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Development of High-Throughput Phenotyping of Metagenomic Clones from the Human Gut Microbiome for Modulation of Eukaryotic Cell Growth▽

Karine Gloux,[1] Marion Leclerc,[1] Harout Iliozer,[1] René L'Haridon,[2] Chaysavanh Manichanh,[1] Gérard Corthier,[1] Renaud Nalin,[3] Hervé M. Blottière,[1] and Joël Doré[1]*

Unité d'Ecologie et Physiologie du Système Digestif[1] and Unité de Virologie et Immunologie Moléculaires,[2] Institut National de la Recherche Agronomique, Domaine de Vilvert, 78352 Jouy en Josas, France, and LibraGen, Bat. Canal Biotech I, 3 rue des Satellites, 31400 Toulouse, France[3]

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Metagenomic libraries derived from human intestinal microbiota (20,725 clones) were screened for epithelial cell growth modulation. Modulatory clones belonging to the four phyla represented among the metagenomic libraries were identified (hit rate, 0.04 to 8.7% depending on the screening cutoff). Several candidate loci were identified by transposon mutagenesis and subcloning.

A long coevolutionary process has led to mutualistic interactions between the gut microbiota and the host (4). Intestinal microbes contribute to gut physiology, metabolism of complex polymers and energy harvest, homeostasis of the immune system, and protection against pathogens (3, 6, 8, 15, 19, 31). The continuous regeneration and proliferative activity of the intestinal epithelium are modulated by the microbiota or its metabolites (1, 5, 17, 20, 22, 27). A better understanding of the microbiota's contribution to human health requires characterization of microbial molecular signals driving interactions with the host. Beyond sequence-based human intestinal microbiome explorations (10, 16, 18), metagenomic libraries may permit functional investigations (9, 12). We describe here functional screening of metagenomic libraries that allowed us to identify candidate loci involved in modulation of eukaryotic cell growth.

Metagenomic libraries from the fecal microbiota of healthy subjects (HSL library) and patients in remission from Crohn's disease (CPL library) were constructed previously (18). The phylogenetic characterization of these libraries was in good agreement with previous studies of human feces using molecular approaches (18). A two-step microplate screening method was validated (Fig. 1) using 565 clones bearing 16S rRNA genes within 40-kb inserts. Lysates of bacterial cell suspensions (optical density at 600 nm in 2YT medium, ~0.6) were broken in a vibrating crusher (4.5 V, three 90-s treatments) using 106-μm glass beads (1 g · ml^{-1}). After filtration (0.2 μm), the lysates were added (1:30, vol/vol) to CV-1 kidney fibroblast cells (in serum-free Eagle's modified essential medium [17a]) or HT-29 human colonic tumor cells (in RPMI 1640 medium containing 2.5% serum). HT-29 and CV-1 cell growth was determined by crystal violet staining (26) after 2 and 4 days, respectively. Modulatory clones were detected by comparison to the median growth and were further validated against insert-free *Escherichia coli* as a control. Populations of 10^3 and 10^4 bacterial cells per eukaryotic cell were used for stimulatory and inhibitory effects, respectively. Active clones were controlled for the absence of growth or quorum-sensing bias (data not shown).

The screens using CV-1 cells were far more sensitive than those using HT-29 cells, but lysates that were significantly inhibitory for HT-29 cells were among the most inhibitory lysates for CV-1 cells. The former yielded 8.7% modulatory inserts (Fig. 2).

Inhibitory and stimulatory inserts were obtained from both the HSL and CPL libraries. The four phyla present in the libraries—*Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Actinobacteria* (18)—were represented among modulatory clones (Fig. 2) with relative proportions similar to those observed in the original libraries (Table 1). Most of the modulatory clones (76%) could not be affiliated to a cultured species, highlighting the potential of the metagenomic approach for mining unconcealed biological resources.

Bacteroidetes inserts predominated, and most were stimulatory. Some of the inserts were from *Prevotella*, *Tannerella*, and *Rikenella*, but the majority (56%) were from the genus *Bacteroides*. Growth-modulating inserts from *Firmicutes* were derived from the families *Clostridiaceae* and *Lachnospiraceae*. The four *Actinobacteria* modulatory inserts were phylogenetically affiliated to the family *Coriobacteriaceae*. The frequency of inhibitory *Proteobacteria* was higher than expected, and all *Proteobacteria* belonged to the genus *Escherichia*.

For five modulatory inserts, the putative loci were localized by transposon mutagenesis (EZ::TN <KAN-2> insertion kit; Epicenter Technologies [11]), followed by screening for revertant behavior. The method revealed two distant loci for each of the two inhibitory inserts and clustered insertion sites for the three stimulatory inserts (Table 2). Subclones corresponding to the transposed regions previously highlighted (3 kb in pcDna2.1) were then screened for their modulatory activity. This demonstrated that single genes or systems could mediate

* Corresponding author. Mailing address: INRA, Unité d'Ecologie et de Physiologie du Système Digestif, Domaine de Vilvert, 78352 Jouy en Josas Cedex, France. Phone: 33 (0)1 34 65 27 09. Fax: 33 (0)1 34 65 24 92. E-mail: joel.dore@jouy.inra.fr.

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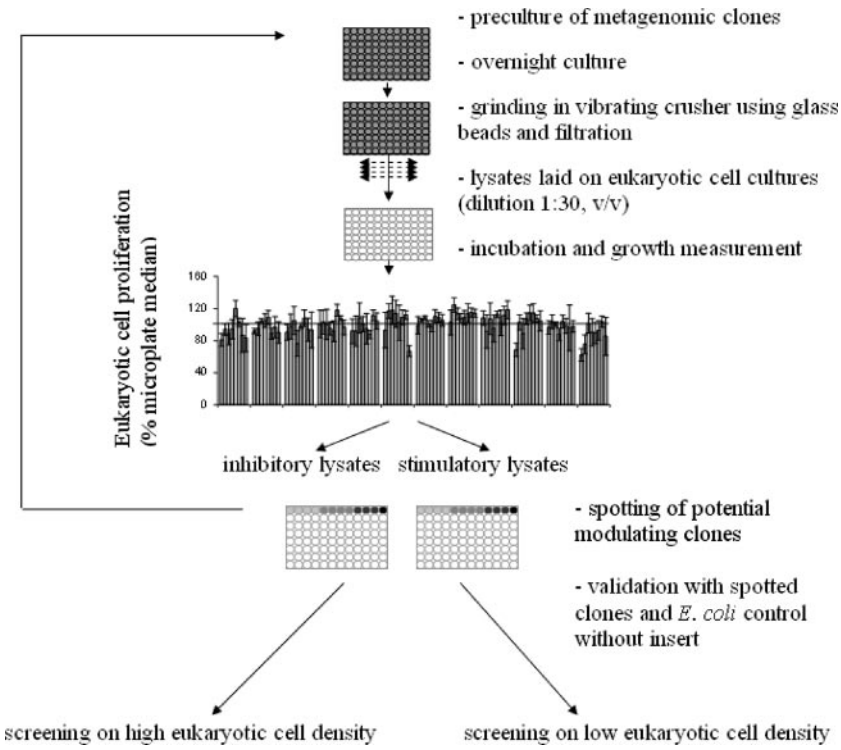

FIG. 1. Screening method for bacterial metagenome-derived lysates modulating CV-1 cell growth. The first screening step was performed on 96-well microplates to identify modulatory lysates compared to the median microplate value for cell growth ($P \leq 0.05$, as determined by Student's *t* test). A second screening step was performed for the highlighted clones compared to the control strain deprived of an insert using low or high initial CV-1 cell concentrations (1×10^3 and 1×10^4 cells per well). The error bars indicate standard deviations for eight replicates.

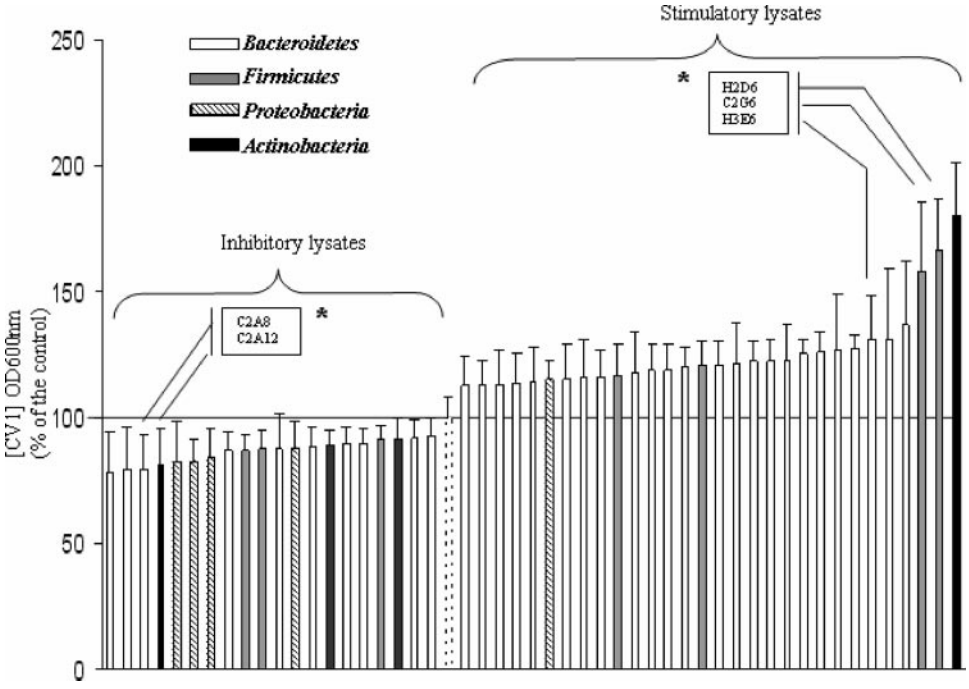

FIG. 2. Distribution of metagenomic clones significantly modulating CV-1 cell growth from the most inhibitory to the most stimulatory. A total of 565 clones bearing 16S rRNA genes on the insert were screened for their lysate activities as described in Fig. 1. Significant effects ($P < 0.05$) were expressed as the percentage of the growth obtained with the control strain (*E. coli* DH10B-Epifos-5) (dashed column). The error bars indicate standard errors of the means. Asterisks indicate modulatory clones chosen for further identification of involved loci on the inserts (see Table 2). OD600nm, optical density at 600 nm.

TABLE 1. Phylogenetic distribution of metagenomic clones modulating CV-1 cell growth

Phylum	Modulatory clones			
	Effect on CV-1	No. of modulatory clones	% of total modulatory clones	% in library for phylum[a]
Bacteroidetes	−	10	20	77
	+	24[b]	48 (68)[c]	
Firmicutes	−	3	6	11
	+	4	8 (14)	
Proteobacteria	−	4	8	5
	+	1	2 (10)[d]	
Actinobacteria	−	3	6	7
	+	1	2 (8)	
Total	−	20	40	100
	+	30	60 (100)	

[a] The distribution was calculated from previous results (18).
[b] $P < 0.05$, as determined by a chi-square test performed between the proportions of phyla in the metagenomic libraries and among stimulatory clones.
[c] The number in parentheses is the total percentage for the phylum.
[d] $P < 0.05$, as determined by a chi-square test performed between the proportions of phyla in the metagenomic libraries and among all modulatory clones.

the modulatory effects observed and that the distant loci tested were independent.

The candidate loci identified in this work corresponded to several ABC systems, a RecD gene homologue, a glutamate synthase subunit, a V-type ATPase subunit, and a specific 16S rRNA gene (Table 2). The common feature of these systems or genes is that they are ubiquitous in nature. They have been proposed to have derived from a common ancestor and have equivalents in eukaryotes (2, 21, 25, 28). The rRNAs have been used for determining deep phylogenetic relationships between *Eukarya*, *Bacteria*, and *Archaea* (30). ABC systems probably constitute the largest superfamily of proteins ever detected in prokaryotes and eukaryotes (13), and ATPases are present in every life form (21). Glutamate synthase (GltS) isoforms, present in bacteria, microorganisms, and plants, result from the coevolution of conserved functional domains. However, specific features of such conserved systems are likely responsible for the effect on eukaryotic cell growth. The modulatory effects with biological significance and those due to nonspecific perturbation of the receiving organism *E. coli* need to be differentiated further. In addition, finer analysis of the eukaryotic cell response should provide a better understanding of the mechanisms involved (cytostasis, cytotoxicity, apoptosis, etc.).

This method was further adapted to initiate high-throughput screening with HT-29 cells. A metagenomic library of 20,160 clones obtained from the mucosal microbial fraction of the ileum of a healthy individual was screened (average insert size, 40 kb). After 24 h of incubation in Dulbecco modified Eagle medium supplemented with 1% serum, HT-29 cells were exposed to filtered clone lysates and maintained for an additional 72 h. Luminescence-based intracellular ATP quantification was used as a cell growth indicator (Cell Titer Glo luminescent kit; Promega). This allowed detection of nine validated clones showing more than 30% inhibition of HT-29 cell growth (hit rate, 0.04%). Despite the threshold used, the hit rates remained high compared to those in previous reports of specific

TABLE 2. Identification of loci or genes involved in the modulatory effects on eukaryotic cell growth for five selected metagenomic clones[a]

Modulatory insert and phylogenetic identification	Gene position(s) on insert (bp)	System or gene (size)	Most similar homologue(s) (accession no.; % identity)	Putative function
C2A12, *Bifidobacterium* inhibitory	5523–4555, 6431–5550	Two integral inner membrane proteins of OSP* ABC system (332 and 278 amino acids)	*Bifidobacterium longum* DJO10A (gi46190851; 88), *Bifidobacterium longum* NCC2705 (gi22775981; 89)	ABC-type sugar import system, OSP family
	18127–17030	One integral inner membrane protein of ABC transport system (365 amino acids)	*Leifsonia xyli* subsp. *xyli* strain CTCB07 (gbAAT89613.1; 66)	ABC transporter, 0228 family[d]
	40417–40815, 42209–40818	Hypothetical protein (131 amino acids) plus downstream helicase RecD (469 amino acids)	*Oceanobacillus iheyensis* HTE831 (gi23465735; 29), *Bifidobacterium longum* DJO10A (gi23335557; 80)	Unknown
C2A8, *Porphyromonadaceae* inhibitory	21060–24374[c]	ND (entire transcription repair coupling factor not tested, 1,053 amino acids)[c]	*Bacteroides thetaiotaomicron* VPI-5482 (gbAAO75359.1; 58)[c]	DNA repair[c]
	33544–34869	Glutamate synthase β-subunit (441 amino acids)	*Thermoanaerobacter tengcongensis* MB4 (gbAAM23955.1; 41)	Amino acid transport and metabolism
H2D6 and C2G6, *Eubacterium biforme* stimulatory[b]	5771–7258,[b] 8527–7499	16S rRNA genes	*Eubacterium biforme* M59230 (gbM59230.1; 98)[e]	Protein synthesis
H3E6, *Bacteroides* stimulatory	11812–11354	V-type ATP synthase subunit K (152 amino acids)	*Bacteroides thetaiotaomicron* VPI-5482 (gbAA076402.1; 92)	Energy production and conversion

[a] Significant modulatory effects of revertant and subclones by comparison to the control strains ($P < 0.05$, as determined by Student's t test).
[b] The same 16S rRNA gene (100% identity) was found to be involved in the effects of both the H2D6 and C2G6 clones, and two repeats of this gene were found in the H2D6 insert.
[c] For the C2A8 clone, a transcription repair coupling factor locus was revealed by transposition experiments, and the subclones tested did not allow the demonstration of the direct implications of the transcription repair coupling factor. ND, not determined.
[d] For C2A12, the integral inner membrane protein involved was in a region of proteins analogous to *Leifsonia xyli* subsp. *xyli* CTCB07 proteins. This locus had the conserved motifs of ABC cassettes (29). The integral inner membrane protein hydrophobic profiles and adjacent ATP binding protein were similar to those of ABC family 0228 (data not shown).
[e] In this instance, the identity refers to DNA identity.

functionality screens with metagenomic libraries (7, 14, 23, 24). This may have been due to the use of eukaryotic cell growth as a very global readout and from the exposure to filtrates of lysed clones which were likely to contain all potential signaling molecules.

In conclusion, we demonstrated the applicability of high-throughput phenotyping for selection of intestinal bacterial metagenomic clones able to modulate the growth of epithelial cells in vitro. The combination of transposon mutagenesis and subcloning appeared to be optimal for identification of the loci involved. Nevertheless, this is only one essential step towards identification of novel, possibly widespread mechanisms of prokaryote-eukaryote signaling in the gastrointestinal tract.

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