Motile Cilia of Human Airway Epithelia Are Chemosensory

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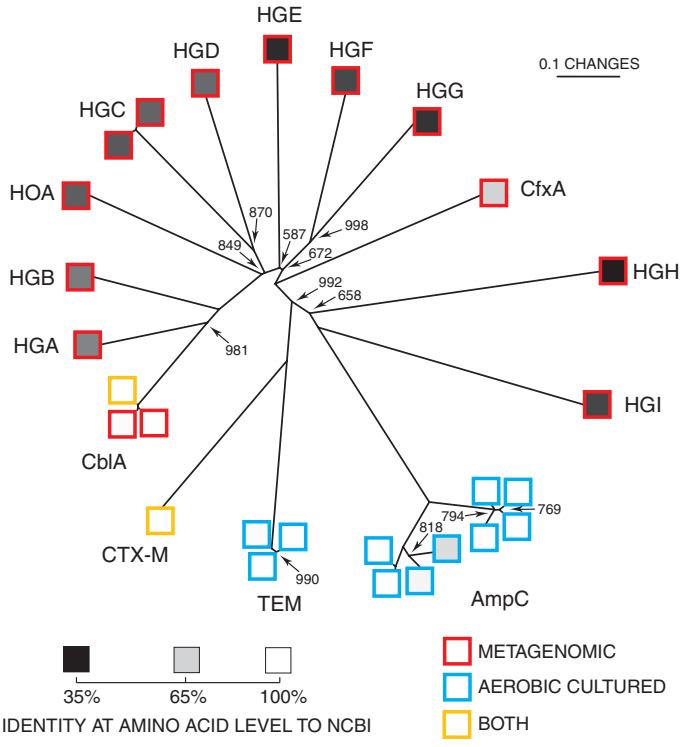

Fig. 4. The phylogenetic relationship of unique beta-lactamases derived from gut and oral microbiomes from healthy humans is displayed as an unrooted neighbor-joining tree (*24*). Except for nodes indicated, bootstrap values = 1000. Scale bar is in fixed amino acid substitutions per sequence position. 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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34. We acknowledge J. Davies, J. Aach, and M. Strong for helpful discussions regarding this manuscript; and the expert assistance of G. Jacoby and K. Bush for beta-lactamase enzyme family nomenclature, L. Kraal and G. Rockwell for sequence manipulations and similarity computations, and S. Caliri, A. Ellison, and T. Ellison for microbial culturing and DNA processing. We acknowledge National Human Genome Research Institute Centers of Excellence in Genomic Science, Personal Genome Project, Bill and Melinda Gates Foundation, Harvard Biophysics Program, Hartmann Foundation, and Det Kongelige Danske Videnskabernes Selskab for funding. GenBank accession numbers GQ342978 to GQ343187 are listed in Table 1 and tables S3, S4, and S7. M.O.A.S. and G.M.C. advise many companies, listed in (*24*); none is working within fields related to the subject of the manuscript.

Supporting Online Material

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Materials and Methods
Figs. S1 to S7
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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

structure (axoneme). In contrast, motile cilia can occur in the tens and hundreds on epithelial cells, have a typical 9+2 axoneme, and serve a mechanical role (*1*–*3*). Although their functions differ, there are exceptions to the primary cilia–motile cilia distinction (*3*); for example, primary nodal cilia exhibit rotational movement (*4*, *5*). In addition, the two types of cilia share many structural and molecular features (*2*, *6*); they are evolutionarily related (*7*); and genetic diseases such as Bardet-Biedl Syndrome can affect both (*8*–*10*). However, whether classic motile cilia can also function as sensory organelles is not clear.

On airway epithelia, motile cilia move mucus out of the lung, and their disruption causes airway disease (*11*, *12*). Because defense of the airway involves detection of danger signals, we hypothesized that motile cilia might sense noxious stimuli. To test this hypothesis, we searched for sensory-related genes in microarray expression data from primary cultures of differentiated human airway epithelia (*13*). Microarray analysis (*14*) and reverse transcription–polymerase chain reaction (RT-PCR) revealed that the epithelia expressed several members of the bitter taste receptor (T2R) family (Fig. 1, A and B). The human genome contains ~25 *T2R* genes. Several T2Rs may detect more than one different bitter compound, and individual taste receptor cells express multiple T2Rs (*15*, *16*). Thus, in the tongue, the system is broadly tuned to detect multiple bitter compounds and warn against ingestion of toxic substances.

We studied several T2Rs for which antibodies are available and/or for which ligands are known, and we identified T2R4, T2R43, T2R38, and T2R46 in human airway epithelia (Fig. 2, A to C, and figs. S1 to S6). Only ciliated epithelial cells expressed these receptors, and they specifically localized in cilia, which were identified by acetylated α-tubulin immunostaining. Almost every ciliated cell expressed each of the four T2Rs, although the intensity varied from cell to cell and in some ciliated cells T2R43 was not detected (Fig. 2B). Thus, single ciliated epithelial cells express multiple T2Rs, similar to individual taste receptor cells that express multiple T2Rs (*17*). The receptors appeared to localize preferentially at different places along the cilium. For example, T2R4 seemed to sit nearer the tips of cilia, whereas T2R43 appeared to reside more proximally in cilia (Fig. 2, A and B, and figs. S1, S3, and S7), which was more apparent

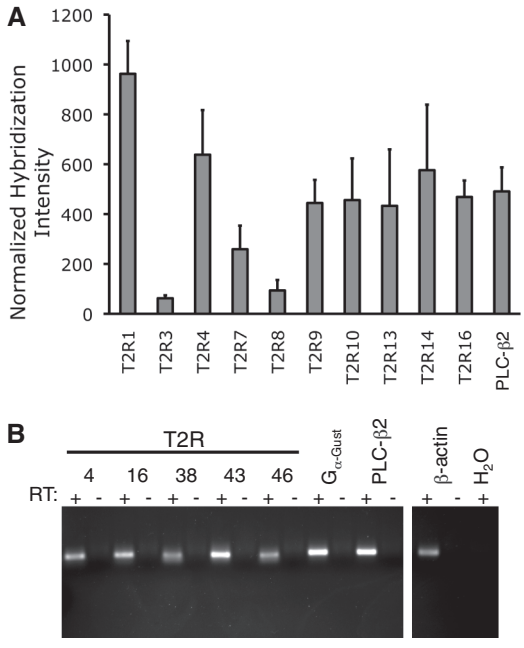

Fig. 1. Bitter taste receptors are expressed in human airway epithelia. (**A**) Microarray analysis of mRNA isolated from airway epithelial samples isolated from eight human donors (*14*, *31*). Data are the mean ± SEM. (**B**) RT-PCR analysis of mRNA isolated from differentiated cultures of human airway epithelia.

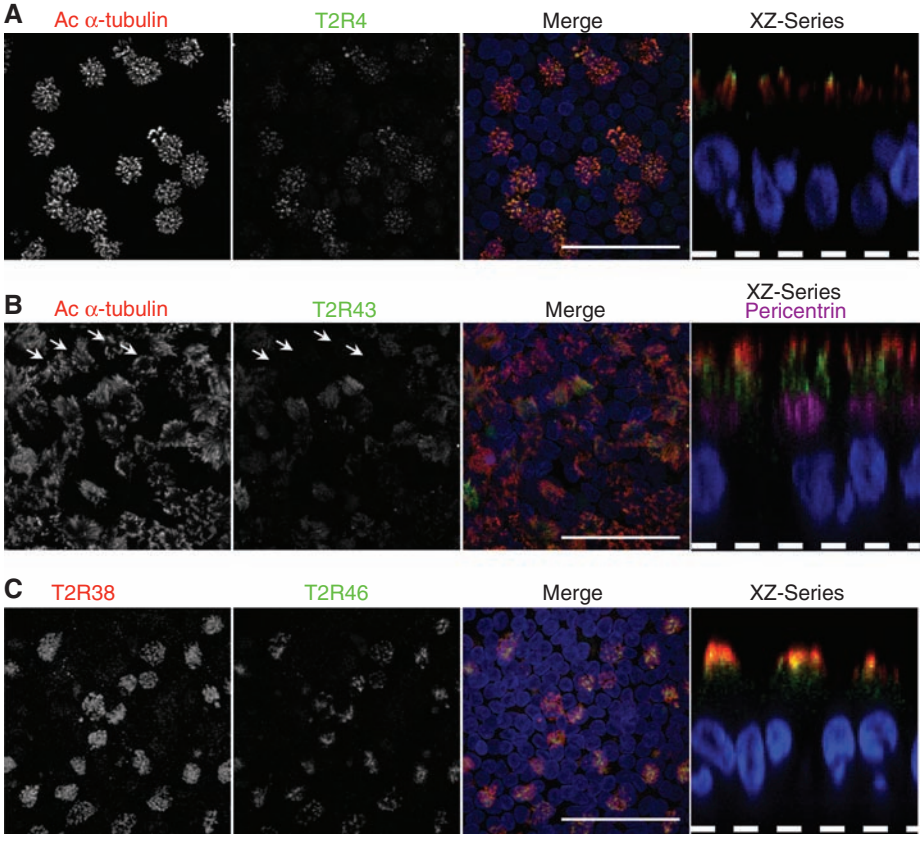

Fig. 2. T2Rs localize along the motile cilia of airway epithelia. Confocal immunofluorescence microscopy of cultured human airway epithelia with antibodies to T2R (anti-T2R) showed that (**A**) T2R4 and (**B**) T2R43 (both green) localize to motile cilia, which were identified by antibodies to acetylated α-tubulin (red). Nuclei stained with DAPI (4′,6-diamidino-2-phenylindole) are in blue. Arrows in (B) indicate the location of ciliated cells that were not labeled by anti-T2R43. (**C**) T2R38 (red) and T2R46 (green) localize to motile cilia. Data are stacks of confocal z-series images in the *X-Y* plane and a single *X-Z* plane image (right; the dashed line indicates the filter). (B) (right) also shows antibodies to pericentrin (purple), which label the basal body below the cilia. Scale bars, 20 μm. See also figs. S1 to S7.

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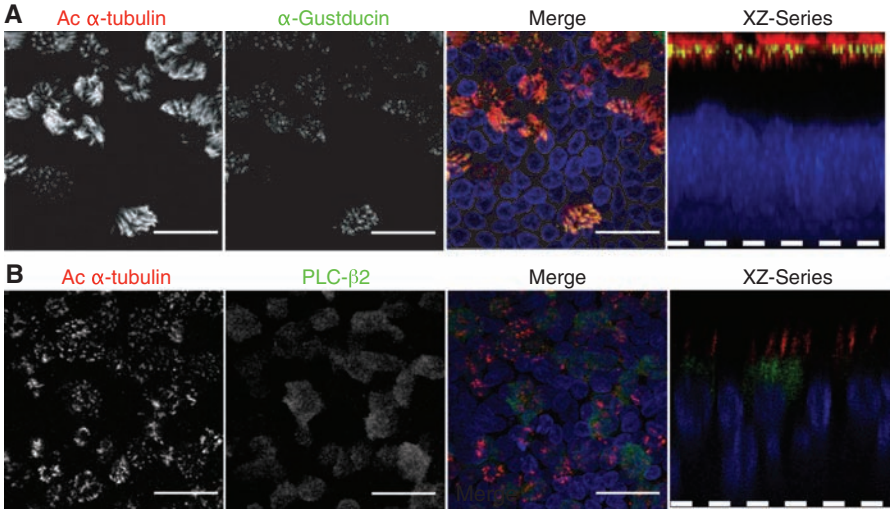

Fig. 3. Components of the bitter taste transduction pathway localize to ciliated cells and to the cilia. Immunolocalization of (**A**) α-gustducin and (**B**) PLC-β2 are in green and acetylated α-tubulin is in red. Other aspects of the figure are as described in the legend to Fig. 2. Scale bars, 20 μm. See also figs. S1 to S3.

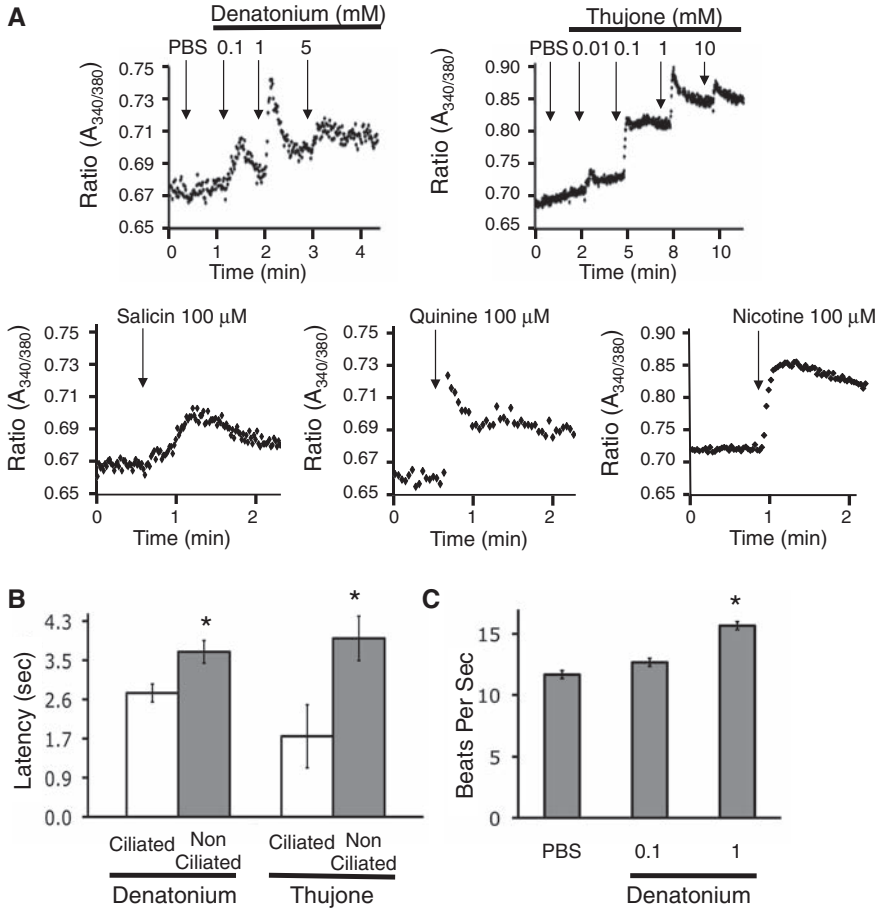

Fig. 4. Bitter compounds increase $[Ca^{2+}]_i$ in ciliated cells. (**A**) Differentiated airway epithelia were loaded with Fura2-AM and fluorescence imaging was used to assess changes in $[Ca^{2+}]_i$, indicated as the ratio of emission when excited at 340 nm versus 380 nm ($A_{340/380}$). Denatonium, thujone, salicin, quinine, or nicotine was added as indicated. "PBS" indicates addition of phosphate-buffered saline and arrows indicate the time of addition. Data points are the average of 20 cells. (**B**) Latency to increase in $[Ca^{2+}]_i$ after addition of 1 mM denatonium or 1 mM thujone; latency includes the time for the compound to diffuse to the cell after its addition. *$P < 0.001$, $N = 20$. See also fig. S8. (**C**) Denatonium increases ciliary beat frequency. Data are the ciliary beat frequency in differentiated human airway epithelia treated with PBS or denatonium. $N = 4$, *$P < 0.01$. See also figs. S8 and S9. The error bars in (B) and (C) denote SEM.

when we immunostained both T2R4 and T2R43 in the same epithelia (fig. S7). Co-labeling T2R38 and T2R46 revealed a similar relation, with T2R38 appearing to sit more distally than T2R46 (Fig. 2C and fig. S7).

The T2R signal transduction pathway comprises several proteins, including the G protein α-gustducin and the enzyme phospholipase C–β2 (PLC-β2) (*15*, *16*). We detected their transcripts in airway epithelia (Fig. 1B). Immunostaining revealed these signaling molecules only in ciliated epithelial cells (Fig. 3, A and B, and fig. S3). Like the T2Rs, α-gustducin resided in cilia. In contrast, PLC-β2 appeared to sit below the cilia in the apical portion of the cell. We speculate that the seeming complexity of T2R and signaling protein localization along the ciliary shaft and in the cell might provide a mechanism for coupling to other signaling pathways or influencing cilia function. Distinct localization may also occur with other proteins that are localized proximally in cilia and flagella (*18*, *19*).

To determine whether the T2R signaling pathway was functional, we applied bitter compounds to differentiated human airway epithelia and measured changes in the T2R-associated second messenger, intracellular Ca^{2+} concentration $[Ca^{2+}]_i$. Denatonium is a very bitter compound and a ligand for T2R4 (*15*). Its application induced transient, dose-dependent increases in $[Ca^{2+}]_i$ (Fig. 4A). Several other bitter compounds, including thujone (activates T2R14), salicin (activates T2R16), quinine, and nicotine, also elicited increases in $[Ca^{2+}]_i$. The denatonium and thujone concentrations inducing increases in $[Ca^{2+}]_i$ were in the same range as those that elicit responses in heterologous cells expressing T2Rs (*20*).

Airway epithelia contain both ciliated and nonciliated cells, and when $[Ca^{2+}]_i$ increases in an individual airway epithelial cell, it can rapidly spread through gap junctions to adjacent cells (*21*). Thus, the $[Ca^{2+}]_i$ increase shown in Fig. 4A could have begun from either cell type. Therefore, we tested if $[Ca^{2+}]_i$ first increased in ciliated or nonciliated cells by applying the bitter compound apically and comparing the latency to an increase in $[Ca^{2+}]_i$ in the different cells. After addition of denatonium or thujone, $[Ca^{2+}]_i$ rose in ciliated cells before increasing in other cells (Fig. 4B), consistent with our finding that only ciliated cells bear receptors for bitter compounds. We also studied cells growing on glass in short-term culture because the $[Ca^{2+}]_i$ increase does not spread; in those cells, denatonium increased $[Ca^{2+}]_i$ only in ciliated cells (fig. S8).

We assessed the functional consequences of activating T2Rs in airway epithelia by measuring the frequency of cilia beating. Because an elevated $[Ca^{2+}]_i$ can stimulate ciliary motility (*22*), we predicted that a bitter compound would increase frequency. Indeed, applying denatonium increased ciliary beat frequency ~25% (Fig. 4C and fig. S9).

Thus, some of the same receptors that detect noxious compounds on the tongue to protect animals from ingesting harmful material may also defend human airways. The receptors are positioned on cilia, a location optimal for sampling air and material entering the lung. Moreover, the bitter compound denatonium stimulated ciliary activity, which should hasten elimination of noxious and harmful substances. Substances that activate T2Rs might enter airways through inhalation or aspiration or be generated within the lung by infection. We speculate that this signaling system might also play a role in airway disease. For example, in cystic fibrosis, lungs are commonly infected with *Pseudomonas aeruginosa*. This bacterium produces quorum-sensing molecules that are lactones, which activate some bitter taste receptors (*23*, *24*). Airway T2Rs might also be activated by cigarette smoke, which contains the bitter-tasting compound nicotine (*25*). In addition, airway cilia are lost in some viral infections and with cigarette smoking (*26*), which would disrupt this defensive system.

Previous work suggested general roles for the two types of vertebrate cilia—primary cilia are sensory and motile cilia are mechanical. Studies of primary nodal cilia in mouse embryos indicated that the distinction is not absolute; those cilia can exhibit a rotational movement different from the planar beating typical of motile cilia (*4*, *5*). In addition, proteomic studies of *Chlamydomonas* flagella identified numerous proteins involved in signal transduction (*27*). Our present data indicate that classical motile cilia also have a sensory function.

T2Rs on taste receptor cells, which are not known to have primary cilia, detect bitter ligands and transmit that information to nerves to elicit a response (*15*, *20*). In cells outside the tongue where α-gustducin and T2Rs have been identified—nasal and laryngeal solitary chemosensory cells and intestinal tract enteroendocrine cells—signals are transmitted to the associated nerve networks (*28*–*30*). In contrast, in airway epithelia, T2Rs enable cell-autonomous detection of a signal followed by a response in the same cell to eliminate harmful substances.

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The E3 Ligase TRAF6 Regulates Akt Ubiquitination and Activation

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Akt signaling plays a central role in many biological functions, such as cell proliferation and apoptosis. Because Akt (also known as protein kinase B) resides primarily in the cytosol, it is not known how these signaling molecules are recruited to the plasma membrane and subsequently activated by growth factor stimuli. We found that the protein kinase Akt undergoes lysine-63 chain ubiquitination, which is important for Akt membrane localization and phosphorylation. TRAF6 was found to be a direct E3 ligase for Akt and was essential for Akt ubiquitination, membrane recruitment, and phosphorylation upon growth-factor stimulation. The human cancer-associated Akt mutant displayed an increase in Akt ubiquitination, in turn contributing to the enhancement of Akt membrane localization and phosphorylation. Thus, Akt ubiquitination is an important step for oncogenic Akt activation.

Protein ubiquitination is an important post-translational modification that regulates various biological functions (*1*, *2*). Although ubiquitination often results in protein degradation, a certain type of ubiquitination is important for signaling activation and protein trafficking (*1*, *2*). Ubiquitination through Lys^{48} (K48) (*3*) of the ubiquitin chain generally targets proteins for degradation, whereas ubiquitination through K63 plays a critical role in signaling activation and protein trafficking (*1*, *2*). Akt (also known as protein kinase B) is an important component of cell signaling pathways that regulate cell survival and metabolism. Although membrane recruitment of Akt by growth-factor stimuli is a critical step for Akt phosphorylation, it is not clear how Akt is recruited to the plasma membrane. Because ubiquitination can regulate protein trafficking (*1*), we tested whether Akt is ubiquitinated in cells. Akt was ubiquitinated in the absence of

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