cell surface. A glass micropipette filled with cell permeabilization/stimulation buffer [139 mM potassium glutamate, 20 mM Pipes, 5 mM EGTA, 2 mM ATP, 2 mM $MgCl_2$, 20 mM digitonin, and 10 μM free $Ca^{2+}$ (pH 6.5)] was positioned on the opposite side from the carbon fiber, ~60 μm from the cell. For stimulation of the cells, the buffer was pressure-ejected onto the cell for 20 s. Amperometric responses were monitored with a VA-10 amplifier (NPI Electronic, Tamm, Germany), collected at 4 kHz, digitized with a Digidata 1200B acquisition system, and monitored online with the Axo-Scope 7.0 program (Axon Instruments, Foster City, CA). Data were subsequently analyzed with an automated peak detection and analysis protocol within the program Origin (Microcal, Northampton, MA). Spikes were selected for analysis if they had a base width greater than 6 ms and an amplitude greater than 40 pA, so that the analyses were confined to spikes arising immediately beneath the carbon fiber.


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# Bakers' Yeast, a Model for Fungal Biofilm Formation


**Todd B. Reynolds and Gerald R. Fink***



**Biofilms are formed by the aggregation of microorganisms into multicellular structures that adhere to surfaces. Here we show that bakers' yeast *Saccharomyces cerevisiae* can initiate biofilm formation. When grown in low-glucose medium, the yeast cells adhered avidly to a number of plastic surfaces. On semi-solid (0.3% agar) medium they formed "mats": complex multicellular structures composed of yeast-form cells. Both attachment to plastic and mat formation require Flo11p, a member of a large family of fungal cell surface glycoproteins involved in adherence. The ability to study biofilm formation in a tractable genetic system may facilitate the identification of new targets for antifungal therapy.**


Many microorganisms have the ability to grow in association with a surface in an aggregate of cells called a biofilm. Biofilms have taken center stage with the increasing recognition of their role in human infections. Pathogenic bacteria and fungi can form biofilms on the inert surfaces of implanted prosthetic devices such as catheters and on fragments of dead tissue. In the protected microenvironment of a biofilm, the pathogens are more resistant to antimicrobial therapies (*1*, *2*).

Little is known about fungal biofilms because many of the organisms that form these structures are not amenable to genetic approaches (*1*, *3*). In search of a model system for fungal biofilms, we investigated whether the well-characterized bakers' yeast *S. cerevisiae* can form biofilms. Bacteria are said to form biofilms if they adhere to plastic (*1*). We found that *S. cerevisiae* adhered to polystyrene plates (Fig. 1), and the cells remained adherent even after repeated washes (*4*). The yeast cells also adhered to polypropylene and, to a lesser degree, to polyvinylchloride (PVC) (*5*). These results suggested that *S. cerevisiae* can initiate biofilm formation.

The adherence of yeast to plastic was enhanced as the glucose concentration was lowered, but it was reduced in the complete absence of glucose, suggesting that there is a requirement for active metabolism (Fig. 1A). Diploid cells did not adhere as well as haploids in this assay (Fig. 1, B and C). Examination of the attached cells by microscopy revealed that they were round yeast-form cells (Fig. 1D).

Because bacterial biofilm formation requires cell surface adhesins (*1*), we disrupted *FLO11*, a yeast gene encoding a cell surface glycoprotein that is required for adhesion to agar (*6*, *7*), and *FLO8*, a yeast gene that encodes a regulatory protein required for *FLO11* expression (*8*). Isogenic strains (*9*) lacking either *FLO11* (*flo11*Δ; Fig. 1, B to D) or *FLO8* (*flo8*Δ) adhered poorly to polystyrene even in low glucose.

The role of Flo11p in the adherence of *Saccharomyces* to plastic may be similar to that of the glycopeptidolipids (GPLs) expressed on the cell surface of *Mycobacterium smegmatis*, a nonflagellated bacterium. *Mycobacterium smegmatis* mutants defective in GPL synthesis are defective in both biofilm formation and in a distinct colonial behavior called "sliding motility," suggesting an intimate connection between the two phenotypes (*10*, *11*). Sliding motility is defined as a form of surface motility " . . . produced by the expansive forces of the growing bacterial population in combination with cell surface properties that favor reduced friction between the cells and the substrate" (*10*, p. 4348).

To determine whether *Saccharomyces* displays a *FLO11*-dependent phenotype similar to sliding motility, we inoculated strains onto YPD plates containing 0.3% rather than 2% agar. On this low agar concentration, *S. cerevisiae* exhibited an elaborate pattern of multicellular growth resulting in a confluent mat (Fig. 2). The low concentration of agar required for formation of this structure is similar to that which triggers the sliding motility of *M. smegmatis* (*10*, *11*).

When inoculated in the center of 0.3% agar plates, *S. cerevisiae* produced a flat mat covering a larger surface than that of the same strain inoculated on 2% agar (Fig. 2, G and H). This structure grew in a radial form both on circular and square petri dishes and ultimately covered most of the agar, achieving a mean diameter of 7.8 ± 0.57 cm after 13 days (*12*). The mature structure had a central hub made of a network of cables (Fig. 3A) and radial spokes emanating from the hub. Spokes formed reproducibly within a defined range with a mean of 14.4 spokes ± 4.5 (*12*). The spokes and hub were more distinct at 25°C than at 30°C. The number of cells produced by mat formation on 0.3% agar was 7.6 times greater (day 12) than that in a colony produced on 2% agar by the same strain (*13*). The formation of mats and spokes, like adherence to plastic, was sensitive to glucose concentration; reduction in the glucose concentration resulted in a more rapid formation of spokes and hubs (*14*).

The ability of *S. cerevisiae* to form the mat structure was *FLO11*-dependent. Growth of a *flo11*Δ strain on a 0.3% agar plate produced a mass of cells with a smaller diameter and without the characteristic morphology of a *FLO11* mat (Figs. 2L and 3B). The *FLO11* mat after 12 days on 0.3% agar contained ~1.6 times the number of cells as a *flo11*Δ strain, whereas on


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2% agar a *flo11*Δ strain produced a colony with ~1.5 times as many cells as an isogenic *FLO11* strain (*13*). *FLO8*, a regulator of *FLO11* expression, was also required for mat formation.

The *FLO11* gene is also required for filamentous growth, a morphological switch from the yeast form to multicellular pseudohyphae (invasive chains of elongated cells), that is induced by conditions of nitrogen starvation. Filamentous growth requires components of a signaling cascade of the mitogen-activated protein kinase (MAP kinase) family for maximal transcription of *FLO11* (*15–17*). Strains carrying mutations (*9*) in genes encoding components of this MAP kinase pathway (e.g., the MAP kinase kinase kinase *ste11* and the transcription factor *ste12*) that reduce filamentation also formed mats more slowly than the wild type. Although *FLO11* expression is required for both filamentous growth and mat formation, the cells in both *FLO11* and *flo11*Δ strains were yeast form and not pseudohyphal (Fig. 3D), as determined by light microscopy and scanning electron microscopy.

The mats formed by isogenic *MAT***a**, *MAT*α, and *MAT***a**/α diploid strains (*9*) had distinguishable morphologies. The mats of the *MAT***a** strain were typically smaller in diameter and formed more spokes than the *MAT*α strain (*12*). In addition, mats formed by the *MAT***a** strain were rougher in texture with more cables, rougher edges, and fewer lobes than mats formed by the *MAT*α strain (compare Fig. 2, I and G, and Fig. 3, A and C).

At early time points, the mats formed by the *MAT***a**/α diploid were markedly different in morphology from that of either haploid (compare Fig. 2, J and E), although the differences lessened with time (compare Fig. 2, G, I, and K). This difference between haploids and diploids is likely to reflect the reduced expression of *FLO11* in *MAT***a**/α diploids versus *MAT***a** or *MAT*α haploids. Previous work has shown that *FLO11* transcription decreases with increasing

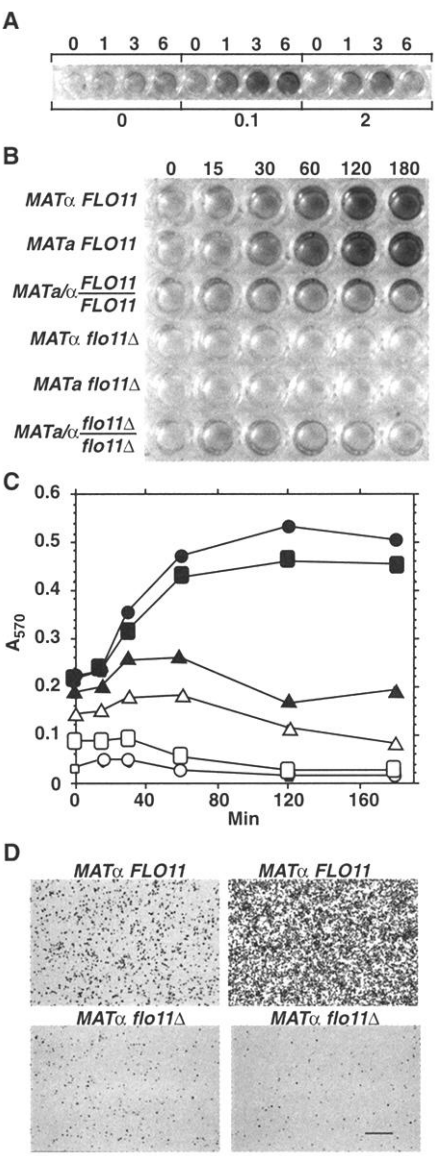


**Fig. 1.** Adherence of *Saccharomyces* to the surface of polystyrene. Yeast was grown in SC [synthetic complete media (*26*)] with 2% (w/v) glucose and harvested at an optical density at 600 nm ($OD_{600}$) of 0.5 to 1.5. Cells were then washed once in sterile $H_2O$, resuspended to 1.0 $OD_{600}$ in SC with 0, 0.1, or 2% glucose, and transferred by pipette (100 μl) into wells of a 96-well polystyrene plate (Falcon Microtest flat bottom plate, 35-1172; Becton-Dickinson Labware). Cells that adhered to polystyrene were visualized by staining with crystal violet (*4*). (**A**) Adherence to plastic at a low glucose concentration. The cells were incubated for 0, 1, 3, or 6 hours at 30°C. The time (hours) after inoculation is indicated above the wells, and glucose concentrations (%) are shown below. (**B**) Flo11p was required for adherence. Yeast strains were resuspended in SC + 0.1% glucose before transfer to the plate. All of the wild-type and mutant strains used were isogenic (*9*). The numbers at the top indicate minutes after addition to the plate. (**C**) Quantitation of the results shown in (B) (*27*). Each data point is the average of three samples: (■) *MAT*α *FLO11*, (●) *MAT***a** *FLO11*, (▲) *MAT***a**/α *FLO11/FLO11*, (□) *MAT*α *flo11*Δ, (○) *MAT***a** *flo11*Δ, (△) *MAT***a**/α *flo11*Δ/*flo11*Δ. (**D**) The cells in the wells shown in (B) were photographed at 100× magnification with a Zeiss Telaval 31 inverted microscope. Incubation in the well was for 0 min (left) and 180 min (right). Bar, 50 μm.

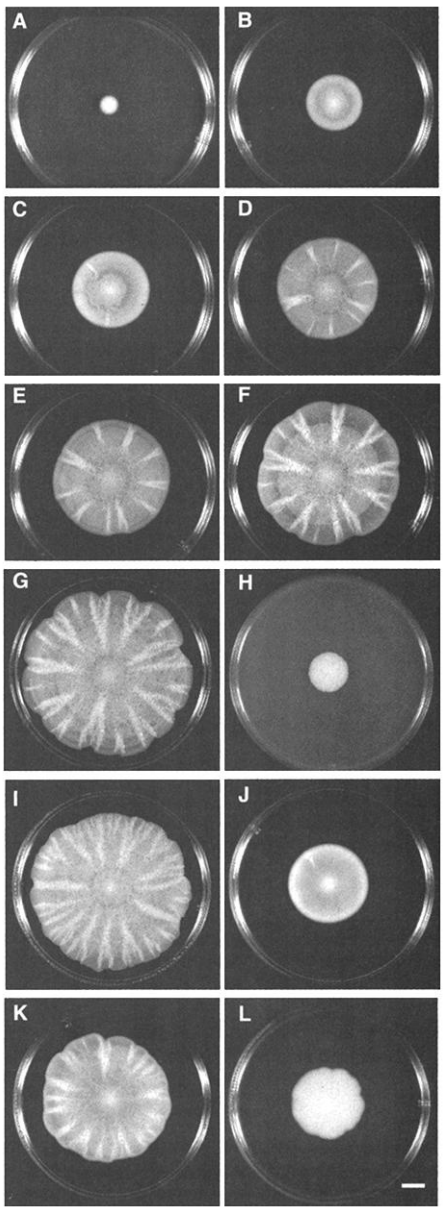


**Fig. 2.** Mat formation by *Saccharomyces*. Isogenic yeast strains (9) were inoculated onto YPD agar plates (0.3 or 2%) with a toothpick 1 to 2 days after the plates were poured. The plates were then wrapped with parafilm and incubated at 25°C. (A) to (G) Formation of a single mat by a *MAT*α strain (9) over time on a 0.3% agar plate. The same plate was photographed after (**A**) 2, (**B**) 4, (**C**) 5, (**D**) 6, (**E**) 7, (**F**) 9, and (**G**) 13 days at 25°C. (**H**) The same *MAT*α strain on a 2% agar YPD plate after 13 days at 25°C. (I) to (K) Mating type affected the morphology of mats. Compare the *MAT***a** strain (**I**) with the *MAT*α strain (G), both grown for 13 days on YPD-0.3% agar. (**J** and **K**) A *MAT***a**/α diploid is delayed in spoke appearance [compare (E, MATα) and (J, MAT**a**/α), both at 7 days of growth]. By 13 days the diploid resembles the haploids [compare (G, MATα), (I, MAT**a**), and (K, MAT**a**/α)]. (**L**) *FLO11* function is required for mat formation. A *flo11*Δ strain after 13 days of growth on a YPD–0.3% agar plate. Bar, 1 cm.

ploidy (*18*). Moreover, the adherence of strains to agar also decreased with ploidy, but could be restored by overexpression of *FLO11* (*18*). We found that the diameter of the mat and the other detailed features of mat architecture decreased as ploidy increased. The alteration in phenotype was pronounced in tetraploid strains, which have four copies of *FLO11* but resembled the attenuated, amorphous structure of *flo11*Δ strains (Fig. 4).

The reproducible structure of the yeast mats raises important questions about its origins. The radial symmetry and the reproducibility of the number of spokes appear to be the hallmarks of a programmed developmental event, but are strongly influenced by the environment. Our data show that the viscosity of the medium, the Flo11p protein on the surface of the yeast cells, and the nutrients in the medium all contribute to the development of this unusual structure.

Although Flo11p is required for both adherence to plastic and mat formation, the molecular basis for this connection remains unclear. One possible explanation for the role of *FLO11* in the adherence of yeast cells to plastic, the multicellular morphological behaviors on 0.3% agar plates, and invasive growth is that Flo11p promotes both cell-cell adhesion and cell-surface adhesion. Previous work has shown that Flo11p is required for cells to adhere to each other in filamentous growth and for cells to adhere to the agar (*6*, *7*). Flo11p may have properties distinct from those of other yeast cell surface proteins that enable it to initiate biofilm formation. For example, Flo1p, a related cell surface protein, promotes avid cell-cell adhesion but not cellular adhesion to an inert surface (*7*).

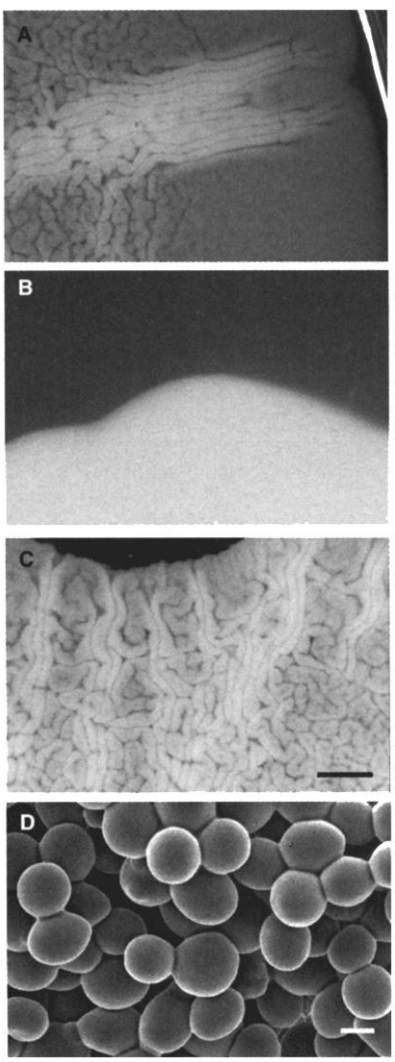


**Fig. 3.** Structure of the *Saccharomyces* mats. (**A**) The parallel cables in this figure formed the white spokes seen in Fig. 2G (*MAT*α). The spokes seemed to emanate from the network of cables originating in the hub. The lighter color of the spoke contrasted with the smoother edge of the mat. (**B**) The *flo11*Δ mat (Fig. 2L) was smooth with no substructure. (**C**) The *MAT***a** strain (Fig. 2I) had a network of cables that extended to the edge of the mat and formed narrower and more frequent spokes. The photographs in (A), (B), and (C) were made at 2.5× magnification through a Technival 2 dissecting microscope. Bar, 2 mm. (**D**) A scanning electron micrograph of the yeast-form cells that comprise the *MAT*α mat was made at 5500× magnification. Bar, 2 μm.

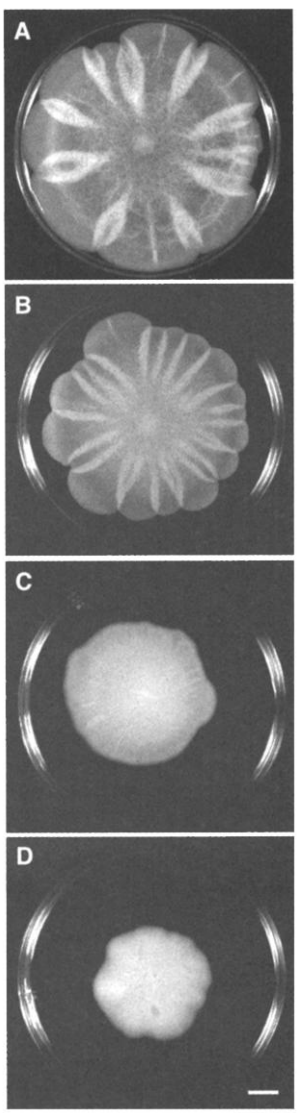


**Fig. 4.** Increased ploidy reduces mat formation by *Saccharomyces*. A series of isogenic strains from haploid to tetraploid (*18*) on YPD-0.3% agar plates after 13 days at 25°C. Previous work (*18*) had shown that the level of *FLO11* as well as the *FLO11*-dependent agar invasion phenotype of Σ1278b decreased as the ploidy increased. (**A**) *MAT*α, (**B**) *MAT*αα, (**C**) *MAT*ααα, and (**D**) *MAT*αααα. Bar, 1 cm.

As noted above, Flo11p may play a role similar to that of the *M. smegmatis* GPLs, which are thought to be required for biofilm formation and sliding function because they increase surface hydrophobicity (*10*, *11*). Indeed, when we measured the hydrophobicity of the *FLO11* and *flo11*Δ strains by their ability to partition between water and octane (*19*), we found that only 12% of the *FLO11* cells partitioned to the aqueous phase as compared with 91% of the *flo11*Δ cells, indicating that *FLO11* cells were more hydrophobic. Flo11p might therefore increase the adherence of *Saccharomyces* to the wells of plastic 96-well plates. The same hydrophobic property may facilitate mat formation on 0.3% agar plates by reducing the interaction of yeast cells with the aqueous surface. Decreasing the adhesion of the cells to the plate's surface would promote the movement of the cells across the plate. Presumably, the patterns arise from a combination of the frictional forces and the cell-cell interactions. The effect of glucose concentration on the development of these various phenotypes is also likely to be related to the repression of *FLO11* transcription by glucose (*20*).

Adherence to plastic is only the initial stage of biofilm formation. In some organisms such as *Pseudomonas aeruginosa* the biofilm matures to form microcolonies of bacteria that are surrounded by an extracellular matrix (*1*). Whether the yeast biofilms described here are a prelude to further developmental events is not yet clear. However, our preliminary analyses of *Saccharomyces* in another assay of biofilm formation (*3*) suggest that the yeast biofilms may undergo some maturation (*21*).

Pathogenic fungi such as *Candida albicans* and *Candida glabrata* have orthologs of Flo11p and form mats (*22*), and *C. albicans* forms biofilms (*1*, *3*). The Flo11p orthologs have been postulated to be virulence genes because when expressed in *Saccharomyces* they confer the ability to adhere to mammalian cells (*23*, *24*). However, because these pathogenic fungi have many redundant copies of the genes encoding these cell surface glycoproteins, it is difficult to investigate their virulence function by mutational analysis (*24*, *25*). The discovery that *Saccharomyces* can undergo the initial steps of biofilm formation suggests that it may be a useful model for the genetic dissection of the

role of these cell surface proteins in pathogenesis. In addition, *Saccharomyces* may be a valuable tool for screening compounds that block fungal adhesion, a possible new avenue to antifungal therapy.

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5. PVC 96 well plates (Falcon Microtest III flexible assay plates, 35-3912; Becton Dickinson) were used to test adhesion by essentially the same assay used for the polystyrene plates. Polypropylene microfuge tubes (Eppendorf flex-tube, 1.5 ml) were used to test adhesion to this type of plastic. In this instance, 400 μl of cells were used because the results were easier to judge macroscopically.
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12. The results were obtained by growing 11 independent mats and measuring the diameter and number of spokes for each mat daily.
13. A *FLO11 MATα* strain grown for 12 days at 25°C produced an average of $8.1 \times 10^9$ cells on YPD−0.3% agar and an average of $1.1 \times 10^9$ cells when grown on YPD−2% agar under the same conditions. A *MATα* *flo11Δ* strain grown for 12 days at 25°C produced an average of $5.3 \times 10^9$ cells on YPD−0.3% agar and an average of $1.6 \times 10^9$ cells when grown on YPD−2% agar. Cell number was determined by transferring the cells from plates to 15-ml conical tubes (Falcon 35-2096) or microfuge tubes. The cell mass was suspended in water, the suspension was diluted, and the optical density at 600 nm ($OD_{600}$) was measured. The number represents the average of three plates for each strain at each agar concentration.
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22. *Candida albicans* formed a mat on 0.3% agar plates with a reproducible morphology that lacked spokes, but had a hub that was distinct from that found in *S. cerevisiae* mats. The mats formed by *C. glabrata* lacked hubs and spokes.
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26. Synthetic complete media and plates and YPD plates were made as described [C. Guthrie, G. R. Fink, *Methods Enzymol.* **194**, 12 (1991)] with the exception of the altered agar or glucose concentrations, which are specified in the text.
27. The adherence of cells was quantitated by solubilizing the crystal violet with 100 μl of 10% SDS. After 30 min, 100 μl of $H_2O$ was added, the solution was mixed by pipetting, and 50 μl was transferred to a fresh polystyrene 96-well plate (Flat-bottom Nunc-MicroWell plate, 269620, Nalge Nunc International). The absorbance at 570 nm ($A_{570}$) was then monitored with a Dynatech MR600 microplate reader. In some experiments, after the cells were added, the plates were centrifuged at 3000 rpm for 5 min before the assay as a control for differences observed between strains due to differences in the rates at which the cells settled to the bottom of the plate. The general trends shown in Fig. 1, B and C, were observed regardless of whether or not the plates were centrifuged before the assay.


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# Molecular Analysis of Commensal Host-Microbial Relationships in the Intestine


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**Human beings contain complex societies of indigenous microbes, yet little is known about how resident bacteria shape our physiology. We colonized germ-free mice with *Bacteroides thetaiotaomicron*, a prominent component of the normal mouse and human intestinal microflora. Global intestinal transcriptional responses to colonization were observed with DNA microarrays, and the cellular origins of selected responses were established by laser-capture microdissection. The results reveal that this commensal bacterium modulates expression of genes involved in several important intestinal functions, including nutrient absorption, mucosal barrier fortification, xenobiotic metabolism, angiogenesis, and postnatal intestinal maturation. These findings provide perspectives about the essential nature of the interactions between resident microorganisms and their hosts.**


Human beings harbor an incredibly complex and abundant ensemble of microbes. We are in contact with components of this microflora from birth, yet little is known about their influence on normal development and physiology. The human intestine is more densely populated with microorganisms than any other organ and is a site where the microflora may have a pronounced impact on our biology. We tested this idea at a molecular level using a simplified mouse model of the interactions between gut commensals and their host. In this model, adult germ-free animals are colonized with *Bacteroides thetaiotaomicron*. This anaerobe was chosen because it can be genetically manipulated and is a prominent member of the adult mouse and human


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