schematic view of technical advancement in the exploration of complex microbial settings:

Phylogenetics

- Metagenomics -

Microbiomics

Diversity, composition

Metagenome

mRNA

Metaprotome

Metabolome
The dominant intestinal microbiota: limited access by culture-based approaches

- Suau et al. 1999: 21-32%
- Hayashi et al. 2002: 21-37%
- Tannock et al. 2000: 21-37%
- Suau et al. 1999: 21-32%

Re-evaluation using culture-independent approaches

~ 30%
Developments towards integrated microbiomics will be illustrated with the human intestinal ecosystem as an example of complex and diverse microbiota.
The human intestinal microbiota

- 100 trillion microorganisms; 10 times the number of cells in the human body (Savage 1977); >150 fold more genes than in the human genome
- Predominantly not yet cultured to date (~70% of dominant species)
- Central to Food-Microbiota-Host inter-actions (microbiome and human genome crosstalks: with the 1st pool of immune cells and the 2nd pool of neural cells of the human body)
- A true organ; geared to « protecting our health and well-being »« throughout all stages of our life »; amenable to modulations
Functions of the intestinal microbiota

- Metabolism of fibers
- Barrier effect
- Maturation of immune system
- Production of vitamins
- Nutrients bioavailability
- Metabolism of xenobiotics
- Fat Storage
- Energy harvest

Intestinal microbiota
Composition, diversity, homeostasis?

= Who is there!

Food

Diversity / Composition

host
The ribosomal RNA approach

Based on cloning

Diversity, composition

Oligo. probes, PCR & FISH, Micro-arrays

Sequence & phylogeny

intact sample

16S rDNA

Extraction d’ADN

PCR 16S rDNA

Vecteurs: plasmid

Clonage

Transformation

E.coli DH10B

16S rDNA libraries in E.coli

Extraction d’ADN

clones d’E.coli
The ribosomal RNA approach

Based on direct pyrosequencing

- Diversity, composition
- Oligo. probes, PCR & FISH, Micro-arrays
- Sequence & phylogeny

intact sample

Extraction d'ADN

PCR 16S rDNA

16S rDNA libraries in E.coli

Sequence & phylogeny

Oligo. probes, PCR & FISH, Micro-arrays

Diversity, composition
Microbiota adaptation to ecosystem / niche
A limited fraction of known bacterial phyla within the dominant human intestinal microbiota

Eckburg et al. 2005
(3 individuals, 11831 rDNA sequences ; 391 species ; ~2500 fecal rDNA sequences)

Wilson et al 1996
Suau et al 1999
Bonnet et al 2002
Hold et al 2002
Hayashi et al 2002
Hayashi et al 2003
Wang et al 2003
Mangin et al 2004
Manichanh et al 2006

1000’s species identified to date ; 3 major Phyla
Phylogenetic view of fecal microbiota (1/2)

- In adults, > 80% of phylotypes belong to 3 major phyla: Bacteroidetes; Firmicutes (Cl. leptum Cl. coccoides) and Actinobacteria (Bifidobacterium Atopobium) Lay et al. AEM 2005

- > 80% sequences in adults (90% in seniors) are not represented in current cultured strain collections. Suau et al. AEM 1999

- The dominant intestinal microbiota is resistant over time and highly resilient upon antibiotherapy. Seksik et al. Gut 2003, De la Cochetière et al, J Clin Mic 2005

- ~ 1000 species make up the dominant microbiota in each individual. Tap & Leclerc. (see below)

- Most dominant phylotypes (70-80%) are subject-specific. (Zoetendahl et al. 1998, Seksik et al. 2003, Tap & Leclerc, see below)

- A few species are altogether more prevalent (conserved between individuals) and more represented, constituting a phylogenetic core; possibly a functional core of the intestinal ecosystem. (Tap & Leclerc 2009)
Taxonomic assignment at Phylum level

ANR-funded AlimIntest Study: 17 healthy volunteers; ~full 16S rDNA cloning and sequencing. As for former studies: few Phyla with 3 most dominant; Firmicutes, Bacteroidetes & Actinobacteria.

Healthy adults fecal microbiota studies:
- Eckburg et al 2006 = 3 individuals. (~2500 seq.)
- Li et al 2008 = 5 ind. (~7000 seq.)
- Manichanh et al 2006 = 6 ind. (~500 seq.)
- Gill et al 2006 = 2 ind. (~2000 seq.)

17 healthy subjects, 10456 sequences grouped into 3180 OTUs at 2%

Julien Tap & Marion Leclerc, EM 2009
Rank distribution shows a phylogenetic core

Core species as defined by their prevalence (>1/2 individual) are both prevalent and highly represented:

- ~2% OTUs (66 / 3180),
- 35% sequences (3740 / 10456)

Microbiota core species

Host specific (80%)

Julien Tap & Marion Leclerc, EM 2009
Phylogenetic view of the fecal microbiota (2/2)

• **phylogenetic core**: a few species are altogether more prevalent (conserved between individuals) and more represented,
  . These include *Faecalibacterium prausnitzii, Eubacterium rectale, Ruminococcus bromii, Allistipes putredinis, Subdoligranulum sp, Bacteroides vulgatus, Bacteroides uniformis rel, Parabacteroides distasonis, Bifidobacterium longum, Dorea formicigenerans,…*

• **Also found in comparable extensive studies from USA, China, France.**

Implication:
  phylogenetic core => core metagenome? => functional core
Limits

• Lack of formal & consensual standards for critical steps

• Representativity of DNA extracts

• Representativity of PCR amplicon mixes
  – Partial versus complete sequence
  – Selectivity of PCR primers
  – Chimera formation

• Choice of threshold sequence similarity for OTU definition

• Overall limit in cross-studies comparisons
At the level of environmental genome ... describe complete gene repertoire of the human intestinal microbiota

Identify core-genes & specificities in diseases?
Metagenome

Rondon et al, 2000
The genomes of the total microbiota found in nature

“The shotgun library, with 10- to -20kb inserts, is also a source of other sequences associated with the rRNA genes or other genes of interest”

http://www.genomesonline.org/gold.cgi

Bacterial genomes 4816
Ongoing metagenomes 240 (41 digestive systems)

As of Sept 1, 2010
Metagenomics

Genome reconstruction
Tyson et al, Nature 2004

Discovery of new functions: rhodospin

Metabolic pathway reconstruction
Hallam et al, Science 2002

New antibiotics
Handelsman, et al, 2004

Functional diversity of a gene or gene family
Venter et al, Science, 2004
Genomic DNA extraction

Heterologous Genomic DNA

Ligation → fosmid + DNA (40Kb)

Transformation

Genomic DNA + Vector → E. coli DH10B

Functionnal analysis

Expression of genes
mRNA
Proteines
secretion

Sequence analysis

ATGCGTTAGCCTTCC
TACGCAATCGGAAGG

From
Handelsman et al, 2004

Sequence-based Metagenomics

Manichanh Gut 2006
Gill Science 2006
Kurokawa DNA Res 2008
Manichanh NAR 2008
Qin Nature 2010

Functional Metagenomics

Gloux AEM 2007
Gloux PNAS 2010*
Duynhoven PNAS 2010*
Lakhdari PLoS One 2010*
Madi BMC Microbiol 2010*
Tasse Genom Res 2010*

* In press
International Human Microbiome Consortium:

Metagenomics of the human intestinal microbiota

Based on cloning

Fr, UK, NL, USA, Japan, China, Canada, Singapore, Korea, …
Metagenomics of the human intestinal microbiota

Based on direct sequencing

Sequences analysis
Phylogenetic binning based on 16S rDNA sequence

16S rDNA reads (%) from sanger reads of cloned ~3kb metagenomic sequences

There is a fairly high inter-individual consistency in representation of expected dominant phyla with variations in relative proportions

n=13 individuals
The microbiota varies in composition between individuals. Based on COG, the functional « make-up » of the fecal microbiota is highly conserved.

A rapidly growing reference gene set

Metagenomic sequences

- 2 american individuals 0.2 Gb (Gill et al. 2006)
- 13 japanese individuals 0.7 Gb (Kurokawa et al. 2008)
- 20 european individuals 2.6 Gb Sanger reads (Genoscope)
- 124 european individuals 500 Gb Solexa reads (BGI-Shenzen)
- 400 europeans total ~1500 Gb Solexa reads (BGI-Shenzen)
- 49 french individuals 200 Gb SoliD reads (INRA)

Full genome sequences

> 250; about 200 000 genes
>1000 to come (HMP & MetaHIT, non-yet-cultured)
Core metagenome in Illumina read set? 
(n=124 test-fecal samples; DK&SP, aged 18-69)

*Qin et al. Nature 2010*

Total: 576 Gb high-quality sequence-tags from the 124 samples.

Assembly …:
6.6 million assembled consensus seq. >500 bp (43% of all reads)
Total length of consensus seq.: 10.3 Gb - largest human metagenome sequence set so far -

unexpectedly, Illumina reads can be assembled allowing de novo metagenomic sequencing

Bioinformatics ‘proof-reading’ tools are currently being refined.
Core metagenome in Illumina read set?
(n=124 test-fecal samples; DK&SP, aged 18-69)
Qin et al. Nature 2010

Total: 576 Gb high-quality sequence-tags from the 124 samples.

Assembly ...:
6.6 million assembled consensus seq. >500 bp (43% of all reads)
Total length of consensus seq.: 10.3 Gb - largest human metagenome sequence set so far -

MetaGene calling:
3.3 million non redundant genes out of 14 million ORFs
0.55 million genes in each individual
prevalence >50% >40% genes of each subject (~9% of all genes)
prevalence <20% 2.4 million rare genes

Core metagenome in Illumina read set?
Prevalence >50% samples

proportion of common genes classified in the bacterial Phyla.
Common genes = present in no less than 50% of 124 samples.

Overall the core metagenome shows:
- expected phylogenetic distribution
- complementarity in coding potential with human genome

ILLUMINA Reads
Enriched KEGG pathways among human gut microbiome genes versus the human genome.

Metagenome highlights the complementarity in coding potential.
Towards core functions in reference gene set:
(n=22 adult-fecal metagenomes vs *B. subtilis* genome)

COGs of essential genes in *B. subtilis* genome => range (#hits/kb)

Distribution of range COGs & NOGs in sequenced bacterial genomes

COGs & NOGs, %

<table>
<thead>
<tr>
<th>Percentage</th>
<th>0%</th>
<th>5%</th>
<th>10%</th>
<th>15%</th>
<th>20%</th>
<th>25%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genomes, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Rare Gut specific COGs?

80 % undefined function

Frequent Housekeeping COGs?

90 % defined function

S.D. Ehrlich
Metagenomic profiling towards molecular diagnostic models

Sanger reads, others

Metagenomic sequence repertoire

Metagenomic Profiles distribution of genes; occurrence & frequency

Metagenomic signatures

HT short tag sequencing (ex. SoliD)
>100 million high fidelity 35-mers / run

Cohort studies
"...we depend on more than the activity of some 30,000 genes ... our existence is critically dependent on the activity of upmost of 1000 bacterial species ... Thus human life depends on additional 2 to 4 million genes, mostly uncharacterized. Until the synergistic activities between humans and their commensals has been elucidated, an understanding of human biology will remain incomplete."

Julian Davies. Science, March 2001
Genomic DNA extraction

Heterologous Genomic DNA

Ligation → fosmid + DNA (40Kb)

Vector

Transformation

E. coli DH10B

Functionnal analysis

Expression of genes

mRNA

Proteins

secretion

Sequence analysis

ATGCCTAGCTTCC

TACGCAATCGGAAGG

Banque métagénomique

Sequence-based Metagenomics

* In press

from
Handelsman et al, 2004

Functional Metagenomics

Gloux AEM 2007
Gloux PNAS 2010*
Duynhoven PNAS 2010*
Lakhdari PLoS One 2010*
Madi BMC Microbiol 2010*
Tasse Genom Res 2010*

* In press

Manichanh Gut 2006
Gill Science 2006
Kurokawa DNA Res 2008
Manichanh NAR 2008
Qin Nature 2010
Functional metagenomics of the human intestinal microbiota

→ culture-independent functional assessments

Functional analysis
Large inserts metagenomic libraries

✓ **France** (INRA & LibraGen S.A.)
  ✓ 50 000cl fecal microbiome; 6 healthy adults & 6 patients (Manichanh et al 2006, 2008)
  ✓ 20 000cl ileal mucosa associated microbiome (Leclerc et al)
  ✓ 180 000cl fecal microbiomes; 3 healthy adults and 3 patients (EU-MetaHIT)

✓ **UK** (Univ College Cork & Univ Aberdeen)
  ✓ 30 000 cl fecal microbiome; 1 adult (Flint et al)
  ✓ 250 000cl fecal microbiome; 1 adult (Marchesi et al)

✓ **NL** (Univ Wageningen)
  ✓ 30 000cl ileal effluent microbiome; 1 patient (De Vos et al)
Functional metagenomics of the human intestinal microbiota

- vitamin production
- plant cell-wall degradation
- β-glucuronidases
- bacteria-host crosstalk

Functional analysis
Vitamin producing metagenomic clone

Clone A6 – “yellow”
Yellow substance in supernatant
No induction by light
Absorbance at 350 and 450 nm
Riboflavin? Confirmed by NMR

Carien Booijink & Marion Leclerc
Gelfand et al 1999, Trends in Genetics

Present in A6 insert:
- ribA → GTP cyclohydrolase II
- ribD → Pyrimidine deaminase
- ribD → Pyrimidine reductase
- ribH → Riboflavin synthase β chain
- ribE → Riboflavin synthase α chain
- Transcription regulator

GTP → 2,5-diamino-6-hydroxy-4-(5’phosphoribosylamino)pyrimidine
- 5-amino-6-(5’phosphoribosylamino)uracil
- 5-amino-6-5’phosphoribityllamino)uracil
- 6,7-dimethyl-8-ribityllumazine

nomenclature E. Coli:
Yellow phenotype - riboflavin producing clone
(2D-DIGE : A6 Cy3 vs DH10B Cy2)

1 spot identified by Maldi-TOF as: Riboflavin synthase
Plant cell wall degradation

Arabinofuranosidase
Galactosidase
Fucosidase
Xylosidase

Esterases

Cellulase
Cellulose-binding

Lignin
Lignin-hemicellulose links
Ramifications

Heteroxylans
Diferulic bridges

Marion Leclerc &
Lena Tasse, Gabrielle Potocki-Veronese et col.
High throughput screens for glycosyl-hydrolases

156,000 clones; 5.10^9 bp

Growth
Protein expression
Substrate hydrolysis

Hit detection
Q-tray
2304 clones

200,000 clones per week and per substrate

- Substrate: Insoluble AZCL-xylan
- Hits: release of soluble AZCL-xylooligosaccharides

Lena Tasse et al. Genome Res 2010
High throughput screens for glycosyl-hydrolases

<table>
<thead>
<tr>
<th></th>
<th>Screened clones</th>
<th>Hits</th>
<th>Hits %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactan</td>
<td>136 000</td>
<td>108</td>
<td>0.79</td>
</tr>
<tr>
<td>β-1,3/1,4-glucan</td>
<td>156 000</td>
<td>97</td>
<td>0.62</td>
</tr>
<tr>
<td>Starch</td>
<td>156 000</td>
<td>64</td>
<td>0.61</td>
</tr>
<tr>
<td>Xylan</td>
<td>156 000</td>
<td>36</td>
<td>0.23</td>
</tr>
<tr>
<td>Inulin</td>
<td>40 000</td>
<td>3</td>
<td>0.075</td>
</tr>
<tr>
<td>Pectin</td>
<td>100 000</td>
<td>3</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>744 000 assays</strong></td>
<td><strong>311</strong></td>
<td></td>
</tr>
</tbody>
</table>

27 clones out of 311 hits were selected for pyrosequencing / potential biotech applications

=> Identification of modules and enzyme families.

Lena Tasse et al. Genome Res 2010
Mining for CAZyme (Carbohydrate Active enZymes)

27 hit clones (850 kb of metagenomic DNA)
88 CAZY modules 0 ≤ E-value ≤ 4.6e⁻⁶ 11 potential multimodular enzymes
74 novel CAZyme proteins
36 CAZY families (20 GH; 7 CE; 2 GT; 1 PL; 5 CBM and 1 Fn; 4 new)

30% Bacteroidetes (β-glucanases, Galactanases, Xylanases, Fructanases); 14% Firmicutes (); 50% bacteria, unassigned

Coupling HTS and pyrosequencing of large metagenomic DNA fragments
→ Enrichment of genes of interest (~1 CAZY every 10 kb)(~5 fold target gene enrichment)
→ Direct access to CAZyme diversity
→ High sequence novelty (71% putative proteins with no similar 3D-structure; < 30% seq-id)
→ Identification of novel enzyme families / 3D structures
→ Observation of horizontal gene transfer

Lena Tasse et al. Genome Res 2010
Example of a xylanase positive clone
Fully equipped with a polyspecific machinery of plant cell wall degradation

Lena Tasse et al. Genome Res 2010
High throughput screens for β-glucuronidases

**Gut microbiota metagenomic library** 96-well microplates
⇒ Random screening of 4608 metagenomic clones from faecal and ileal libraries (*E.coli* DH10B as receiving strain)

**Qualitative screen**
⇒ 1.79% of clones over-expressing the activity

**Quantitative validation**
(*E.coli* DH10B with empty fosmid as control)
⇒ 0.73% of positive clones

**Selection of fosmids generating β-glucuronidase activity**
(β-glucuronidase⁻ strain transformation)
⇒ 0.41 % of all clones tested

⇒ **Identification of genes involved**
(subcloning, sequencing and bioinformatic analysis)

⇒ 1 « beta-galactosidase » (insert H11G11)
⇒ 4 proteins with unexpected fonctions

Karine Gloux et al., PNAS 2010
Inducible expression of H11G11 β-glucuronidases in pRSF-BL21

H11G11 β-glucuronidase sequence:

⇒ different from known beta-glucuronidases (<33% AA sequence identity)
⇒ longer sequence
⇒ lacking the conserved patterns of corresponding glycosyl hydrolases families

Are H11G11-like β-glucuronidases:
Widespread in commensals?  
In the gut? or
In other environments?

Karine Gloux et al., PNAS 2010
H11G11-like proteins:

⇒ H11G11-like BG sequence mainly recovered in Firmicutes (Ruminococcaceae, Lachnospiraceae and Clostridiaceae)
⇒ some strains have several paralogs ⇒ adaptability/resilience?

Karine Gloux et al., PNAS 2010
Faecalibacterium prausnitzii M21/2
ZP_02090684 (45%id, 59%sim)

Subdoligranulum variabile DSM 15176
ZP_03774451 (45%id, 57%sim)

Shuttleworthia satelles DSM 14600
ZP_04455048 (41%id, 56%sim)

Roseburia inulinivorans DSM 16841
ZP_03753494 (44%id, 57%sim)

Bryantella formategens DSM 14469
ZP_03686296 (41%id, 53%sim)

Subdoligranulum variabile DSM 15176
ZP_03776204 (43%id, 54%sim)

Bacteroides capillosus ATCC 29799
ZP_02038110 (44%id, 56%sim)

Ruminococcus gnavus ATCC 29149
ZP_02042987 (45%id, 57%sim)

Parabacteroides johnsonii DSM 18315
ZP_03478350 (34%id, 46%sim)

Parabacteroides merdae ATCC 43184
ZP_02031489 (34%id, 46%sim)

Bacteroides ovatus ATCC 8483
ZP_02065512 (34%id, 46%sim)

β-glucuronidase => All strains tested are active

Karine Gloux et al., PNAS 2010
The novel H11G11-like BG is:

- specific of the human gut & more frequent than the formerly known BGs
- present in all adults fecal microbiota but rare in infants (Kurokawa data)

Karine Gloux et al., PNAS 2010
Metagenomic screening for bacteria-cell crosstalk

Bacterial fraction

DNA extraction → Metagenomic DNA

Cloning → Fosmid Vectors

Transformation → E.coli DH10B

Large homogenous DNA library

Recombinant clones

European program

Hervé Blottière, Omar Lakhdari, Tomas de Wouters, Gosia Nepelska
Strategy: screening for the modulation of transcriptional pathways

- 3 reporter cell lines with SEAP:
  - HT29/SEAP-25
  - Caco-2/SEAP-7
  - THP-1 blue® CD14+ (Invivogen)

- 1 reporter cell lines with luciferase
  - HT29/LUC-E

Stable transfections
Easily detectable proteins
Expression level ↔ gene activation

Hervé Blottière and col.
EU Integrated Program MetaHIT & ITN Crosstalk
Metagenomic screening for bacteria-cell crosstalk - workflow -

NF-κB modulation

Culture of reporter cell line in microplates
+ 48 h

Addition of metagenomic clone fractions to cultured cells.
+ 24 h

Luminescence or colorimetric assessment of reporter gene activity

Genetic characterization of bioactive clones

Validation of bioactive clones requires: primary + secondary + tertiary screens and final octoplicates

Hervé Blottière and col.
EU Integrated Program MetaHIT & ITN Crosstalk
Application of metagenomics approach to the identification of bacteria-host crosstalk molecules

- **Proliferation-apoptosis and differentiation**
  - Cell growth *Gloux et al. 2007*
  - Activation of AP1 pathway

- **Modulation of immunity**
  - Activation of NF-kB pathway *Lakhdari et al. 2010*
  - Modulation of TSLP

- **Modulation of metabolism**
  - PPAR, CAR, NR4A regulation
  - Modulation of Fiaf

Hervé Blottière and col.
EU Integrated Program MetaHIT & ITN Crosstalk
Metagenomic screening for NF-κB modulation

2640 clones

- Metagenomic library derived from the intestinal microbiota of CD patients (Manichanh et al 2006)
- Lysate preparation using glass beads
- 24 hours contact (10% vol/vol) with NF-κB reporter cells (HT-29/kb-seap-25)

a. 2640 lysates screened
171 positive hits

b. 5 stimulatory and 5 inhibitory clones selected for validation (8 independent cultures)
6 clones with reproducible effects

Hervé Blottièrè
Omar Lakhdari et al PLoS One 2010
52B7 - the most stimulatory clone identified

• 37,006 base pairs long metagenomic DNA insert: 40 genes and 23 transcriptional units (GeneMark.hmm and FGENESB predictions)

• Best blastp results for predicted genes => member of genus Bacteroides (99% of predicted genes coverage)

• Closest cultivated relative is B. vulgatus strain ATCC8482 (40.8% of predicted genes coverage)

• Also stimulatory on other NF-kB reporter models (HT-29/kb-luc-E and THP-1 blue®)

• Most active culture fraction is the culture supernatant => soluble factor ?

Hervé Blottièrè
Omar Lakhdari et al PLoS One 2010
Genes involved in 52B7 bioactivity

Random mutagenesis by insertion of EZ-TN5 transposon. 192 transposed clones of 52B7 obtained and screened

3 clones with revertant phenotype identified. Insertion loci determined by sequencing

*52B7/A and C: transposon targeted the same gene encoding a putative ABC transporter permease

*52B7/B transposon targeted a putative lipoprotein with unknown function.

the 2 targeted genes are part of 2 putative distinct transcription units

Omar Lakhdari et al
PLoS One 2010
current limitations in the metagenomics approach include:

- DNA extraction and cloning biases
- Restricted to dominant compartments (microbes & genomes)
- Expression so far only in E.coli
- Restricted to DNA (later RNA, proteins, metabolites & integrated microbiomics)
Post-Metagenomic perspective 1/3

- Profiling based diagnostics
  Based on *Reference-Gene-Set*
  - Identification of core-metagenome
  - Metagenomic signatures of pathologies

- Ecosystems – Systems Ecology
- Bacteria-host crosstalk
Post-Metagenomic perspective 2/3

- Profiling based diagnostics
- **Ecosystems – Systems Ecology**
  known: microbes present -------- metabolisms at play
  unknown: who is doing what = guilds

Challenges:
- Reconstruct microbial food chain
- Identify key functional groups
- Design and validate metabolic models
- Identify signatures of functional dysbiosis

- **Bacteria-host crosstalk**
Post-Metagenomic perspective 3/3

• Profiling based diagnostics
• Ecosystems – Systems Ecology
• Food-Bacteria-host crosstalk/interactions

known: host functions responding to food-microbe signalling
unknown: active molecular structures and mechanisms

Challenges:
  – Identify crosstalk genes & molecules
  – Decipher mechanisms (bacteria and host side)
  – Design strategies of modulation with relevance in nutri/pharma applications
INRA Jouy-en-Josas
Marion Leclerc
Hervé Blottière
Patricia Lepage
Catherine Juste
Karine Gloux
Fabien Dumetz
Florence Levenez
Florence Blon
Chaysavanh Manichanh
Omar Lakhdari
Antonella Cultrone
Tomas de Wouters
Gosia Nepelska
Karine Leroux

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