Chapter 3
Exploring the Bioactive Landscape of the Gut Microbiota to Identify Metabolites Underpinning Human Health

Páraic Ó Cuív, Sriti Burman, Sian Pottenger and Mark Morrison

1 Introduction

North America, Europe and Australasia have amongst the highest incidences of chronic gastrointestinal and metabolic diseases including inflammatory bowel diseases (IBD), colorectal cancer (CRC) and obesity (Molodecky et al. 2012; Stevens et al. 2012; Bray et al. 2013). Although once considered rare in large parts of the world the incidences of IBD and obesity in particular have also been steadily increasing in Asia, South America and the Middle East (Ng et al. 2014; Kaplan 2015). These diseases are associated with considerable socioeconomic costs; for example, the estimated costs to the global economy from obesity approaches US$2 trillion per annum, which equates to 2.8 % of global gross domestic product (Dobbs et al. 2014). Thus, there is an urgent need to develop more effective preventative and therapeutic strategies to ameliorate the impacts of these diseases.

Genomic studies have revealed that IBD, CRC and obesity are underpinned by specific host genetic susceptibilities that are considered to be necessary but often not sufficient for disease to develop (Jostins et al. 2012; Peters et al. 2015; Locke et al. 2015), and it is now recognised that environmental factors and lifestyle choices also affect disease risk. Epidemiological studies also suggest that host genetic, environmental factors and lifestyle choices either alone or in combination does not fully explain disease risk implying that other risk factors remain to be identified. With that context, the human gastrointestinal tract harbours a diverse microbial community (gut microbiota) that provides a range of ecological and metabolic functions relevant to host health and well-being (reviewed by Backhed et al. 2005). Human- and animal-based studies have now also identified the gut microbiota as an important risk factor in the aetiology of chronic gut diseases. First,

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human studies have revealed that the microbiota varies between healthy and diseased individuals (e.g. Turnbaugh et al. 2009; Qin et al. 2010; Nakatsu et al. 2015) and these variations are associated with changes in the disease state (Cotillard et al. 2013; Nakatsu et al. 2015; De Cruz et al. 2015). Second, germ-free animals are protected from disease but become susceptible following microbiota transfer (Turnbaugh et al. 2006; Zackular et al. 2013; Schaubeck et al. 2016). Third, both human- and animal-based studies have revealed these diseases are responsive to interventions that modulate the activity of the gut microbiota including antibiotics (Zackular et al. 2013; Murphy et al. 2013; Schaubeck et al. 2016), diet (Donohoe et al. 2014; Quince et al. 2015), probiotics (Kadooka et al. 2010; Bassaganya-Riera et al. 2012) and faecal microbiota transfers (Suskind et al. 2015).

Even though IBD, CRC and obesity are a heterogeneous group of diseases, they are all characterised by an activated inflammatory response. Nuclear factor-kappa B (NF-κB) is a master regulator of gut epithelial integrity and inflammation, and activation of the NF-κB signalling pathway plays a key role in driving the inflammatory response during the onset and progression of these diseases. Consistent with this, the NF-κB signalling pathway is a validated therapeutic target for the treatment of IBD (Atreya et al. 2008) (Fig. 1), and it is also a recognised therapeutic target for CRC (Sakamoto and Maeda 2010), and for obesity and its co-morbidities (Donath 2014; Esser et al. 2015). The NF-κB pathway is particularly well recognised as a therapeutic target for IBD, however, many of the current therapeutics are only partially effective and/or have significant side effects. For instance, glucocorticosteroids can affect linear growth and bone health in paediatric subjects; methotrexate can cause hepatotoxicity, and as a teratogen, the treatment of female subjects is complicated; salicylates are associated with an increased risk of bleeding. Similarly, the newer biologics (e.g. anti-TNFα factors) are expensive, increase the risk of infection and suffer from a loss of response. Interestingly, the gut microbiota plays a central role in modulating the host immune response and specific gut microbes have been shown to possess potent NF-κB suppressive capabilities that can ameliorate the inflammatory response (Ménard et al. 2004; Sokol et al. 2008; Heuvelin et al. 2009; Petrof et al. 2009; Eeckhaut et al. 2012; Khokhlova et al. 2012; Kaci et al. 2013). This suggests that exploiting gut microbe-derived NF-κB suppressive bioactives may provide new opportunities to maintain host health. In this Chapter, we examine our current understanding of the host-microbiota interaction and outline strategies to identify and characterise the NF-κB suppressive capabilities of the gut microbiota. In particular, we propose that an integrated approach combining culture-dependent and independent approaches with a more mechanistic dissection of the microbiota provided by improved cultivation techniques, high-throughput functional screens and metabolomic and genetic dissections is necessary to transform our understanding of gut health and support the development of new preventative and therapeutic strategies.

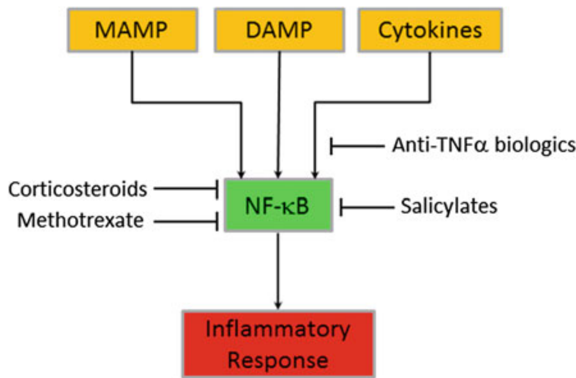

Fig. 1 The NF-κB pathway as a validated drug target for the treatment of chronic gut diseases. The NF-κB pathway can be activated by several mechanisms including microbe-associated molecular patterns (MAMP; e.g. via lipopolysaccharide, flagellin from the gut microbiota), damage associated molecular patterns (DAMP; e.g. via extracellular detection of normally intracellular proteins) or cytokines. Targeting of the NF-κB pathway for the treatment of chronic gut diseases is best recognised for IBD with glucocorticosteroids (corticosteroids), methotrexate, salicylates (e.g. mesalazine, sulfasalazine) and anti-TNFα biologics interfering with pathway signalling. However, this pathway is also increasingly targeted for CRC, and obesity and its co-morbidities

2 The Human Holobiont: An Emergent Paradigm of Human Health

The publication of the human genome sequence was a seminal milestone in our history. Published with much excitement in 2001, it promised new insights and understanding of what it means to be human (Venter et al. 2001; Lander et al. 2001). Initial estimates of the number of protein-coding genes deemed necessary to explain the biological and phenotypic complexity characteristic of humans varied widely, however, there was considerable surprise when it was revealed that the human genome is comprised of as few as 25,000 genes (International Human Genome Sequencing Consortium 2004). Humans are not autonomous and following a period of introspection it was increasingly recognised that our associated microbiota provides a range of functions relevant to health and disease. Thus, in its aftermath, there was an increasing call to sequence our second genome—that of the human microbiota (Davies 2001; Relman and Falkow 2001). This international effort to sequence the human microbiome has principally been led by the Human Microbiome Project (HMP) funded by the US National Institutes of Health (Peterson et al. 2009) and the MetaHIT Project (Ehrlich 2010) funded by the European Union, with additional coordination of other global efforts mediated through the International Human Microbiome Consortium.

Humans and their associated microbiota co-exist as a symbiotic multispecies assemblage termed a "holobiont" that is defined as a physical association between a

host and its associated microbiota for significant portions of their life history (Bordenstein and Theis 2015). The emergence of the holobiont concept has dramatically altered our perception of human health—where the role of microbes was traditionally viewed from the perspective of infectious diseases—to one where the microbiota is viewed as an integral component that contributes essential functionalities relevant to the fitness of the holobiont. The assembly of a holobiont is a dynamic process that impacts both the host and microbiota (Gilbert 2014). For instance, the human gut provides a wide variety of ecological niches that are characterised by a constant temperature, oxygen tension, humidity and nutrient supply. This supports colonisation by a numerically abundant and diverse microbiota that in return helps prevent colonisation by potential pathogens, detoxifies harmful compounds, produces essential nutrients and catalyses the biotransformation of dietary substrate so they can be utilised by the host (Fig. 2). The "hologenome" then is comprised of the genetic potential encoded by the host's genome and their associated microbiota (microbiome) and can thus be considered as an extension of the host genotype itself. Notably, the hologenome is dynamic in terms of its composition with the potential to change more rapidly than the host genome alone via gene acquisition or loss which also confers a greater adaptive potential to the holobiont (Quercia et al. 2014).

The holobiont concept provides a new paradigm for a more holistic understanding of the aetiology of chronic gut diseases. For instance, some of the genetic

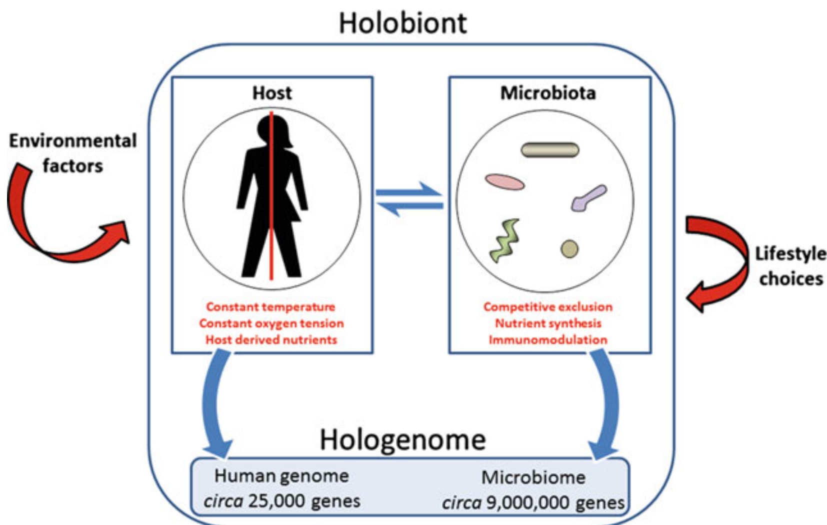

Fig. 2 The emergent holobiont model of human health. The holobiont assembly impacts the fitness of the host and its associated microbiota and is characterised by specific host–microbiota interdependencies. The hologenome is comprised of the genetic capacity of both the human genome and microbiome. The holobiont provides a new model to examine the impact of environmental factors and lifestyle choices on host health and disease risk

susceptibility loci for these diseases also affect the ability of specific microbes to colonise the gut (reviewed by Spor et al. 2011) suggesting that the contribution of genetic susceptibility and microbiota composition to disease risk may be intrinsically linked. In addition, the holobiont has provided a framework on which the impact of environmental factors and lifestyle choices on health and disease risk can be dissected and this has informed the development of new strategies to rationally modulate the holobiont phenotype to improve host health (Zeevi et al. 2015). The holobiont may also represent an optimum biological system to bioprospect for novel NF-κB suppressive bioactives as the gut microbiota has co-evolved with the development of the host mucosal immune system. In particular, we hypothesise that select microbes produce bioactives that actively suppress the NF-κB-mediated immune response perhaps as an essential capability to allow for successful colonisation and persistence. These NF-κB suppressive bioactives may have specific attributes that are relevant to the development of new therapeutics including high bioactivity, bioavailability and target site specificity, as well as stability in the gut environment. Thus, these bioactives could potentially be used directly or serve as lead molecules for the development of novel NF-κB suppressive therapeutics. Alternatively, determining the mechanism by which they exert their suppressive effects could help to identify new cellular targets that could be drugged by existing or new therapeutics. Taken together the identification and characterisation of these bioactives may help realise new opportunities to prevent or treat chronic gut diseases.

3 Insights into the Structure: Function Capacity of the Human Gut Microbiota

Our understanding and appreciation of the diversity and functional capacity of the gut microbiota are largely based on distinct yet complementary culture-dependent and culture-independent analyses of the gut environment (Fig. 3). Many of the current reference strains used in gut microbiota research were first isolated in the mid-twentieth century following the advent of techniques in anaerobic microbiology (for a historical perspective see Rajilić-Stojanović and de Vos 2014). However, it has long been recognised that the vast majority of gut microbes are resistant to cultivation as revealed by the discordance between microscopic counts of microbial cells and those recovered using traditional laboratory based cultivation. This phenomenon was first described in aquatic environments and termed “the great plate count anomaly” (reviewed by Staley and Konopka 1985). Instead, advances in molecular biology and DNA sequencing technology culminated in the establishment of culture-independent approaches to study the microbiota, based largely on 16S rRNA gene community profiling and metagenomics. A crucial discovery was that the microbial 16S rRNA gene could be used as a molecular clock to infer phylogeny and provide an estimate of microbial diversity (Woese and Fox 1977).

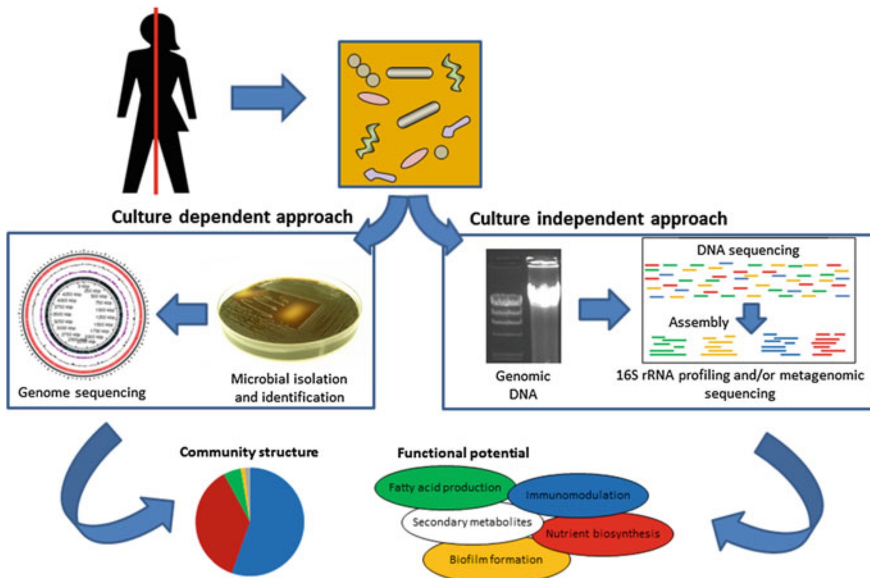

Fig. 3 The analysis of the gut microbiota by culture-dependent and culture-independent approaches. For culture-dependent approaches gut microbes are ideally recovered from gut samples (e.g. faeces, biopsy tissue) as axenic cultures. The phylogeny and functional potential of the isolates can then be assessed by 16S rRNA gene and/or genome sequencing. The functional characteristics of the isolates can be assessed by phenotypic profiling. For culture-independent approaches bulk DNA is typically recovered directly from gut samples and the DNA is then used for 16S rRNA gene profiling and/or metagenomic sequencing. The diversity and functional capacity of the microbiota can be assessed from the resultant sequence data

The 16S rRNA gene is approximately 1550 bp in length and has a divergence rate of 1–2 % per 50 million years (Ochman et al. 1999). The gene is comprised of conserved and (hyper)variable regions and this architecture has been exploited in culture-independent studies to assess microbial diversity. Here, near full-length or subsections of the 16S rRNA gene are amplified by polymerase chain reaction (PCR) using primers targeting the conserved regions and the intervening variable regions are used to infer phylogeny (Klindworth et al. 2013). The length of the 16S rRNA gene sequence can affect phylogenetic assignment (Kim et al. 2011; Franzén et al. 2015) and it does not provide any information on the functional potential of the taxa identified. However, the development of metagenomic approaches, facilitating the sequencing of bulk DNA recovered from microbial communities has now provided new opportunities to both assess microbial diversity through sequencing of defined phylogenetic marker genes (Sunagawa et al. 2013) and the functional capacity of the microbiome (Qin et al. 2010; Li et al. 2014a). In a landmark study Qin et al. (2010) examined the microbiome of 124 subjects and determined that it is comprised of a genetic pool of up to 3.3 million non-redundant genes that is as much as 150× that of the human genome. In practical terms the

functional activity of an individual's gut microbiome is supported by ~500,000 non-redundant genes. In line with an earlier estimate (Yang et al. 2009), a subsequent study by Li et al. (2014a) identified over 9.8 million non-redundant genes in the human gut microbiome. It is likely that the number of non-redundant genes remains underestimated, however, metagenomics may have reached a point of diminishing returns where a greater effort has to be expended to provide an even deeper insight into the gene repertoire of the gut microbiota. Together, both 16S rRNA-based profiling and metagenomic sequencing have provided a unique insight into the gut microbiome and revealed that the vast majority of gut microbes remain uncultured (reviewed by Rajilic-Stojanovic et al. 2007; Rajilić-Stojanović and de Vos 2014).

The adult human gut microbiota is comprised of viruses, bacteria, archaea and eukaryotes with the number of microbial cells inhabiting the adult human gut outnumbering host cells by an order of magnitude (Savage 1977). The gut environment is characterised by a host driven top-down pressure on the microbiota that selects for a community of distantly related microbes with similar functional capabilities ensuring redundancy of microbial processes essential for the host. In contrast, intra-microbiota competition results in a bottom-up pressure that selects for functional specialisation. Consequently, the structure of the gut microbiota is characterised by distinct inter-subject variability although the core functional capabilities of the microbiota (e.g. short chain fatty acid (SCFA) production, vitamin biosynthesis) are largely conserved (Turnbaugh et al. 2009; Lozupone et al.2012). The diversity and functional attributes of the bacterial and archaeal communities in the human gut is best understood. The human gut microbiota is dominated by bacteria affiliated with the phyla Firmicutes and Bacteroidetes with smaller numbers of other phyla including Actinobacteria, Fusobacteria, Proteobacteria and Verrucomicrobia also present (Rajilic-Stojanovic et al. 2007; Lozupone et al. 2012). The diversity of the microbiota becomes increasingly complex at deeper phylogenetic levels and the human gut can harbour up to several hundred individual strains that vary substantially between individuals (Greenblum et al. 2015; Yassour et al. 2016). The gut archaea have a low abundance and are comprised of methanogenic and non-methanogenic archaea (Rieu-Lesme et al. 2005; Gill et al. 2006; Nam et al. 2008; Oxley et al. 2010; Ó Cuív et al. 2011a). The methanogenic gut archaea are dominated by strains affiliated with *Methanobrevibacter* and *Methanosphaera* spp. although the diversity of human gut methanogenic archaea may be underestimated (Gill et al. 2006; Nam et al. 2008; Scanlan et al. 2008; Mihajlovski et al. 2008; Ó Cuív et al. 2011a). Despite the substantial inter-subject variability, the healthy gut microbiota has been shown to be comprised of a core microbiota that is widely shared between individuals and that includes some of the most abundant members of the microbiota (Tap et al. 2009; Qin et al. 2010; Jalanka-Tuovinen et al. 2011; Sekelja et al. 2011; Rajilic-Stojanovic et al. 2012; Martínez et al. 2013; Li et al. 2013), and an accessory microbiota that is less widely shared and typically comprised of low abundance taxa that are nonetheless metabolically active (Peris-Bondia et al. 2011).

Both culture-dependent and culture-independent approaches have helped to identify important differences to the structure-function activity between the gut microbiota of healthy individuals and those with chronic gut diseases. For instance, perturbations of signature bacterial species from the core microbiota have been associated with chronic gut diseases (Qin et al. 2010; Nakatsu et al. 2015). In CD, the abundance of specific core bacteria differs from the healthy gut (Kang et al. 2010; Mondot et al. 2011; Prideaux et al. 2013; De Cruz et al. 2015), these differences are coincident with the onset of active disease (De Cruz et al. 2015) and a restoration of their abundance may support remission (Dey et al. 2013; De Cruz et al. 2015). Furthermore, in vivo and in vitro-based experiments have demonstrated that structure-function differences between the healthy and diseased gut microbiota are associated with variations in biological activities that are relevant to gut health. While the immunoregulatory capacity of the microbiota remains largely unknown several representative isolates from the core microbiota produce bioactive factors that can suppress NF-κB activation (Ménard et al. 2004; Sokol et al. 2008; Heuvelin et al. 2009; Khokhlova et al. 2012; Quevrain et al. 2016), modulate the balance and/or activity of regulatory and effector T-cell populations (Atarashi et al. 2011; Atarashi et al. 2013; Qiu et al. 2013; Li et al. 2014b) and restore barrier function (Martin et al. 2015), thus attenuating the host inflammatory response and helping to maintain gut homeostasis. Based on these observations, members of the core gut microbiota have been proposed as “next-generation” probiotics for the treatment of chronic gut diseases (Neef and Sanz 2013). There continues to be a growing appreciation of the NF-κB suppressive capabilities of individual members of the core microbiota and other gut bacteria. However, while some of the NF-κB suppressive factors produced by gut bacteria have been identified, in many instances they remain to be determined (Kelly et al. 2004; Lakhdari et al. 2011; Kaci et al. 2011; Santos Rocha et al. 2012; Kaci et al. 2013).

Advancements in DNA sequencing technologies continue apace and the cost and speed at which sequence data can be produced and annotated continues to dramatically improve (Loman et al. 2012; Land et al. 2015). However, despite the wealth of gut microbiome associated sequence data now available in the public databases, the overwhelming majority of gene products have not been functionally characterised. Indeed, it is estimated that up to 75 % of protein families are assigned to uncharacterised orthologous groups and novel families (Qin et al. 2010; Ellrott et al. 2010; The Human Microbiome Project Consortium 2012). This challenge is further compounded by the fact that DNA sequence data are typically annotated using automated pipelines with little manual curation resulting in the introduction and propagation of annotation errors, and ultimately spurious function prediction (Schnoes et al. 2009; Promponas et al. 2015). It is widely acknowledged that the ability to functionally dissect the gut microbiome has not kept pace with DNA sequencing technology and it is notable that the functions of over one-third of the gene complement of the model organism and best characterised gut bacterium *Escherichia coli* (*E. coli*) K-12 remain undetermined (Hu et al. 2009). This shortcoming is increasingly being addressed (Nichols et al. 2011; Meng et al. 2012; Paradis-Bleau et al. 2014; Rajagopala et al. 2014) supported largely by *E. coli*’s

ease of propagation and its amenability to genetic dissection. In contrast, the vast majority of gut microbes are fastidious anaerobes that are not known to be amenable to genetic dissection and hence their genetic potential remains cryptic. This has led to suggestions that an increased effort must be expended to functionally characterise existing gene sets as this will provide new insights into the microbial factors supporting gut health or driving disease (Roberts 2004; Galperin and Koonin 2010; Anton et al. 2014; Joice et al. 2014).

Based on these collective observations, we contend that new advances in microbial isolation coupled with parallel developments in functional characterisation and dissection approaches will provide the best opportunities to develop streamlined strategies to identify NF-κB suppressive and other types of bioactives produced by gut microbes. In particular, considering the complexity of the gut microbiota these strategies must be cost-effective, scalable and amenable to automation, and the following sections provide an overview of each of these aspects.

4 Bringing the Microbiome to Life: Culture-Dependent Analysis of the Gut Microbiota

It is indisputable that the development of new approaches to isolate and propagate fastidious gut microbes has not kept pace with those of culture-independent approaches. In particular, microbial culturing is widely perceived to be a time and labour intensive process and much information can now be provided without having to isolate individual microbes (Table 1). Nonetheless, both approaches are complementary and in some instances culture-dependent approaches provide the best opportunity to dissect the functional capacity of the microbiota. For instance, culture-dependent approaches allow specific axenic isolates to be directly linked with NF-κB suppressive capabilities and, moreover, they provide a valuable resource to test experimental hypotheses (e.g. Koch's postulates).

The vast majority of gut microbes are strict anaerobes and require an environment with a low redox potential in which to grow. The history of isolating and cultivating fastidious gut microbes extends from the late-nineteenth century (Rajilić-Stojanović and de Vos 2014). Many of the techniques used in contemporary laboratories were developed and adapted by Hungate (1969) and colleagues (Eller et al. 1971; Macy et al. 1972; Bryant 1972; Balch et al. 1979) and have been used to isolate and propagate facultative anaerobic microbes (e.g. *E. coli*), microaerophilic microbes (e.g. *Lactobacillus* spp.), aerotolerant anaerobic microbes (e.g. *Bacteroides* spp.) and obligate anaerobic microbes (e.g. *Clostridium* spp.) (Virginia Polytechnic Institute and State University Anaerobe Laboratory 1975; Dowell et al. 1981). These techniques can be readily established and remain relevant today although the ability to isolate and propagate fastidious anaerobic microbes has been advanced by the development of anaerobic chambers for microbiological culturing

Table 1 Culture-dependent analysis of the gut microbiota—opportunities and challenges

Culture-dependent approach	Advantage	Disadvantage
Microbial isolation	• Enables experimental hypotheses to be evaluated (e.g. Koch's postulates) • Provides a resource for further experimentation	• Time-consuming and labour intensive
Genomic characterisation	• Enables the functional potential of an isolate to be assessed • The 16S rRNA gene sequence can be associated with specific functional genes • Intraspecies genetic variability can be assessed where multiple isolates are available	• Genomic data can be provided by culture-independent means • Genome annotations can result in a high number of genes of unknown function
Functional characterisation	• Facilitates phenotypic profiling (e.g. metabolic, physiological characteristics) • Functional attributes can be linked with the 16S rRNA gene and/or genomic content	• Limited ability to genetically dissect microbial isolates

which further reduce the risk of inadvertent oxygen contamination and allow many standard techniques (e.g. spread plates, streak plates) to be used to isolate target microbes.

The distinct ecological niches present along the human gut can be challenging to replicate in a laboratory environment particularly as the nutritional requirements of many target microbes are unknown. The use of "habitat simulating" media has been widely used to circumvent this challenge and typically includes sterile aqueous extracts of faecal or rumen digesta, in addition to sources of amino acids, carbohydrates and other nutrients (Eller et al. 1971; Barcenilla et al. 2000; McSweeney et al. 2005; Lagier et al. 2012), leading to the isolation of phylogenetically diverse gut microbes including bacteria that have specific host dependencies such as *Akkermansia muciniphila* (Derrien et al. 2004) and the obligate symbiont segmented filamentous bacterium (Schnupf et al. 2015). Although habitat simulating media often support the growth of subdominant populations, their enrichment and isolation is often complicated because of rapid overgrowth by fast growing, numerically abundant microbes. More selective media have been developed for the isolation of specific gut taxa including *Bacteroides* spp. (Livingston et al. 1978), *Bifidobacterium* spp. (Ferraris et al. 2010) and *Enterococcus* spp. (Isenberg et al. 1970) by identifying specific nutritional dependencies, and promoters/inhibitors of growth (e.g. antibiotics, bile salts, sodium azide). Alternatively, subdominant populations can be enriched by selecting for a specific phenotype (e.g. spore formation) and this has enabled taxonomically novel microbes to be directly recovered on nutrient-rich habitat simulating media (Atarashi et al. 2013; Browne et al. 2016).

Accordingly, based on these, our own (Ó Cuív et al. 2011b, 2015) and other (Rettedal et al. 2014; Ma et al. 2014) observations, many "uncultured" microbes grow reproducibly well in vitro when isolated as axenic cultures. Thus, many more novel gut microbes could be recovered if the practical considerations involved with screening large numbers of microbial isolates under strict anaerobic conditions could be overcome.

To improve the throughput of microbial isolation, Stevenson et al. (2004) developed an approach called "Plate wash PCR" to recover axenic isolates of previously uncultured bacteria from agricultural soil and the guts of wood-feeding termites. Briefly, an inoculum is plated in duplicate on solid medium and following growth the colonies are re-suspended *en masse* from one of the replicate plates and the sample extracted DNA is screened using specific PCR primers. By this approach, a broad range of growth parameters can be rapidly screened to determine conditions supporting the growth of target taxa. Once identified, colonies from the matching replica plate are grown in multiwell plates and screened with specific primers to identify the target isolate. Plate wash PCR was successfully used to isolate a Lachnospiraceae affiliated bacterium that inhibits colonisation of the murine gut by *Clostridium difficile* VPI 10463 (Reeves et al. 2012), and it has been adapted to support the isolation of human gut bacteria affiliated with the HMP's most-wanted taxa using a microfluidic platform (Ma et al. 2014). Goodman et al. (2011) described a similar approach but determined the diversity of microbial isolates recovered on the replica culture plate by 16S rRNA-based microbial profiling. In addition, to further improve the throughput of the isolation process, a most probable number (MPN) approach was used to create, in 384 well plates, personalised archived culture collections of axenic isolates directly from faecal samples without picking individual colonies. The MPN approach is based on extinction culturing, whereby diluting microbial cells so that ≤1 culturable cell is used as an inoculum supports the production of axenic cultures (Button et al. 1993). This favours the isolation of the most abundant rather than the fastest growing or most culturable microbes and the MPN method has also been used to produce axenic cultures of previously uncultured rumen bacteria (Kenters et al. 2011). Rettedal et al. (2014) also used 16S rRNA profiling to profile gut bacteria recovered on a broad range of solid growth media. Then, by a process termed cultivation-based multiplex phenotyping, they combined growth on solid medium with antibiotic selection and 16S rRNA profiling to selectively target and recover target bacteria including members of the HMP's most-wanted taxa (Fodor et al. 2012). Recently, Browne et al. (2016) applied a similar approach to isolate spore forming bacteria from the human gut.

Separately, Raoult and colleagues (Lagier et al. 2012) coined the term "culturomics" and demonstrated that increasing the throughput of microbial isolation greatly extended the number of cultured isolates from the human gut. By this approach, 32,500 colonies representing 340 bacterial species and including 31 previously unidentified species were obtained using 212 culture conditions and

three human faecal samples. Culturomics was also shown to be superior to culture-independent approaches in its ability to detect bacteria that were below the detection threshold of 16S rRNA profiling approaches (Lagier et al. 2012; Dubourg et al. 2013). It is notable that these studies were performed using methodologies that could be readily established in a standard microbiological laboratory (e.g. the use of anaerobic jars to produce microaerobic or anaerobic conditions). It is likely that the use of an anaerobic chamber could have further increased the recovery of fastidious obligate anaerobes, however, manipulating large numbers of isolates in multiwell plates and a confined environment is challenging. Interestingly, Raoult and colleagues (La Scola et al. 2014; Dione et al. 2015) discovered that the addition of antioxidants to the growth medium permitted the growth of strict anaerobic bacteria under atmospheric conditions. This observation could revolutionise our ability to isolate fastidious gut bacteria particularly if it can be verified that their growth and metabolic activity is similar under aerobic and anaerobic conditions, and it complements advancements in automated colony picking robotic platforms that are capable of operating in an anaerobic chamber.

The wealth of sequence data now available for gut microbes has also helped to direct the isolation of gut microbes. For instance, Pope et al. (2011) described the successful isolation of an uncultured bacterium affiliated with the Succinivibrionaceae from foregut digesta samples collected from Tammar wallabies. Here, metagenomic data were used to partially reconstruct and model the bacterium's metabolism and physiological features, and then tailored culture conditions were developed to direct the axenic cultivation of the bacterium by a process termed metagenome directed isolation. Bomar et al. (2011) similarly used metatranscriptomic data to direct the isolation of an abundant *Rikenella*-like bacterium from the gut of a medicinal leech. Recently, Oberhardt et al. (2015) developed a web-based platform that uses a database of microbe-medium combinations to predict media for microbes based on their 16S rRNA sequence. The exploitation of sequence data to help bring the microbiome to life is a vital development as much of these data languishes mostly unused in online databases.

The throughput of microbe identification has also been expedited by developments in matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF-MS) based analyses. The early classification of microbes was primarily based on physiological and morphological characteristics (Virginia Polytechnic Institute and State University Anaerobe Laboratory 1975), however, the development of 16S rRNA-based phylogenetics allowed the genetic relatedness of these isolates to be determined. The gold standard of 16S rRNA-based phylogeny taxonomy is based on the production of near full-length gene sequences that are used to infer relatedness (Kim et al. 2011; Franzén et al.2015). The identification of microbial isolates is typically achieved by low-throughput Sanger sequencing of the 16S rRNA gene, however, due to its low rate of divergence, it is widely recognised that the 16S rRNA gene is limited in its ability to provide

phylogenetic resolution of the microbiota at lower phylogenetic levels. Other genes can also be used as phylogenetic markers [e.g. *gyrB, rpoB* (Sunagawa et al. 2013; Fish et al. 2013)] but these are less well established and not routinely used. Instead, MALDI-TOF-MS-based analyses now provide an alternative and in many respects a superior means to identify microbial isolates. The ability to identify specific isolates is typically based on the mass patterns of ribosomal or other abundant housekeeping proteins and is determined by reference to a database of spectra produced using representative isolates. This approach is particularly valuable in providing a cost-effective rapid and sensitive assessment of intraspecies variability without any prior knowledge of the strains being tested, although the ability to distinguish between very closely related strains can be challenging (Sandrin et al. 2013). We anticipate that MALDI-TOF-MS-based identification of microbial isolates will increasingly supplant 16S rRNA gene-based identification as the reference databases become more comprehensive and the technology more robust and affordable.

In summation, meaningful progress has been made to increase the efficiency and throughput of microbial isolation and these have increased the diversity of gut microbes that are available in international biorepositories (e.g. Biodefense and Emerging Infections Research Resources Repository (BEI Resources), Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) GmbH) or that are held in private laboratory culture collections. Continued advances in automated microbial isolation and identification will further expedite these efforts and support a more mechanistic dissection of the gut microbiota in the maintenance of gut homeostasis and the prevention of chronic gut diseases, although important challenges remain. Much of our understanding of the diversity and functional capability of the gut microbiota is based on analyses of faecal associated microbiota, which can be collected in a non-invasive manner and up to 54 % of the faecal mass is comprised of microbial biomass (Stephen and Cummings 1980; Rose et al. 2015) thus providing copious material for experimental interrogation. However, it has been long recognised that the faecal and mucosa associated microbiota differ (Zoetendal et al. 2002; Ott et al. 2004; Lepage et al. 2005; Eckburg et al. 2005) and it is now also recognised that the mucosa associated microbiota is also characterised by a distinct biogeography (Obata et al. 2010; Aguirre de Carcer et al. 2011; Pedron et al. 2012; Sonnenberg et al. 2012; Zhang et al. 2014) that likely reflects different ecological niches driven by variations in nutrient availability, oxygen tension, pH and immune activation (reviewed by Donaldson et al. 2015). The aetiology of several chronic gut diseases is characterised by site-specific differences with CD predominantly affecting the ileum and proximal colon, and UC and CRC predominantly affecting the distal colon. Thus, the spatial distribution of the gut microbiota may have implications for our understanding of host–microbe interactions and their relationship to health and disease, and future efforts should seek to preferentially culture gut microbes from sites relevant to disease.

5 Dissecting the Functional Potential of the Gut Microbiota: Advances in In Vitro Approaches to Identify NF-κB Suppressive Gut Microbes

In 2014 it was reported that over 1000 cultured gut microbial species had been described in the scientific literature and this number continues to increase rapidly due to the new advances in microbial cultivation techniques (Rajilić-Stojanović and de Vos 2014). The NF-κB suppressive activities of the vast majority of existing isolates have not been assessed but taken together with the increasing rate of microbial isolation there is a need for improved functional screening strategies to effectively identify these strains. Strategies to identify immunomodulatory microbes should address three key criteria. First, the assays should be biologically relevant, sensitive and specific, facilitating the identification of virulent or cytotoxic microbes at an early point in the screening process. Second, the assays should allow the extent of immunomodulatory activity to be quantified and the host pathways affected to be readily identified and dissected to determine the target of the bioactive. Third, the assays should be cost-effective and robust, easy to perform and amenable to scaling to an automated high-throughput format. Historically, the use of well-established cell lines to identify NF-κB suppressive microbial isolates broadly fulfils these criteria.

The NF-κB pathway has been extensively characterised and transcription can be activated via two alternate pathways, called the canonical and non-canonical pathways. These pathways can be activated either independently (e.g. TNFα/IL-1β activates the canonical pathway, B-cell activating factor activates the non-canonical pathway) or in tandem (e.g. CD40L/Lipopolysaccharide activate both pathways). Many gut bacteria are considered to be pathobionts—symbionts that are capable of acting as pathogens under certain environmental conditions—and are capable of stimulating an immune response. Consequently, the ability of gut bacteria to supress NF-κB activation is often initially assessed using peripheral blood mononuclear cells (PBMC) as several studies have reported that peripheral blood cells predict the in vivo immunomodulatory potential of different bacteria (Foligne et al. 2007; Sokol et al. 2008). Alternatively, NF-κB suppressive capability can be assessed using peripheral blood derived cell lines (e.g. human monocyte-like THP-1 cell line, murine RAW macrophage cell line) stimulated with a specific NF-κB pathway agonist. These cell lines have rapid and reproducible growth characteristics and they express a broad range of Toll-like receptors (TLR) [e.g. THP-1 cells expresses all TLRs including the surface TLRs (i.e. TLR1/2, TLR2, TLR4, TLR5 and TLR6/2)]. These characteristics can be used to identify microbes that modulate NF-κB activity, or that express virulence or cytotoxic factors, in a high-throughput manner.

Despite the usefulness of immune cell lines the ability of gut microbes to suppress NF-κB activity is typically assessed using intestinal epithelial cell culture lines. Numerous epithelial cell lines are widely used by researchers, however, the HT-29, Caco-2 and T84 cell lines and their derivatives are amongst the most widely used to assess immunomodulatory activity. Gut epithelial cells are constantly

exposed to microbial factors and are thus broadly unresponsive to stimulation by the healthy gut microbiota. Consistent with this, HT-29, Caco-2 and T84 cells express a subset of functional TLRs (e.g. TLR2, TLR3, TLR4 and TLR5) (Cario et al. 2000; Melmed et al. 2003; Lakhdari et al. 2010), and the cell surface receptors are predominantly expressed basolaterally. Several gut epithelial cell lines carrying NF-κB reporter genes including secreted embryonic alkaline phosphatase (Lakhdari et al. 2010), luciferase (Kaci et al. 2011) and green fluorescent protein (Mastropietro et al. 2015) have been described. Using reporter cell lines, Blottière and colleagues at the Institute National de la Recherche Agronomique (France) have led efforts to identify gut bacteria and metagenomic clones capable of modulating NF-κB expression using high-throughput screening approaches (e.g. Lakhdari et al. 2010, 2011; Santos Rocha et al. 2012). In the most exhaustive study to date, Lakhdari et al. (2011) used a series of immune and intestinal epithelial reporter cells to determine the NF-κB suppressive capabilities of 49 strains of well-described gut bacteria. Interestingly, thirteen NF-κB suppressive strains were identified although their activity was cell line-dependent (one isolate suppressed NF-κB activation in HT-29 cells whereas the other twelve isolates suppressed activation in Caco-2 cells) suggesting that the responsiveness may be affected by the genotype of the cell lines.

While cancer derived intestinal epithelial cell lines may provide biological insights relevant to CRC, a major criticism is that they typically lack the genetic susceptibilities relevant to IBD and obesity. For instance, IBD is associated with over 160 genetic susceptibility loci (McGovern et al. 2010; Jostins et al. 2012) and is characterised by disease heterogeneity with differences in location, severity and extent that may change over time. Host genetics can also influence therapeutic responsiveness and CD carriers of the *nod2* mutation are more likely to be refractory to glucocorticosteroid treatment although they can be effectively treated by TNFα biologics (Niess et al. 2012). While primary cells can be used as an alternative to immortal cell lines, they have a finite life span which typically precludes long-term study. Also, the diversity in cell lineages found in the gut epithelium (e.g. epithelial, goblet, enteroendocrine, Paneth cells) is not reflected in homogenous primary cells or immortal cell lines. This issue has been addressed by recent advances in gut epithelial culture methods from human and laboratory animals which have resulted in the generation of "mini-guts" from intestinal samples containing adult, human embryonic or inducible stem cells that retain the phenotype of the tissue of origin. Mini-guts produced from embryonic or induced pluripotent stem cells are termed induced intestinal organoids while those produced from adult stem cells are termed enteroids (small intestinal) or colonoids (colonic) (Stelzner et al. 2012). Induced intestinal organoid cultures take longer to establish and retain a foetal phenotype and consequently enteroids/colonoids are considered to be a more representative model for human disease. Enteroids/colonoids are derived from intestinal samples containing adult stem cells following cultivation in the presence of growth factors and ultimately form three-dimensional cultures containing differentiated epithelial cells (Sato and Clevers 2013; VanDussen et al. 2015; Mahe et al. 2015). The cultured cells can be grown as spheroids with the apical membrane

facing a single internal lumen compartment or, alternatively, they can be grown as monolayers in a transwell system. These cell cultures can also be stably maintained through repeated rounds of propagation and freezing thus recapitulating the main elements of cancer cell culture lines and providing a superior in vitro model to assess NF-κB suppressive capabilities. For instance, the impact of NF-κB suppressive bioactives on individual epithelial cell subtypes could be assessed by fluorescence-activated cell sorting using antibodies targeting the NF-κB complex and lineage specific markers. Enteroids/colonoids can be generated from animals carrying reporter genes or, alternatively, Schwank et al. (2013) reported that the CRISPR/Cas9 system could be used to edit organoid genome sequences. Along with new developments in CRISPR/Cas9-based large fragment deletions and insertions (Wang et al. 2015; Zhang et al. 2015a), this may provide new opportunities to produce patient-specific reporter cell lines. Together, these developments offer new opportunities to identify and dissect disease-specific pathways as well as assess their responsiveness to different therapeutics.

6 Metabolomic-Based Strategies to Identify NF-κB Suppressive Bioactives

The healthy gut microbiota produces a diverse array of factors including proteins (Rieu et al. 2014), peptides (Kaci et al. 2011; Quevrain et al. 2016), polysaccharide-peptidoglycans (Matsumoto et al. 2009) and secondary metabolites (Bansal et al. 2010; Gonzalez-Sarrias et al. 2010; Lim et al. 2015; Lee et al. 2015) that are capable of suppressing NF-κB, revealing this capability is characterised by a high degree of functional redundancy. Metabolomic approaches have played a central role in the identification of these factors although they have been challenged by the sheer diversity of metabolites produced by gut microbes. In addition, many of these metabolites are produced at low concentrations and include novel metabolites that are not represented in existing databases, further hindering identification efforts. Nonetheless, effective bioassay guided fractionation strategies that typically involve successive fractionation coupled with functional assays to track the fraction(s) retaining suppressive activity can be devised to identify NF-κB suppressive bioactive factors (Fig. 4).

Microbes in the healthy gut environment are physically separated from epithelial cells by a mucus layer and bioactive factors must be capable of traversing this barrier to reach their cellular target. Many NF-κB bioactives are secreted into the extracellular milieu and the first stage of the screening process involves the preparation of a cell-free supernatant fraction of spent medium that can be assessed for suppressive activity. The supernatant fraction of most fastidious gut microbes is likely to contain SCFA which are produced by anaerobes as an end product of fermentation, and are amongst the most abundant metabolites produced. Acetate, propionate and butyrate are produced at the highest concentrations with other SCFA

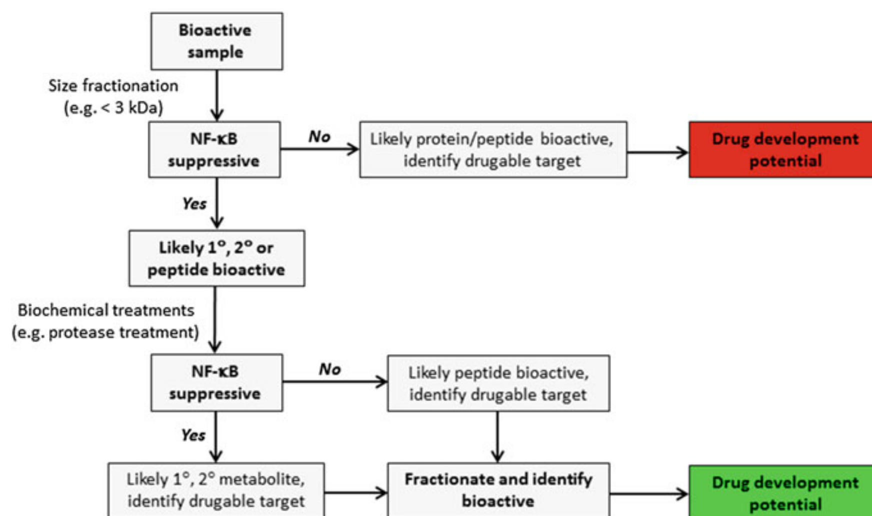

Fig. 4 Bioassay guided fractionation strategy to enrich and purify NF-κB suppressive bioactives. The bioactive fractions are successively fractionated and the NF-κB suppressive activity in the fractions is assessed after each treatment. Fractions enriched in primary and secondary metabolites (1° and 2° respectively), and peptides are typically produced by size fractionation. Fractions >3 kDa are typically not considered to be suitable for drug development but may help to identify new drugable targets. Metabolites and peptide bioactives can be further fractionated using biochemical treatments (e.g. protease, denaturant, thermal treatment). Peptide bioactives can be further fractionated and used to identify new drugable targets or as lead molecules for drug development. The 1° and 2° metabolites can be further fractionated (e.g. solid phase extractions, HPLC based fractionation) to identify the bioactive factor. Secondary metabolites can also be used to identify new drugable targets or as lead molecules for drug development

produced at lower concentrations. SCFA bind to G-protein coupled receptors including GPR41 and GPR43 (acetate, butyrate, propionate), GPR109A (butyrate) and OLFR78 (lactate, propionate). These receptors are found on a range of cells including immune and epithelial cells (Karaki et al. 2008; Pluznick et al. 2013; Tazoe et al. 2009; Thangaraju et al. 2009; Vinolo et al. 2011). SCFA are amongst the most bioactive metabolites produced by the microbiota and they affect a variety of cellular process including NF-κB activity (Inan et al. 2000; Tedelind et al. 2007; Lakhdari et al. 2011). To identify culture supernatants possessing non-SCFA suppressors of NF-κB activity, the SCFA concentrations in the spent culture supernatant are typically first determined and the ability of similar concentrations of SCFA to suppress NF-κB activation is then assessed (Lakhdari et al. 2011).

A variety of fractionation strategies have been described to differentiate between bioactives with specific biochemical characteristics. For instance, small peptides and secondary metabolites are considered to be more conducive to drug development as they are more likely to possess desirable characteristics and be less costly to produce (Uhlig et al. 2014; Fosgerau and Hoffmann 2015; Harvey et al. 2015). These bioactives can often be readily separated from larger macromolecules on the

basis of size and a simple 3 kDa molecular weight cut-off filter allows peptides up to 27 amino acids long (assuming an average amino acid size of 110 Da) to be easily separated from larger molecules. Bioactive secondary metabolites and peptides can subsequently be distinguished using routine (e.g. protease, denaturing, thermal) treatments and the sample can subsequently be further fractionated (e.g. solid phase extractions, HPLC based fractionation) to further reduce the complexity of the samples. This approach has been used effectively to identify NF-κB suppressive peptides produced by *Faecalibacterium prausnitzii* (Quevrain et al. 2016). However, the identification of secondary metabolites can be more challenging and it can be necessary to fractionate large sample volumes to identify the metabolites of interest, although this process can be expedited if isogenic mutant or non-suppressive strains can be processed in parallel (Donia et al. 2014). Once sufficiently enriched and concentrated the bioactives can be identified using specialist metabolomic methodologies and equipment.

7 Genetic-Based Strategies to Identify NF-κB Suppressive Bioactives

Much of our understanding of the functional capacity of the microbial world has been provided by the genetic dissection of clinically and agriculturally relevant bacteria that are only distantly related to the microbes that typically inhabit the human gut. The ability to conclusively link genes and function is a central challenge in elucidating the functional potential of the microbiota. However, with limited exceptions (Rey et al. 2013; Ichimura et al. 2013), few molecular tools have been described for the characterisation of gut microbes. The vast majority of the currently available microbial isolates are not known to be amenable to genetic transformation although (meta)genomics has revealed evidence of extensive lateral gene transfer within the gut microbiome. To address this challenge, we recently developed an innovative approach termed metaparental mating that expedites the directed isolation of genetically tractable gut bacteria from mixed microbial communities (Ó Cuív et al. 2015). The metaparental mating approach is based on the well-established biparental mating approach (Simon et al. 1983; Simon et al. 1986) and uses RP4 (RK2)-mediated bacterial conjugation and a broad host range mobilisable shuttle vector. Metaparental mating has several advantages over alternative natural (i.e. transduction, transformation) or contrived (e.g. electroporation, sonoporation) genetic transformation approaches. First, RP4-based conjugation is very promiscuous and has been shown to mediate the transfer of DNA to a diverse range of bacteria (Whitehead and Hespell 1990; Picardeau 2008; Tolonen et al. 2009; Dominguez and O'Sullivan 2013) and also to archaea (Dodsworth et al. 2010), fungi (Nishikawa et al. 1990) and animal cells (Waters 2001). Second, the metaparental mating can be performed under anaerobic conditions and stably transformed recipients can be recovered by selection of a vector encoded marker. In

addition, as the antibiotic resistance phenotype of the recipients may not be known, the laboratory *E. coli* ST18 donor strain can be efficiently counter selected without antibiotics by nutritional auxotrophy (i.e. the omission of δ-aminolevulinic acid from the selection medium). Third, the RP4-based conjugation can be readily scaled and automated (Clarke et al. 2005) to increase the throughput of the metaparental mating mediated isolation process.

We used the metaparental mating approach to specifically target bacteria affiliated with the Firmicutes as these comprise the majority of the human gut microbial core although they are underrepresented in microbial culture collections. Furthermore, few of these bacteria, and in particular those affiliated with the Clostridia, have been genetically characterised although many strains are capable of modulating the host immune response (Sokol et al. 2008; Ivanov et al. 2009; Atarashi et al. 2011, 2013; Li et al. 2014b; Quevrain et al. 2016). In support of this effort we developed a series of modular vectors termed pEHR5 that can be conjugated from an *E. coli* host to a pool of potential recipients. As the efficiency of conjugation can be affected by the size of the vectors, their modular architecture helps minimise their overall size. In addition, it allows individual modules to be easily exchanged ensuring that the base vectors are flexible and can be readily re-purposed. Similar modular vectors have been used in a broad range of non-*E. coli* hosts to support protein expression and the construction of fluorescently labelled bacterial strains (Herrero et al. 1990; Charpentier et al. 2004; Fodor et al. 2004; Heap et al. 2009; Dammeyer et al. 2013; Wright et al. 2015). By this approach, we recovered a broad suite of axenic fastidious gut bacteria affiliated with the Firmicutes that were stably transformed with pEHR5-based vectors. In addition, we demonstrated that the metaparental mating approach and the pEHR vectors can be used for heterologous protein expression by constructing fluorescently labelled gut bacteria (Ó Cuív et al. 2015).

The pEHR5 vector system is freely available to the research community without the need for a restrictive material transfer agreement and it offers a basis for the development of a uniform and streamlined set of molecular tools for the isolation and functional genetic characterisation of fastidious microbes. Nonetheless, the metaparental mating approach can plausibly be applied with any RP4 mobilisable vector bearing an appropriate resistance marker(s) and origin(s) of replication, thus allowing genetically tractable bacteria to be recovered from complex microbial communities. We confirmed this hypothesis by using the narrow host range vector pJQ200sk(+) (Quandt and Hynes 1993) to demonstrate that *E. coli* transconjugants bearing pJQ200sk(+) could be selectively recovered from an anaerobic enrichment from human faeces by metaparental mating (Fig. 5). In addition, we used the vector pGusA*mob* [(Girbal et al. 2003), pGusA modified to carry an *oriT*] to target the recovery of Firmicutes affiliated bacteria and isolated transconjugants affiliated with *Blautia hathewayi*, *Streptococcus pleomorphus* and *Anaerococcus vaginalis* on M10-based medium. We have now also demonstrated that the pEHR vectors can be cured using standard molecular techniques to yield naïve strains (Pottenger and Ó Cuív, Unpublished data).

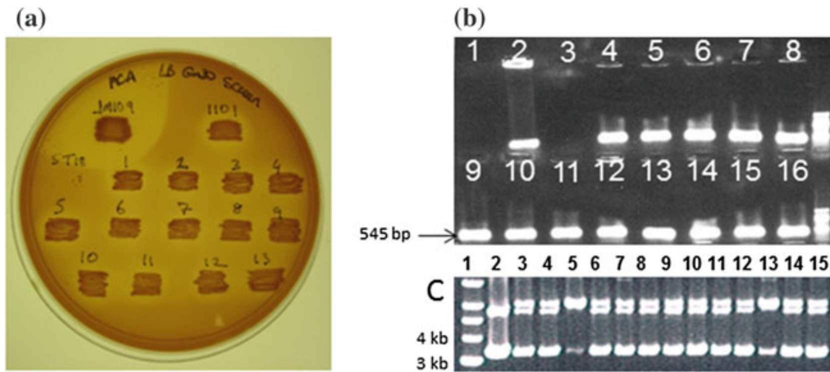

Fig. 5 **a** Identification of *E. coli* transconjugants carrying pJQ200sk(+) (5.4 kb) recovered by metaparental mating. The *E. coli* transconjugants were recovered on LB medium and replica plated onto MacConkey Agar supplemented with 30 μg.ml^{-1} gentamicin sulphate to differentiate between the different laboratory and commensal strains. The laboratory strain *E. coli* JM109 carrying pJQ200sk(+) was characterised by a clear zone around the patched culture consistent with its inability to ferment lactose. In contrast, the recent human gut isolate *E. coli* PC1101 was capable of fermenting lactose and the 13 transconjugants recovered exhibited a similar phenotype. As expected, *E. coli* ST18 did not grow on MacConkey agar due to its nutritional requirement for δ-aminolevulinic acid. **b** The identity of the transconjugants was confirmed by PCR using *E. coli* specific primers (Sabat et al. 2000). Successful confirmation is indicated by a 545 bp product and, as expected, *E. coli* ST18 carrying pJQ200sk(+) (*Lane 2*) and all of the transconjugants (*Lanes 4–16*) produced a product of the correct size. In contrast, no products were observed for PCR lacking DNA template (*Lane 1*) or containing *Campylobacter jejuni* DNA template (*Lane 3*). **c** The presence and integrity of the plasmid vector was assessed by agarose gel electrophoresis. Plasmid vector prepared from each of the 13 transconjugants (*Lanes 3–15*) exhibited similar mobility to plasmid DNA prepared from *E. coli* JM109 (*Lane 2*) confirming that they were stably transformed

The metaparental mating approach and the pEHR vector series are significant developments for the genetic dissection of the gut microbiota by forward and reverse genetic approaches. For instance, it is now known that many gut bacteria carry putative biosynthetic gene clusters for secondary metabolites (Letzel et al. 2013; Donia et al. 2014; Cimermancic et al. 2014; Donia and Fischbach 2015; Hadjithomas et al. 2015), some of which may encode for NF-κB suppressive bioactives. The specific gene clusters underpinning the production of NF-κB suppressive bioactives could potentially be identified by comparative genomics of suppressive and non-suppressive strains, however, reverse genetic approaches can now also plausibly be applied to specifically disrupt target genes and conclusively confirm their role in the production of specific bioactives (Donia et al. 2014). Consistent with this, mutagenesis strategies based on homologous recombination (Al-Hinai et al. 2012; Heap et al. 2012; Faulds-Pain and Wren 2013) and the Ll. *ltrB* group II intron (Chen et al. 2005; Heap et al. 2007; Tolonen et al. 2009) have been described for a diverse range bacteria affiliated with the Firmicutes. While the specific factors underpinning NF-κB suppressive activity have been identified in some cases (e.g. Rieu et al. 2014; Quevrain et al. 2016) in most instances, they

remain cryptic and forward genetic approaches including transposon mutagenesis (Liu et al. 2013; Ichimura et al. 2013; Zhang et al. 2015b) and in vivo transposomics (Vidal et al. 2009; Veeranagouda et al. 2012) have been successfully developed for fastidious bacteria. While forward genetic approaches can be applied with fastidious gut microbes they are constrained by the number of mutant clones that have to be screened to achieve good coverage of the genome. For example, assuming a genome size of 4 Mb and an average gene size of 753 bp (Li et al. 2014a), over 12,000 mutants would have to be screened to achieve 90 % coverage of the genome (Clarke and Carbon 1976).

An alternative approach involves the construction of medium/large insert gene libraries (e.g. plasmid, cosmid, fosmid, BAC libraries) that are screened for NF-κB suppressive activity in a suitable microbial host. Using this approach, approximately 230 clones would have to be screened to achieve 90 % coverage of the genome assuming a genome size of 4 Mb and an average insert size of 40 kb. Gene libraries generally assume that all of the genetic elements supporting the immunomodulatory activity are linked, expressed and functional in the microbial host. *E. coli* has relaxed requirements for promoter recognition and this approach has been exploited to identify metagenomic fosmids derived from human gut microbiota that are capable of suppressing/activating NF-κB (Lakhdari et al. 2010; Cohen et al. 2015). Nonetheless, as few as 40 % of heterologous genes are expressed in *E. coli* (Aakvik et al. 2011) and new cloning vectors have been developed that have extended the host range of large insert vectors (e.g. Aakvik et al. 2009; Kakirde et al. 2011). Currently, the replication range of these vectors is mostly limited to proteobacteria and examples of vectors for more distantly related phyla, especially the Firmicutes, are limited (Hain et al. 2008; Liu et al. 2009).

8 Bioprospecting for NF-κB Suppressive Bioactives: *Faecalibacterium prausnitzii* as a Case Study

The butyrate producing gut bacterium, *F. prausnitzii*, comprises part of the core microbiota in healthy adult humans and is ubiquitously found in the gut of mammals and insects (Foglesong et al. 1984; Bjerrum et al. 2006; Castillo et al. 2007; Qin et al. 2010; Nava and Stappenbeck 2011; Miquel et al. 2013; Oikonomou et al. 2013). This suggests that *F. prausnitzii* plays a critical role in host metabolism and physiology and consequently it is widely considered to be a model gut bacterium with relevance to health and disease. In that context, much progress has been made in identifying the true metabolic potential of *F. prasunitzii* and its contribution to health and well being (Sokol et al. 2008; Quevrain et al. 2016). Swidsinski et al. (2008) first reported a reduced population of *F. prausnitzii* in CD subjects and Sokol et al. (2008) subsequently demonstrated a low abundance of *F. prausnitzii* in ileal biopsies from CD subjects at the time of surgery was associated with recurrence six months postoperatively, and that the abundance at six months was

consistently lower in subjects with recurrent disease in comparison to those in remission. In support of this observation a longitudinal study with an Australian CD cohort examined the mucosa associated microbial communities in subjects undergoing ileal resection and determined that patients who were in remission 6 months postoperatively had a higher population of *F. prausnitzii* and other members of the Firmicutes at surgery (De Cruz et al. 2015). Notably, changes in the abundance of *F. prausnitzii* in CD subjects have been reported in different ethnic populations (Prideaux et al. 2013) and perturbations have also been reported in other inflammatory and metabolic disorders like ulcerative colitis, coeliac disease, juvenile spondyloarthritis and type 2 diabetes (Sokol et al. 2009; De Palma et al. 2010; Remely et al. 2014; Gill et al. 2015) suggesting that it plays an important role in maintaining gut homeostasis.

The immunomodulatory potential of *F. prausnitzii* A2-165 was first identified by Sokol et al. (2008) who demonstrated that the bacterium exerted an anti-inflammatory effect in PBMCs by inducing IL-10 and suppressing IL-12 and INFγ secretion. They also showed that spent culture supernatant but not sterile medium, UV-killed *F. prausnitzii* or cellular fractions were able to block the activation of NF-κB and reduce IL-8 secretion in Caco-2 cells. Butyrate exerts physiological and anti-inflammatory effects in the gut (Canani et al. 2011; Ploger et al. 2012), however, the presence of butyrate in the spent culture supernatants did not suppress NF-κB activation in Caco-2 cells suggesting that other bioactive factors were responsible for the anti-inflammatory effects (Sokol et al. 2008). Critically, *F. prausnitzii* whole cells as well as filter-sterilised culture supernatant could attenuate the overall severity of trinitrobenzene sulphonic acid induced colitis in BALB/c mice by both a gut-dependent and gut-independent route. Separate studies have also supported these observations and revealed that *F. prausnitzii* and/or its supernatant can induce T_{reg} proliferation (Qiu et al. 2013; Martin et al. 2014), modulate T-cell responses (Rossi et al. 2016) and improve gut barrier function (Carlsson et al. 2013; Martin et al. 2015; Laval et al. 2015), thus also contributing to the suppression of inflammation. Together, these observations indicated that the anti-inflammatory activity could be largely attributed to a secreted bioactive.

In addition to butyrate, it is now known that *F. prausnitzii* produces a range of distinct immunomodulatory bioactives relevant to host health including peptides and secondary metabolites. Using a peptidomic approach, Quevrain et al. (2016) identified 7 peptides derived from a 15 kDa protein termed MAM (Microbial Anti-inflammatory Molecule) that is phylogenetically narrowly distributed. Intracellular expression of the MAM protein in human epithelial cells suppressed NF-κB activation in a specific and dose-dependent manner possibly by affecting IκκB function. Furthermore, *Lactococcus lactis* expressing MAM was capable of ameliorating dinitrobenzene sulphonic acid induced colitis in BALB/c mice. The mechanism of action of the MAM protein remains to be determined including whether its NF-κB suppressive activity is mediated by the intact protein and/or its derived peptides, and how these are delivered to the cell. In addition to MAM, *F. prausnitzii* produces a range of (precursor) anti-inflammatory secondary

metabolites. Using a gnotobiotic mouse model, Miquel et al. (2015) revealed that the protective effect of *F. prausnitzii* following colonisation was associated with the presence of salicylic acid and shikimic acid in gut and serum metabolomic profiles. Salicylic acid is a precursor of 5-aminosalycilic acid and is capable of suppressing IL-8 secretion from TNFα stimulated HT-29 cells. In contrast, shikimic acid is not capable of suppressing IL-8 secretion from TNFα stimulated HT-29 cells, however, this molecule is a precursor of anti-inflammatory aromatic compounds including salicylic acid and 3,4-oxo-eisopropylideneshikimic acid (Xing et al. 2013).

These observations underline the role played by *F. prausnitzii* in the maintenance of gut homeostasis and reveal the evolution of a variety of strategies to affect specific aspects of gut function and the immune response. Consistent with this, *F. prausnitzii* has been suggested as a candidate next generation probiotic for the treatment of gut inflammatory diseases (Sokol et al. 2008; Neef and Sanz 2013). Critically, the characterisation of *F. prausnitzii* has provided a template by which the contribution of other microbes to gut health can be examined. It should be noted that although *F. prausnitzii* A2-165 has been known to suppress NF-κB since 2008 and its genome was sequenced in 2009, the specific bioactives supporting this activity remained unidentified until 2015, highlighting the limited capacity of -omic approaches to identify novel functional capabilities. We have now used meta-parental mating to isolate genetically tractable strains of *F. prausnitzii* (Ó Cuív et al. 2015), and we anticipate that these will further expedite the functional dissection of this important gut bacterium.

9 Concluding Remarks and Future Perspective

The healthy gut microbiota plays a vital role in helping to maintain gut homeostasis and preventing the onset of chronic gut disease. Surprisingly, little is known about the essential functionalities that underlie this capability and how they might be exploited to develop more effective therapeutic interventions. The rapid advances in DNA sequencing technologies continue to provide an unprecedented insight into structure-functional activity of the gut microbiome. In contrast, the development of complementary approaches including microbial culturing, functional assays, metabolomics and genetic technologies have not kept pace with these developments. This has hindered efforts to realise the functional potential of the microbiota, however, the successful metabolomic dissection of *F. prausnitzii* will encourage and inform the development of improved methodologies for other gut bacteria. Similarly, new advances in microbial culturing and genetic techniques will provide new opportunities to support a more mechanistic dissection of these functionalities. We anticipate that the effective integration of these disparate yet complementary approaches will afford the best opportunity to effectively bioprospect the gut microbiota and support the discovery of novel bioactives, and the development of new therapeutics.

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Competing interest

The authors declare no competing interest.

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