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Functional Characterization of the Antibiotic Resistance Reservoir in the Human Microflora

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To understand the process by which antibiotic resistance genes are acquired by human pathogens, we functionally characterized the resistance reservoir in the microbial flora of healthy individuals. Most of the resistance genes we identified using culture-independent sampling have not been previously identified and are evolutionarily distant from known resistance genes. By contrast, nearly half of the resistance genes we identified in cultured aerobic gut isolates (a small subset of the gut microbiome) are identical to resistance genes harbored by major pathogens. The immense diversity of resistance genes in the human microbiome could contribute to future emergence of antibiotic resistance in human pathogens.

Multiple antibiotic resistance in human pathogens has increased over the past decades and challenged our ability to treat bacterial infections (*1*, *2*). For example, methicillin-resistant *Staphylococcus aureus* (MRSA) caused 18,964 mortalities in the United States in 2006 (*3*). The comparison with 14,627 AIDS-related mortalities that occurred in the same year (*4*) highlights the public health importance of just one multiresistant bacterial pathogen in an industrialized nation. Whole-genome sequencing of bacteria has revealed that many of the resistance genes harbored by these strains have not evolved within the sequenced strain but were acquired by lateral gene transfer events (*5*). Antibiotic resistance determinants encoded on mobilizable elements move between diverse bacteria to disseminate resistance genes into a variety of interacting microbial communities (*6*, *7*). Consequently, there is an increasing interest in elucidating reservoirs of mobile antibiotic resistance genes that may be accessible to clinically relevant pathogens (*8*–*10*).

The human microbiome substantially impacts human health and plays beneficial roles in dietary processing and prevention of pathogen intrusion (*11*–*15*). The widespread use of antibiotics in human medicine and agriculture has likely induced substantial responsive changes in this community. Phylogenetic analysis of human and mice gut microbiomes reveal substantial transient increases in the Proteobacteria at the expense of Firmicutes and Bacteroidetes during antibiotic treatment (*16*, *17*). Although the phylogenetic distribution of the human gut microbiome was found to largely reestablish after ciprofloxacin treatment (*18*), it has likely been enriched in resistance to this antibiotic. Indeed, persistent clarithromycin resistance in commensal enterococci has been observed years after cessation of antibiotic therapy (*19*), and resistance toward amoxicillin has been observed in cultured oral bacterial isolates from children not exposed to antibiotic therapy (*20*). Previous work has also shown that the abundance of *ermB* and *tetQ*, encoding resistance to erythromycin and tetracycline, respectively, has increased in frequency in cultured human *Bacteriodes* isolates during the past three decades and that *ermB* has been exchanged between distantly related cultured commensal gut bacteria (*21*). These findings suggest that the human microflora could constitute a substantial reservoir of antibiotic resistance genes accessible to pathogens (*22*, *23*). We used functional selections coupled with metagenomic analysis to characterize the antibiotic resistance genes in the oral and gut microflora of healthy individuals.

We isolated DNA directly from saliva and fecal samples from two unrelated healthy humans who had not been treated with antibiotics for at least 1 year (*24*). One- to three-kilobase fragments of the purified metagenomic DNA were cloned into an expression vector and transformed into an *Escherichia coli* host strain, resulting in libraries of a total size of 9.3×10^9 base pairs. Antibiotic-resistant clones from each library were selected by plating on Luria broth agar containing one of 13 antibiotics belonging to the classes amino acid derivatives, aminoglycosides, amphenicols, beta-lactams, and tetracyclines, at concentrations where the wild-type host strain is susceptible (table S1) (*24*). Inserts conferring resistance to all 13 antibiotics tested were sequenced and annotated, enabling the discovery of 95 unique inserts containing functional antibiotic resistance genes (table S3) (*24*).

On average, the sequence similarity of the resistance genes we obtained and the closest related gene in GenBank is 69.5% at the nucleotide level (Fig. 1A) and 65.3% at the amino acid level (Fig. 1B) (*24*). A minority (22%) had high homology (>90% amino acid identity) to previously known genes, including identical matches to the *tet(Q)-3* gene previously identified in cultured *Bacteriodes* isolates from the human colon (*21*) and the *CTX-M-15* enzyme, which over the past decade has become one of the most prevalent extended-spectrum beta-lactamases (Table 1) (*25*). Most of the closely related homologs are derived from commensals including nonpathogenic species such as

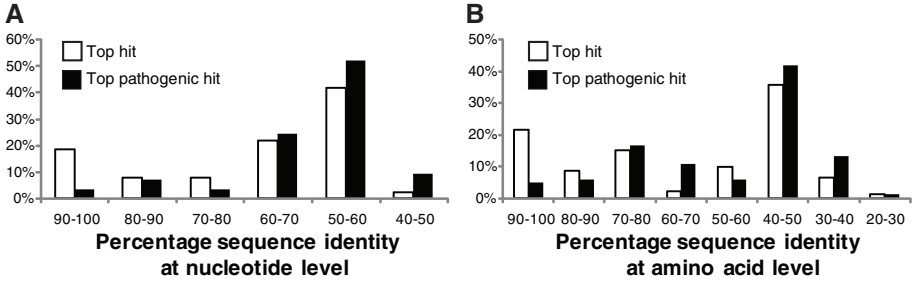


Fig. 1. Distributions of (**A**) nucleotide identities and (**B**) amino acid identities for 93 resistance genes identified from DNA extracted directly from saliva and fecal samples to the most similar resistance gene from any organism (white bars) as well as the most similar resistance gene harbored by a pathogenic isolate (black bars) in GenBank (table S3) (*24*). Two resistance genes with no significant similarity to sequences in GenBank are not shown.

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Bifidobacterium longum (*26*), as well as commensals with the capacity to become opportunistic pathogens such as *Bacteroides fragilis* and *Bacteroides uniformis* (table S3) (*21*). Interestingly, we also identified genes that encode proteins that are 100% identical to hypothetical proteins of unverified function in GenBank, for example, BACUNI_02013 from *Bacteroides uniformis* ATCC 8492, which we show encodes resistance to broad-spectrum beta-lactams such as amoxicillin and carbenicillin, and the third-generation oxyimino-cephalosporin cefdinir (table S3) (*27*). This highlights the utility of a functional selection approach to improve annotation of genomic and metagenomic sequencing data from the human microbiome project (*28*).

Most of the antibiotic resistance genes harbored by the human microflora were distantly related (60.7% at the nucleotide level and 54.9% at the amino acid level) to antibiotic resistance genes so far detected in pathogenic isolates (Fig. 1 and table S3) (*24*). In total, we identified 78 unique inserts with genes with low homology (<90% amino acid identity) to proteins in GenBank, encoding resistance to the 13 antibiotics profiled (tables S1 and S3). This may imply that the resistance genes of the human microbiome are inaccessible or infrequently exchanged with human pathogens; however, all the resistance genes we identified in this study were functional in *E. coli*, which suggests that if a barrier to gene transfer exists between the constituents of the human microbiome and pathogens, it must stem from processes other than functional compatibility.

Phylogenetic analysis of the inserts using PhyloPythia (*29*) indicates that they predominantly originate from the phyla Bacteroidetes and Firmicutes, which dominate the gut flora (*14*). However, the majority of the genes we discovered have low sequence identity to resistance genes previously identified in pathogens from these phyla (e.g., *Staphylococcus aureus* and *Streptococcus pneumoniae*), as well as from the numerous pathogens that are readily culturable facultative anaerobic bacteria from the phylum Proteobacteria. Although commensal Proteobacteria constitute less than 1% of the human gut microflora (*14*), they increase in abundance during antibiotic treatment at the expense of the normally abundant Bacteroidetes and Firmicutes (*16*, *17*). As a consequence of their normal low abundance in healthy individuals, they are not well represented in unbiased metagenomic libraries.

We isolated 572 bacterial strains on rich media under aerobic conditions from fecal samples from two healthy individuals (*24*). Phylogenetic profiling revealed that they belonged primarily to Proteobacteria, with a few Firmicutes and Actinobacteria (fig. S1). The isolates from individuals 1 and 2 were on average resistant to 9 and 5 out of 13 antibiotics, respectively (Fig. 2, C and D). Chloramphenicol and minocycline were the only antibiotics tested that were able to prevent the growth of more than 99% of the isolates (Fig. 2, A and B, and figs. S2 and S3).

Functional selections identified 115 unique inserts encoding transferable antibiotic resistance genes from the cultured aerobic gut microbiome isolates (Fig. 3 and table S4) (*24*). We found that 95% of these genes are over 90% identical at the nucleotide level to resistance genes in pathogenic isolates, and almost half of these genes were 100% identical (Fig. 3A), indicating an evolutionarily close relationship to the resistance genes harbored by clinical pathogens.

The group of resistance genes identical to those in pathogens belong to one class of tetracycline efflux pumps (*TetA*), two classes of aminoglycoside-modifying enzymes [*AAC(3)-II* and *AAC(6)-Ib*], and three classes of beta-lactam–inactivating enzymes (*TEM*, *AmpC*, and *CTX-M*) (Fig. 4). We identified a *TEM-1* gene variant (Fig. 4 and Table 1) in cultured isolates from one gut microbiome on every sampling time (*24*) that has recently been reported in pathogenic strains of

Table 1. Unique beta-lactamase genes identified from gut and oral microbiomes from healthy humans. Gene ID refers to unique identifier in tables S3 and S4, and enzyme names use established nomenclature (*24*, *32*). Percentage amino acid identity to the closest related gene in GenBank is calculated using the global alignment program clustalW (*24*, *33*). NCBI, National Center for Biotechnology Information.

Beta-lactamase family	Enzyme name	Gene ID	GenBank ID	Source	Amino acid identity to NCBI (%)
AmpC	AmpC-EcolK12	AX_iG2_08	GQ343010	Aerobic gut isolate	100.0
	AmpC-EC6	PE_iG1_02	GQ343155	Aerobic gut isolate	100.0
	AmpC-EC31	PE_iG2_05	GQ343162	Aerobic gut isolate	100.0
	AmpC-HG1	AX_iG2_21	GQ343018	Aerobic gut isolate	99.7
	AmpC-HG2	CA_iG2_12	GQ343059	Aerobic gut isolate	99.5
	AmpC-HG3	CF_iG2_01	GQ343071	Aerobic gut isolate	90.0
	AmpC-HG4	CF_iG2_06	GQ343073	Aerobic gut isolate	97.4
	AmpC-HG5	CA_iG1_06	GQ343055	Aerobic gut isolate	99.2
TEM	TEM-1b	AX_iG1_01	GQ343004	Aerobic gut isolate	100.0
	TEM-168	PE_iG2_13	GQ343167	Aerobic gut isolate	99.7
	TEM-169	PI_iG2_05	GQ343173	Aerobic gut isolate	99.1
CTX-M	CTX-M-15	AX_iG1_04	GQ343005	Aerobic gut isolate and metagenomic gut sample	100.0
CblA	CblA-1	AX_iG2_02	GQ343019	Aerobic gut isolate and metagenomic gut sample	100.0
	CblA-2	AX_mG2_03	GQ342999	Metagenomic gut sample	99.7
	CblA-3	PE_mG2_02	GQ343154	Metagenomic gut sample	99.0
CfxA	CfxA6	AX_mG2_01	GQ342996	Metagenomic gut sample	87.2
HGA	HGA-1	CA_mG1_02	GQ343038	Metagenomic gut sample	61.4
HGB	HGB-1	AX_mG2_05	GQ343000	Metagenomic gut sample	58.5
HOA	HOA-1	AX_mO1_01	GQ343035	Metagenomic saliva sample	49.5
HGC	HGC-1	CA_mG1_01	GQ343037	Metagenomic gut sample	48.1
	HGC-2	CA_mG1_04	GQ343039	Metagenomic gut sample	51.0
HGD	HGD-1	CA_mG2_04	GQ343044	Metagenomic gut sample	52.9
HGE	HGE-1	AX_mG2_11	GQ343003	Metagenomic gut sample	37.1
HGF	HGF-1	AX_mG2_09	GQ343002	Metagenomic gut sample	43.3
HGG	HGG-1	PE_mG1_01	GQ343153	Metagenomic gut sample	38.8
HGH	HGH-1	PI_mG1_01	GQ343170	Metagenomic gut sample	34.5
HGI	HGI-1	CA_mG2_07	GQ343045	Metagenomic gut sample	42.6

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E. coli, Salmonella enterica, Klebsiella pneumonia, Haemophilus parainfluenzae, Serratia marcescens, Pseudomonas aeruginosa, and *Neisseria meningitidis* isolated around the globe (table S2). Nearly 80% of the depositions of this *TEM-1* variant to GenBank have occurred between 2007 and 2008, which seems to indicate a relationship between the emergence of this resistance gene variant in the clinic and its occurrence in healthy humans.

The *AmpC* and *CTX-M* family of enzymes are extended-spectrum beta-lactamases that hydrolyze a wider variety of later generation beta-lactams (Table 1) (*30*). We identified the *CTX-M-15* beta-lactamase (Fig. 4 and Table 1) in libraries from cultured gut microbiome isolates across multiple sampling days, as well as in our metagenomic libraries from the same microbiome.

Global sequence alignments of each of the 27 unique beta-lactamase sequences from our study identified 15 distinct sequence groups (Fig. 4). Of these groups, 5 were previously characterized (*CblA*, *CfxA, CTX-M*, *TEM*, and *AmpC*) (*31*), whereas 10 constitute previously unknown beta-lactamase sequence families because they are between only 35% and 61% identical at the amino acid level to any gene products in GenBank (Fig. 4). Interestingly, the known beta-lactamase genes we identified were from the cultured microbiome isolates, but the 10 previously unidentified gene families were identified solely by our culture-independent characterization (Fig. 4). In general, we found that the metagenomically derived resistance genes in our study were more distantly related to previously identified genes than those derived from aerobic gut isolates (Figs. 1 and 3 and figs. S5 and S6).

Of our 210 unique microbiome-derived inserts encoding antibiotic resistance, we found a subset of 29 that also contained genes similar (>96% nucleotide sequence identity) to previously characterized transposases (table S6). Of these, 14 transposases were identical to those previously identified on resistance and conjugative plasmids from *Bacteroides fragilis* and clinical pathogenic isolates of *Klebsiella pneumoniae*, *Escherichia coli*, and *Salmonella enterica* (table S6). Interestingly, the proportion of identified transposases derived from the culturable aerobic isolates (90%) was significantly higher than those derived from metagenomic sampling (Pearson's chi-square test, $P < 0.0005$).

Nearly half of the resistance genes identified in the cultured human gut isolates were identical at the nucleotide level to resistance genes from human pathogenic isolates. Although this identity provides no information regarding the direction or mechanism of transfer, we can offer some speculation regarding the implications of our findings. First, the human microbiome may constitute a mobilizable reservoir of antibiotic resistance genes that are accessed by a pathogenic bacterium to acquire antibiotic resistance, although direct experimental proof of in vivo transfer of antibiotic resistance genes within the human microbiome remains to be shown. Second, despite selecting samples from untreated healthy humans, the aerobic cultured isolates may be dormant pathogens inhabiting the human microbiome. Third, by contrast with the cultured isolates, the resistance genes discovered by the culture-independent approach were distantly related to resistance genes from even closely related pathogenic isolates, which may reflect an unappreciated barrier to lateral gene transfer in vivo between the dominant commensals in healthy humans and disease-causing isolates. Fourth, this work exposes previous substantial undersampling of antibiotic resistance genes in the human microbiome.

We found many microbial DNA fragments encoding resistance genes that have never before been described, and our analysis suggests that we have just begun to scratch the surface of the im-

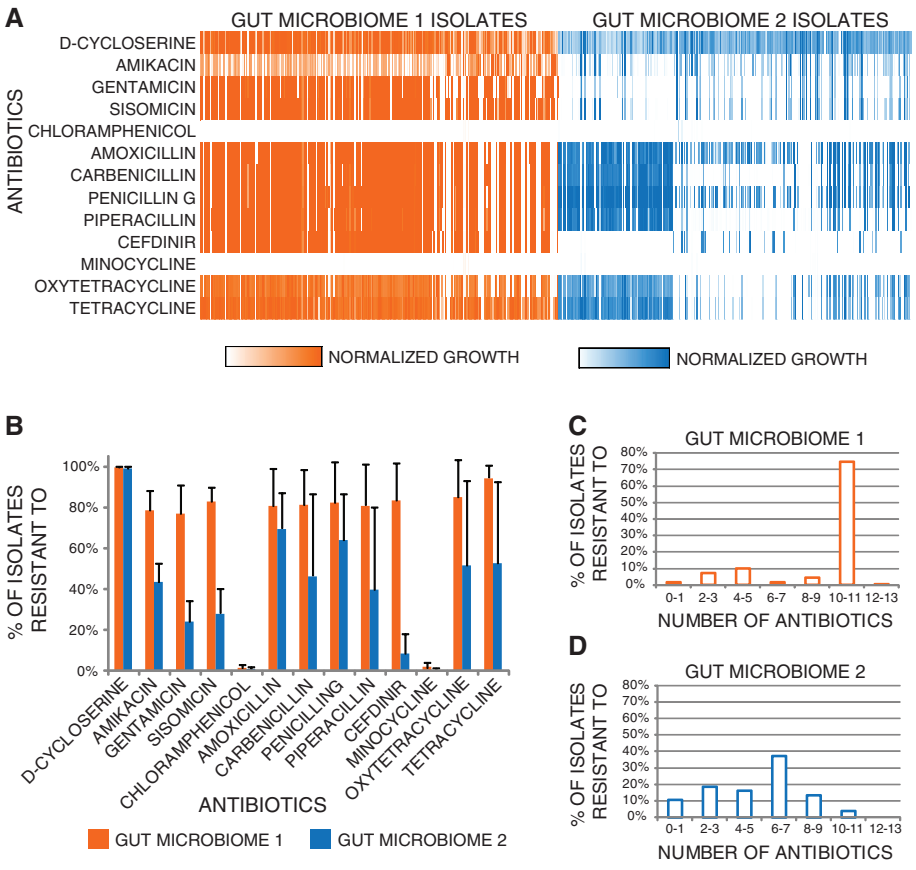


Fig. 2. Antibiotic resistance profiles of cultured aerobic gut microbiome isolates. (**A**) Heat map displaying resistance profiles of 572 aerobic bacterial isolates obtained across three different sampling times from two human gut microbiomes. Linear color-scaled bars display 7436 growth measurements of aerobic cultured microbiome isolates after 24 hours at 37°C in Luria broth containing one of 13 antibiotics at concentrations between 20 and 100 μg/mL that prevent the growth of wild-type *E. coli* (table S1). White denotes no growth, and color intensity is proportional to growth in the presence of antibiotic, scaled to growth in the absence of antibiotic per individual isolate. (**B**) Percentage of aerobic gut isolates resistant to each of 13 antibiotics. Each data point represents the mean number of isolates resistant to each antibiotic, and error bars represent the standard deviation of this mean value from each of the three sampling times. Histograms depict the distribution of the number of different antibiotics that aerobic (**C**) gut microbiome 1 and (**D**) gut microbiome 2 isolates are resistant to.

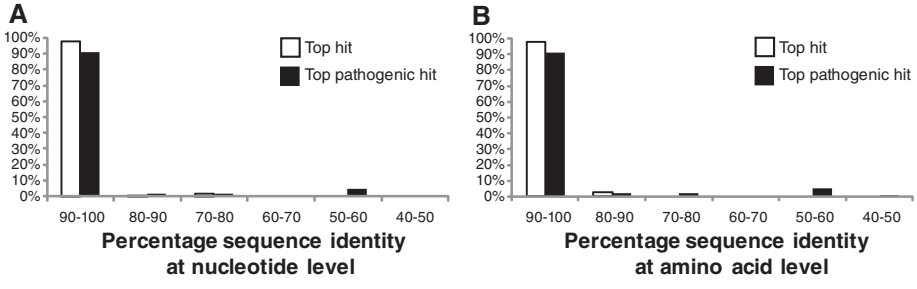


Fig. 3. Distributions of (**A**) nucleotide identities and (**B**) amino acid identities for 114 resistance genes identified from cultured aerobic gut isolates to the most similar resistance gene from any organism (white bars) as well as the most similar resistance gene harbored by a pathogenic isolate (black bars) in GenBank (table S4) (*24*). One resistance gene with no significant similarity to sequences in GenBank is not shown.

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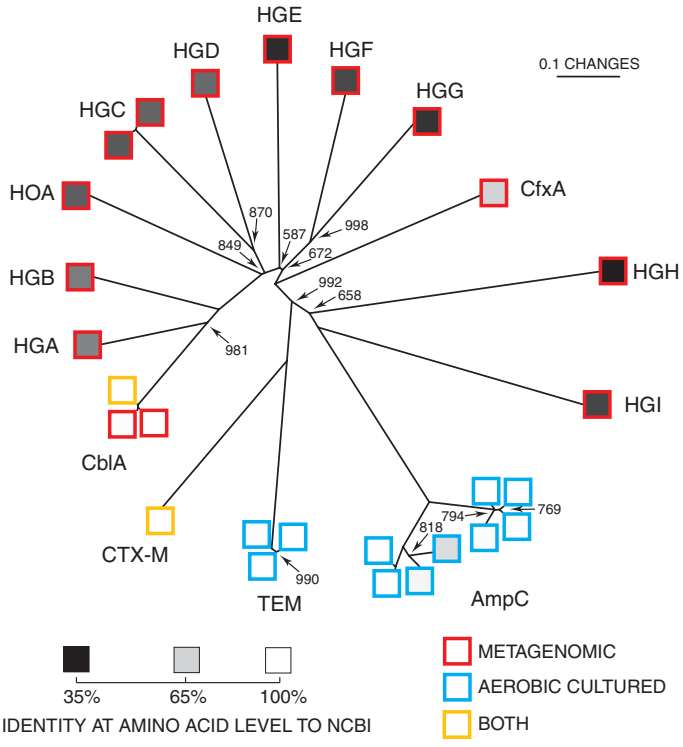


Fig. 4. The phylogenetic relationship of unique beta-lactamases derived from gut and oral microbiomes from healthy humans is displayed as an unrooted neighbor-joining tree (*24*). Except for nodes indicated, bootstrap values = 1000. Scale bar is in fixed amino acid substitutions per sequence position. Border colors of squares denote sequences derived from cultured aerobic gut isolates (blue), metagenomic DNA (red), or both (yellow). Internal shading of each square represents percentage amino acid identity to the most similar sequence in GenBank, with a linear gradient between 100% identity (white) and 35% identity (black). Sequence groups are labeled according to standard nomenclature (Table 1) (*24*, *32*).

mense diversity of antibiotic resistance machinery in the human microbiome. More than half of the inserts that were derived from metagenomic libraries and libraries from cultured gut aerobes were sequenced only once in our experiment (fig. S4), and we estimate that complete sequencing of these libraries would yield hundreds more resistance genes (*24*). Interestingly, when we compared the resistance genes derived from the microbiomes of the two different individuals, we found that over 65% of the resistance genes derived from cultured aerobes were highly similar (>90% nucleotide sequence identity) between the two individuals, whereas less than 10% of the metagenomically derived resistance genes were highly similar between the individuals (table S7) (*24*).

Many commensal bacterial species, which were once considered relatively harmless residents of the human microbiome, have recently emerged as multidrug-resistant disease-causing organisms (*7*). In the absence of in-depth characterization of the resistance reservoir of the human microbiome, the process by which antibiotic resistance emerges in human pathogens will remain unclear.

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Supporting Online Material

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Motile Cilia of Human Airway Epithelia Are Chemosensory

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Cilia are microscopic projections that extend from eukaryotic cells. There are two general types of cilia; primary cilia serve as sensory organelles, whereas motile cilia exert mechanical force. The motile cilia emerging from human airway epithelial cells propel harmful inhaled material out of the lung. We found that these cells express sensory bitter taste receptors, which localized on motile cilia. Bitter compounds increased the intracellular calcium ion concentration and stimulated ciliary beat frequency. Thus, airway epithelia contain a cell-autonomous system in which motile cilia both sense noxious substances entering airways and initiate a defensive mechanical mechanism to eliminate the offending compound. Hence, like primary cilia, classical motile cilia also contain sensors to detect the external environment.

Cilia can be divided into two general types, primary and motile (*1*–*3*). Primary cilia are sensory organelles that contain a variety of receptors, including G protein–coupled receptors (*1*). Cells typically sprout a single primary cilium with a characteristic 9+0 cytoskeletal

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