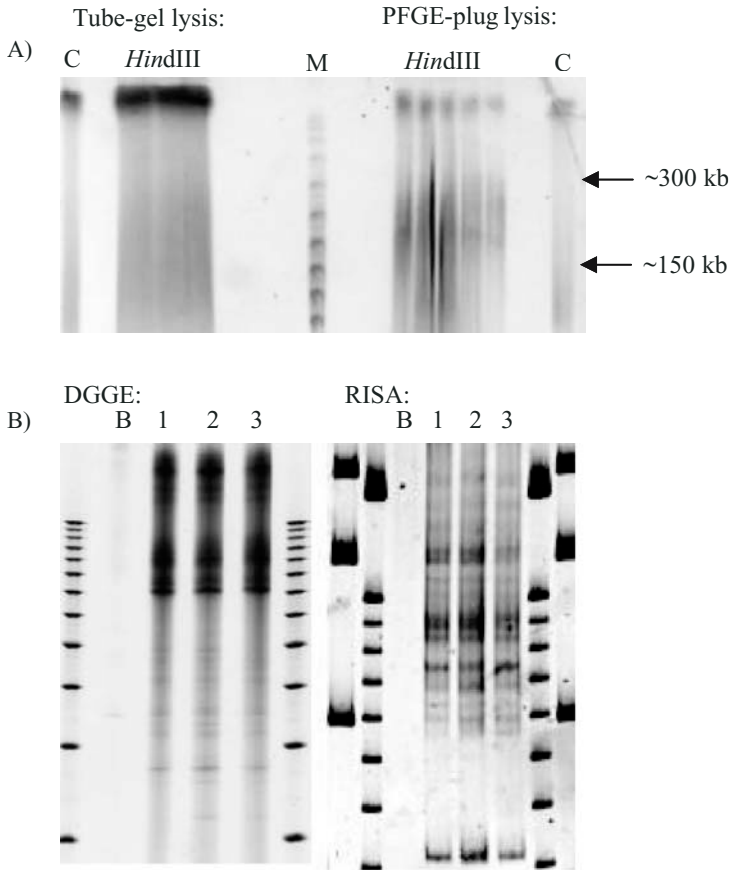


Figure 1. Release and partial *Hin*dIII digestion of metagenomic DNA from rumen microorganisms, and the diversity profiles produced from these samples using either PCR-denaturing gradient gel electrophoresis (DGGE) or ribosomal intergenic spacer analysis (RISA). Panel A illustrates the PFGE results following in-gel cell lysis and partial *Hin*dIII digestion, using either the tube-gel method described in Table 2 or the PFGE-plug lysis method described in Table 3. Lanes marked C represent metagenomic DNA released by the respective in-gel lysis method, but without partial *Hin*dIII digestion. Lane M shows the DNA-sizing ladder (New England BioLabs MidRange Marker I), and the region representing DNA fragment lengths between 150 and 300 kb are marked. Panel B shows the DGGE and RISA profiles produced from the metagenomic DNA recovered from the PFGE gels in the 150–300 kb size range, following either tube-gel lysis (lane 1) or PFGE-plug lysis (lane 2), and compared with community DNA extracted by the bead beater-based method (RBB + C) described by Yu and Morrison [22], lane 3. Lanes marked B represent the PCR negative controls, with no DNA added to the PCR reaction. The lanes flanking the DGGE and RISA profiles contain DNA-sizing ladders.

preparing metagenomic libraries from particulate and surface-associated members of gut microbiomes.

The original BAC and fosmid vectors employed for the construction of metagenomic libraries are maintained in *E. coli* in low copy number, usually one to several

copies per cell. Although this is inherently valuable for minimizing toxicity problems and thereby improving the stability of the DNA inserts, further downstream applications were constrained by the relatively poor yields of BAC or fosmid DNA selected for more detailed analysis. These problems have been largely overcome by the development of the pCC1BAC and pCC1FOS vectors, available from Epicentre Technologies. Both vectors possess an inducible *oriV* for increased plasmid copy number.This affords routine growth of the clones at single copy number to avoid toxicity problems, but the clones can be induced up to 50 copies per cell for clone purification and sequencing. Another key development is the creation of BAC and fosmid vectors by Tom Schmidt and John Breznak at Michigan State University, which contain I-*Ceu*I cloning sites. An I-*Ceu*I site is positioned within the genes encoding 23S rRNA in many prokayotes [11, 17]; so, these newly developed vectors, therefore, provide the advantage of producing libraries that are enriched for clones encoding a specific phylogenetic marker. The investigator can produce a detailed inventory of biodiversity, as well as selectively recover large fragments of DNA flanking *rrn* operons that can be sequenced to gain additional insights into genome composition and organization. Hybrid BAC vectors that can replicate in other host bacteria have also been developed, such as one that has been constructed for replication in *Streptomyces* spp. [15] and superBAC1, which can be stably maintained in *Bacillus subtilis* and other Gram-positive bacteria [6]. Thus, if genes from a particular bacterial phylum are being targeted, the BAC vector can be adapted to provide increased versatility in host strain choice. Considering the *Cytophaga–Flexibacter–Bacteroides* phylum contributes much to microbial diversity, and thereby the genetic potential, present in environments such as soil [7] and gut microbiomes, may also be useful to examine the use of a *Bacteroides*-based shuttle vector and *B. thetaiotaomicron* as a potential host strain.

Concluding remarks and future perspectives

In a little more than 10 years, microbial biology has advanced from the sequencing of individual genomes to comprehensive assessments of microbial diversity and genetic potential resident within entire microbial communities. This has been driven largely by (i) the widespread appreciation that culturable microbes represent only a small percentage of the total microbial world; (ii) development of cloning vectors that support the stable maintenance of large DNA inserts in *E. coli* and other bacteria and (iii) advances in high throughput sequencing technologies. The BAC and fosmid libraries reported in the literature range from 10 000 to 25 000 clones, representing 0.5−1.0 Gbp of DNA (1 Gbp = 10^9 basepairs) [2, 13]. Although these libraries might initially seem immense, millions of clones are estimated to be required for representative coverage of soil metagenomes [6]. Given that gut microbiomes possess a similar breadth in biodiversity as many soil microbiomes, a comprehensive inventory of the metagenome is currently out of reach for many investigators interested in rumen and gut microbiomes. In that context, the selective enrichment of subpopulations could be achieved via physical fractionation (e.g. recovering cells tightly adherent to digesta particles), cell sorting or the establishment of enrichment cultures.

Additionally, BAC and(or) fosmid libraries may contain a disproportionate number of clones derived from the numerically most predominant specie(s). Therefore, normalization strategies, similar in context to those used to produce cDNA libraries from higher eukaryotes, might need to be developed to enhance the representation in the libraries of less-abundant species. The meta analysis of rumen microbiomes may need to be approached in incremental steps, perhaps with a coordinated effort involving multiple research teams throughout the world.

Acknowledgements

Sarah Adams was supported by a scholarship from the Ohio State University Nutritional Sciences Program, and the DNA isolation procedures and analysis described here were developed with funding from the Ohio Agricultural Research and Development Center (OARDC) matching grant program (500486 OHOG0592). The North American Consortium for Genomics of Fibrolytic Ruminal Bacteria was created through financial support provided by the United States Department of Agriculture's Initiative for Future Agriculture and Food Systems and the United States Department of Agriculture's and National Science Foundation's Microbial Genome Sequencing Program. The authors also acknowledge the efforts of other principal members of the North American Consortium for Fibrolytic Ruminal Bacteria (W. Nelson, S. Daugherty and E. Mongodin, TIGR; C.W. Forsberg, University of Guelph; D.B. Wilson and J.B. Russell, Cornell University; B.A. White, R.I. Mackie, and I.K.O. Cann, University of Illinois).

References

1. Antonopoulos, D., K.E. Nelson, M. Morrison, and B.A. White. 2004. Strain specific genomic regions of *Ruminococcus flavefaciens* FD-1 as revealed by combinatorial random-phase genome sequencing and suppressive subtractive hybridization. *Environ. Microbiol.* **6:**335–346.
2. Beja, O., M.T. Suzuki, E.V. Koonin, L. Aravind, A. Hadd, L.P. Nguyen, M. Amjadi, C. Garrigues, S.B. Jovanovich, R.A. Feldman, and E.F. DeLong. 2000. Construction and analysis of bacterial artificial chromosome libraries from a marine microbial assemblage. *Environ. Microbiol.* **2:**516–529.
3. Felske, A. 2002. Streamlined representational difference analysis for comprehensive studies of numerous genomes. *J. Microbiol. Methods* **50:**305–311.
4. Fleischmann, R.D., M.D. Adams, O. White, R.A. Clayton, E.F. Kirkness, A.R. Kerlavage, C.J. Bult, J.-F. Tomb, B.A. Dougherty, J.M. Merrick, K. McKenney, G. Sutton, W. FitzHugh, C. Fields, J.D. Gocayne, J. Scott, R. Shirley, L.-I. Liu, A. Glodek, J.M. Kelley, J.F. Weidman, C.A. Phillips, T. Spriggs, E. Hedblom, M.D. Cotton, T.R. Utterback, M.C. Hanna, D.T. Nguyen, D.M. Saudek, R.C. Brandon, L.D. Fine, J.L. Fritchman, J.L. Fuhrmann, N.S.M. Geoghagen, C.L., Gnehm, L.A. McDonald, K.V. Small, C.M. Fraser, H.O. Smith, and J.C. Venter. 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* **269:**496–512.
5. Fraser, C.M., T.D. Read, and K.E. Nelson. 2004. *Microbial Genomes*. Humana Press, Totowa, NJ, USA.
6. Handelsman, J., M. Liles, D. Mann, C. Riesenfeld, and R.M. Goodman. 2002. Cloning the metagenome: culture-independent access to the diversity and functions of the uncultivated microbial world, pp. 241–256. *In* B. Wren and N. Dorell (eds.), *Functional Microbial Genomics*. *Methods of Microbiology*, Vol. 33. Academic Press, London.

7. Hugenholtz, P., B.M. Goebel, and N.R. Pace. 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J. Bacteriol.* **180:**4765–4774.
8. Kim, C.C., E.A. Joyce, K. Chan, and S. Falkow. 2002. Improved analytical methods for microarray based genome composition analysis. *Genome Biol.* http://genomebiology.com /2002/3/11/research/0065.1–0065.17.
9. Knietsch, A., T. Waschkowitz, S. Bowien, A. Henne, and R. Daniel. 2003. Construction and screening of metagenomic libraries derived from enrichment cultures: generation of a gene bank for genes conferring alcohol oxidoreductase activity on *Escherichia coli. Appl. Environ. Microbiol.* **69:**1408–1416.
10. Krause, D.O., B.P. Dalrymple, W.J. Smith, R.I. Mackie, and C.S. McSweeney. 1999. 16S rDNA sequencing of *Ruminococcus albus* and *Ruminococcus flavefaciens*: design of a signature probe and its application in adult sheep. *Microbiology* **145:**1797–1807.
11. Liu, S.-L., A. Hessel, and K.E. Sanderson. 1993. Genomic mapping with I-*Ceu*I, an intron-encoded endonuclease specific for genes for ribosomal RNA, in *Salmonella* spp., *Escherichia coli,* and other bacteria. *Proc. Natl Acad. Sci. USA* **90:**6874–6878.
12. Parrish, K.D., and E.P. Greenberg. 1995. A rapid method for extraction and purification of DNA from dental plaque. *Appl. Environ. Microbiol.* **61:**4120–4123.
13. Rondon, M.R., P.R. August, A.D. Bettermann, S.F. Brady, T.H. Grossman, M.R. Liles, K.A. Loiacono, B.A. Lynch, I.A. MacNeil, C. Minor, C.L. Tiong, M. Gilman, M.S. Osburne, J. Clardy, J. Handelsman, and R.M. Goodman. 2000. Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. *Appl. Environ. Microbiol.* **66:**2541–2547.
14. Rondon, M.R., S.J. Raffel, R.M. Goodman, and J. Handelsman. 1999. Toward functional genomics in bacteria: analysis of gene expression in *Escherichia coli* from a bacterial artificial chromosome library of *Bacillus cereus. Proc. Natl Acad. Sci. USA* **96:**6451–6455.
15. Sosio, M., F. Giusino, C. Cappellano, E. Bossi, A.M. Puglia, and S. Donadio. 2000. Artificial chromosomes for antibiotic-producing actinomycetes. *Nat. Biotechnol.* **18:**343–345.
16. Stein, J.L., T.L. Marsh, K.Y. Wu, H. Shizuya, and E.F. DeLong. 1996. Characterization of uncultivated prokaryotes: isolation and analysis of a 40-kilobase-pair genome fragment from a planktonic marine archaeon. *J. Bacteriol.* **178:**591–599.
17. Toda, T., and M. Itaya. 1995. I-*Ceu*I recognition sites in the *rrn* operons of the *B. subtilis* 168 chromosome: inherent landmarks for genome analysis. *Microbiology* **141:**1937–1945.
18. Tyson, G.W., J. Chapman, P. Hugenholtz, E.E. Allen, R.J. Ram, P.M. Richardson, V.V. Solovyev, E.M. Rubin, D.S. Rokhsar, and J.F. Banfield. 2004. Community structure and metabolism through reconstruction of microbial genomes from the environment. *Nature* **428:**37–43.
19. Venter, J.C., K. Remington, J.F. Heidelberg, A.L. Halpern, D. Rusch, J.A. Eisen, D. Wu, I. Paulsen, K.E. Nelson, W. Nelson, D.E. Fouts, S. Levy, A.H. Knap, M.W. Lomas, K. Nealson, O. White, J. Peterson, J. Hoffman, R. Parsons, H. Baden-Tillson, C. Pfannkoch, Y.H. Rogers, and H.O. Smith. 2004. Environmental genome shotgun sequencing of the Sargasso Sea. *Science* **304:**66–74.
20. Wren, B., and N. Dorrell. 2002. *Functional Microbial Genomics. Methods in Microbiology*, Vol. 33. Academic Press. London.
21. Youssef, G., A. El-Osta, A.J. Hillier, B.E. Davidson, and M. Dobos. 2002. Pulsed-field gel electrophoretic analysis of the genome of *Lactobacillus gasseri* ATCC33323, and construction of a physical map. *Electrophoresis* **23:**3321–3331.
22. Yu, Z., and M. Morrison. 2004. Improved extraction of PCR-quality community DNA from digesta and fecal samples. *Biotechniques* **36**:808–812.
23. Zimmer R., and A.M. Verrinder Gibbins. 1997. Construction and characterization of a large-fragment chicken bacterial artificial chromosome library. *Genomics* **42:**217–226.

Chapterwise keywords

1.1. Experimental design, treatments, experimental unit, rumen environment, digesta fractions, microbial diversity, sampling, variability, heterogeneity, dynamics
2.1. Rumen, microbiology, media, culturing, anaerobic, enzyme assays
2.2. Bacteriophage, rumen, virus, bacteria, DNA, lysis, isolation, characterization
2.3. Methanogens, isolation, culture, enumeration, storage, identification, diversity, archaea, hydrogen utilization, methane, mycoplasmas
2.4. Rumen, micro-organism, anaerobe, anaerobic fungi, rumen fungi, growth, maintenance, cultivation, culture, batch, continuous
2.5. Counting, culture, identification, preservation, protozoa, rumen, sampling, staining
3.1. DNA, RNA, extraction, real-time PCR, RT-PCR, RAP-PCR, probes, primers, rumen
3.2. Real-time PCR, SYBR Green, relative expression, quantification, anaerobic, rumen, fungi, bacteria
4.1. DGGE, genomic DNA isolation, TGGE, 16S-rDNA fingerprinting techniques
4.2. Rumen, virus, phage enumeration, DNA extraction, nucleic acid characterization
4.3. Fungal community, ribosomal markers, ITS1, DGGE, molecular fingerprints
4.4. RAPD, RFLP, T-RFLP, RISA, rumen, ecology, 16S rDNA, PCR
5.1. Rumen bacteria, rumen fungi, rumen methanogens, rumen protozoa, small subunit rRNA, phylogenetic analysis, riboprinting, RFLP
6.1. Northern blot analysis, 16S-rDNA probes, oligonucleotoide probes
6.2. FISH, rumen, oligonucleotide probe, *in situ* 16S rDNA probes
6.3. FISH–MAR, microautoradiography, *in situ* physiology, *in situ* microbial activity
7.1. Rumen microbiology, biotechnology, genomics, rumen function, subtractive hybridization, cloning techniques, DNA isolation, BAC vectors, fosmid vectors, unculturable microbes

Keyword Index