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10. The meteorological time series is derived from the land-only gridded SAT anomalies assembled by P. D. Jones, T. J. Osborn, and K. R. Briffa [*J. Clim.* **10**, 2548 (1997)] (also available from the Climatic Research Unit at the University of East Anglia, Norwich, UK, as a data set at www.cru.uea.ac.uk/cru/data/temperat.htm).
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Isolation of Acidophilic Methane-Oxidizing Bacteria from Northern Peat Wetlands

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Acidic northern wetlands are an important source of methane, one of the gases that contributes to global warming. Methane oxidation in the surface of these acidic wetlands can reduce the methane flux to the atmosphere up to 90 percent. Here the isolation of three methanotrophic microorganisms from three boreal forest sites is reported. They are moderately acidophilic organisms and have a soluble methane monooxygenase. In contrast to the known groups of methanotrophs, 16*S* ribosomal DNA sequence analysis shows that they are affiliated with the acidophilic heterotrophic bacterium *Beijerinckia indica* subsp. *indica*.

The methane (CH_4) concentration in the atmosphere has more than doubled over the last 300 years (*1*) and is currently increasing at an annual rate of 0.8 to 1.0% per year (*2*). About half of the total annual flux of CH_4 to the atmosphere is contributed by wetlands (*3*). The massive northern wetlands account for 50% of the global wetland area (*4*), and their most extensive type, found in northern Europe, West Siberia, the United States, and Canada, is the acidic *Sphagnum* peat bogs, which have pH values ranging from 3.5 to 5.

The primary barrier that limits the release of CH_4 from methanogenic peatlands is its in situ consumption by indigenous methane-oxidizing bacteria (MOB). MOB inhabit a spectrum of diverse environments and have the unique ability to use CH_4 as a sole carbon and energy source (*5*). The colonization of acidic bogs by MOB has been established by measurement of methanotrophic activity (*6*), MOB signatures in phospholipids (*7*), and DNA (*8*, *9*) extracted from peat. Nevertheless, virtually all MOB available in pure culture are neutrophiles, and there are no reports of methanotrophs that grow at pH values below 5.0 (*10*).

We recently reported on the enrichment of methanotrophic communities from acidic peat bogs of four boreal forest sites in West Siberia and European North Russia (*11*). The key to successful enrichment was the use of a medium of very low ionic strength and low pH (3 to 6), and incubation under CH_4-air mixture at moderate temperature (20°C). These communities were moderately acidophilic with growth and activity optima at pH 4.5 to 5.5. We have now isolated in pure culture MOB from three of these four enrichments (*12*). The colonies of MOB developed after 4 to 5 weeks of incubation. We selected three strains (strains K, M131, and S6), each representing one enriched community, and confirmed their purity (*13*). The cells of these three strains were Gram-negative, nonmotile, polymorphic, straight or curved rods with a diameter of 0.7 to 1.0 μm and length of 1.0 to 2.0 μm. They shared an identical morphotype, that is, a specific flattened shape with a concave center and round, bent ends (*14*) (Fig. 1). The same morphotype was observed as one of the dominant components of the primary communities (*11*).

Strains S6, K, and M131 grew on minimal mineral medium with the addition of a vitamin mixture and CH_4 as a sole source of carbon and energy (*15*). Growth did not occur in control experiments on the same mineral medium containing vitamins and no CH_4. The isolates were slow growing with a specific growth rate $\leq$ 0.8 to 1.0 day^{-1}, which is consistent with the in situ growth rate for MOB of 0.02 day^{-1} (*16*). The temperature range for growth of isolates was from 10° to 25°C with the optimum at ~20°C. The same optimum was found for CH_4 consumption by native peat samples (*17*). No growth of isolates occurred at 30°C. Clearly these bacteria are adapted to conditions of their natural habitat where the temperature never exceeds 25°C, even during summer.

Methane consumption peaked at pH 5.1 for all three strains (Fig. 2). The same pattern of pH dependence was also found for the original peat samples and the methanotrophic enrichments (*11*, *17*–*19*). The acidophilic nature of the isolated bacteria was confirmed by observing exponential growth without a lag phase and the highest specific growth rate at pH 4.8, whereas no growth was recorded in a medium at initial pH 7.4 (Fig. 2). Furthermore, growth of isolates was sustained in serial transfers in pH 5.0 to 5.5 medium.

Polymerase chain reaction (PCR) assays for the *mmoX* and *mmoY* genes, encoding the α and β subunits of the soluble methane monooxy-

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genase (sMMO) hydroxylase component, yielded products of the predicted size of 524 and 602 bp, respectively, with genomic DNA of the strains K, M131, and S6 (*20*). The nucleotide sequences of the PCR-amplified *mmoX* gene fragments from the three strains were identical to each other and to the sequence of *mmoX* clones obtained previously from the methanotrophic communities (*21*). The amino acid sequence deduced from these *mmoX* gene fragments corresponds to positions 300 to 458 of the homologous sequence of *Methylococcus capsulatus* (Bath), *Methylosinus trichosporium* OB3b, and *Methylocystis* sp. strain M (*22*) (Fig. 3). Although the level of homology is high, the *mmoX* gene fragment in bog methanotrophs diverged significantly from these gene fragments in known methanotrophs. The level of sequence divergence indicates that the *mmoX* gene of the acidophilic isolates forms a branch distinct from the *mmoX* sequences of the *Methylocystis-Methylosinus* and the *Methylococcus* groups.

The 16*S* ribosomal DNA (rDNA) was sequenced to establish the phylogenetic affiliation of acidophilic strains (*23*). The 16*S* rRNA gene sequence was identical for all three strains. The phylogenetic analysis revealed that acidophilic strains are members of the α subclass of *Proteobacteria* and are most closely related to *Beijerinckia indica* subsp. *indica* (Fig. 4); the sequence identity was

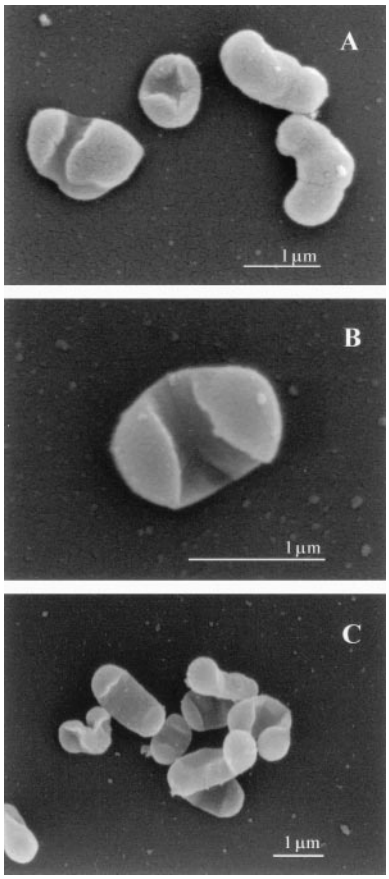

Fig. 1. Cell morphology of acidophilic methanotrophic isolate (strain K). (**A**) Polymorphic cell appearance, (**B**) cell bipolarity, and (**C**) formation of shapeless aggregates.

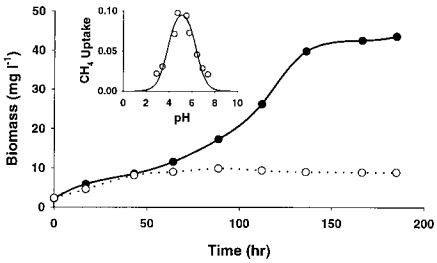

Fig. 2. Effect of medium pH on the growth and methanotrophic activity of isolated strains. Growth at initial pH 4.8 (●) and pH 7.4 (○). (**Inset**) The dependence of instant methanotrophic activity on medium pH.

```
                                                                    53
stK-M-S   PWVKTWNRWVYEDWGGIWIGRLAKYGVNSPPSLRDAKKDAYWAHHDLFLLAYA
Ms.tri    ----------S-----------G----E-.RV-----R---------A-A---
Mcy.stM   ----------------------G----E--RV-----T---------A-A---
Mc.cap    ----------------------G----E--R--K---Q---------Y-----

                                                                   106
stK-M-S   LWPTGFFRLSLPDEEDMEWFEANYPGWDAHYGKILREWKALGCEDPSSGFIPI
Ms.tri    M--LA-A--A------QA---------AD-----FN---K--Y---K-----Y
Mcy.stM   ---LA-A--A------Q----------AD-----YN---K--Y---K-----Y
Mc.cap    ---------A---Q-E-----------YD-----YE--R-R-----------L

                                                                   159
stK-M-S   QWLIQHGHKVYVDRTSQVPFCPTLAKCSGSLRVHEFNGQKHSFSDDWGERQWL
Ms.tri    ---LAN--D--I--V-----I-S---GT----------K---LT---------
Mcy.stM   A--LEN--D--I--V-----I-S---G----------K---LT------M--
Mc.cap    M-F-ENN-PI-I--V-------S---GAST-----Y---M-T---Q----M--
```

Fig. 3. Alignment of partial amino acid sequences deduced from *mmoX* gene fragments of the acidophilic isolates K, M131, and S6 (stK-M-S) (GenBank accession number AF004554), *Methylosinus trichosporium* OB3b (Ms.tri), *Methylocystis* sp. strain M (Mcy.stM), and *Methylococcus capsulatus* (Bath) (Mc.cap). The sequence stretch corresponds to positions 300 through 458 of the open reading frame published for the *mmoX* gene of *M. capsulatus* (Bath) (*22*). For this stretch, the strains K, M131, and S6 share identical primary structures at the nucleotide and amino acid levels. The identity and similarity values between the amino acid sequence shared by the three strains and these homologous fragments were, for *M. capsulatus* (Bath), 78.0 and 88.7%, respectively; for *Methylosinus trichosporium*, OB3b 76.5 and 80.5%, respectively, and for *Methylocystis* sp. strain M, 78.6 and 83.6%, respectively. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

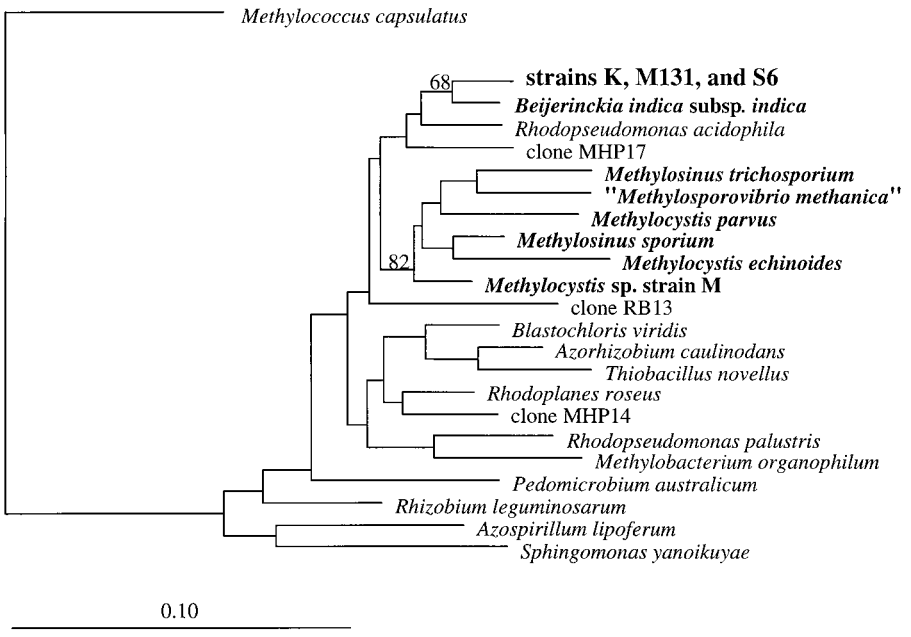

Fig. 4. 16*S* rDNA-based maximum-likelihood tree constructed for the three acidophilic methanotrophs and 18 reference organisms of the α subclass of *Proteobacteria* and three environmental clone sequences [clones MPH14, MPH17 (*9*), and RB13 (*26*)] that have been retrieved from acidic environments. *Methylococcus capsulatus* (Bath) was used as the outgroup. The numbers indicate the statistical significance (percentage of outcome) of the respective interior nodes in 1000 neighbor-joining tests. The scale bar represents the estimated number of base changes per nucleotide sequence position.

96.5%. The identity to the previously known methanotrophic genera, *Methylocystis* and *Methylosinus*, was only 90.5 and 92.6%, respectively. *Methylocystis* spp. and *Methylosinus* spp. always formed a coherent group in the treeing analyses, which is related to, but clearly distinct from, the branch characterized by the strains K, M131, S6, and *B. indica* subsp. *indica*. Apart from *B. indica* subsp. *indica*, *Rhodopseudomonas acidophila* is the next most closely related organism in the rDNA database, with a similarity value of 93.5%. This organism also is found in acidic, boggy waters and soils and has a pH optimum for growth of 5.5 (*24*). One of the environmental sequences (clone MPH17) (Fig. 4) included in the phylogenetic analyses was retrieved from a peat core sampled at the Pennine Hills in the north of England (*9*); it had an identity value of 94.5% to the acidophilic isolates.

Both the *mmoX* and 16*S* rRNA gene sequence data indicate that the acidophilic strains represent a lineage of CH_4-oxidizing bacteria only moderately related to the known cluster of α-proteobacterial methanotrophs, that is, *Methylosinus-Methylocystis* spp. Furthermore, the acidophilic strains turned out to be closely affiliated with the heterotrophic bacterium *B. indica* subsp. *indica*, contrary to the generally accepted notion that neither known group of methanotrophs has any close relatives that are not methanotrophs (*10*). *Beijerinckia indica* is a common inhabitant of acidic soils and has a pH optimum at 5.0 (*25*). Our isolates and *B. indica* have many similar features, such as cell morphology, temperature response, pH optimum, and low growth rate. Nevertheless, *B. indica* is a typical representative of heterotrophic bacteria that uses a large spectrum of sugars and other organic substrates, whereas our strains do not grow on sugars. We attempted to amplify the sMMO gene from *B. indica* DNA (*27*), but found no sMMO-like products.

The phylogenetic tree (Fig. 4) is suggestive of a common methanotrophic ancestor for the three acidophilic methanotrophs and the *Methylocystis-Methylosinus* cluster with a subsequent evolutionary loss of methanotrophic activity in the ancestors of *B. indica* and *R. acidophila* because the new strains usually grouped next to the *Methylocystis-Methylosinus* cluster when the 16*S* rRNA sequences of *B. indica*, *R. acidophila*, and clone MHP17 were excluded from the treeing analyses. However, the bootstrap values in neighbor-joining and maximum parsimony tests were above 80 (percentage of outcome) for the relevant interior node indicating the *Methylosinus-Methylocystis* group as a monophyletic cluster (82 for the tree shown in Fig. 4), whereas these values were always below 35 for the node shared by the strains K, M131, and S6 and the *Methylosinus-Methylocystis* group in the same analyses. Thus, considering the known metabolic traits of the acidophilic methanotrophs and of the phylogenetically related bacteria, the hypothesis of a common methanotrophic ancestor for the three acidophilic methanotrophs and the *Methylosinus-Methylocystis* group appears to be very unlikely, but may not be completely discarded. Discussion about the evolutionary history of these two α-proteobacterial methanotrophic groups becomes even more speculative when the amino acid sequence deduced from the partial *mmoX* gene fragment of the three acidophilic methanotrophs (Fig. 3) is taken into account. This *mmoX* sequence is clearly distinct from both the *Methylocystis-Methylosinus*–like *mmoX* sequences and the *M. capsulatus* (Bath) *mmoX* sequence as indicated by the overall identity values between 76.5 and 79% (legend to Fig. 3) to each of the other two *mmoX* sequence groups.

Most likely, the indigenous methanotrophs of acidic bogs are quite diverse, and our isolates may be the first representatives of a group of previously unculturable MOB. Nevertheless, the ecological fitness of these bacteria to the environmental conditions of *Sphagnum* bogs suggests that they play a major role in the CH_4 cycle in the vast acidic northern wetlands, which are the largest natural source of atmospheric CH_4.

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12. We used the fivefold dilution of minimal mineral medium M1 (*11*). The solid medium was prepared by adding 1.5% (w/v) of purified agar (Difco). After sterilization the medium was adjusted to pH 5.0 with 0.1 M H_3PO_4. Cell suspensions of exponentially growing methanotrophic communities were spread plated onto the surface of agar medium, and the plates were incubated at 20°C in desiccators under CH_4–air 30:70 mixture.
13. Phase contrast and electron microscopy revealed only one morphotype. No growth was observed on all tested complex organic media and on mineral media with individual sugars. A PCR-amplified 16*S* rDNA library was constructed for strain K grown on CH_4 and methanol and was screened by digestion with two sets of tetrameric endonucleases (Rsa I + Msp I and Hha I + Hae III). No differences were observed among the 86 clones obtained.
14. The microscopic examination was done with batch cultures in late exponential growth phase. Cells were fixed at 4°C for 1 to 2 hours in 4% glutaraldehyde buffered with 0.1 M sodium phosphate (pH 7.4). Samples were prepared with poly-L-lysine to adhere bacteria to the cover slip. After a brief rinse in the buffer, samples were dehydrated in an ethanol series (25, 50, 75, 95%) for 15 min in each solution and three times for 15 min in 100% ethanol. After dehydration, samples were dried in a Balzers critical point dryer to prevent any shape alterations and were then coated with gold in an Emscope Sputter Coater model SC 500. Cells were examined with a JEOL JSM-6400V scanning electron microscope at the Center for Electron Optics, Michigan State University.
15. Mineral medium M1 (*11*) was supplemented with 0.5% (v/v) of a vitamin stock solution containing (milligrams per 100 ml): biotin (0.4), nicotinic acid (10), riboflavin (10), and inositol (10). The cultivation was performed in screw-cap 500-ml serum bottles with 100 ml of culture medium. Incubation was on a rotary shaker at 20°C. CH_4 and CO_2 were measured with a gas chromatograph. The cell biomass was measured by nephelometry at 410 nm followed by conversion to cell carbon from the respective calibration curve. Isolates were subcultured at 3- to 4-week intervals. The relation between CH_4 uptake, biomass, and CO_2 produced yielded a stoichiometric growth equation of $CH_4 + 1.13O_2 = 0.39CH_{5.9}O_{0.5} + 0.61CO_2 + 0.85H_2O$, which is very close to that of the enriched methanotrophic communities (*11*). The average value of the biomass yield was 0.39 g C per gram of CH_4–C.
16. We stimulated exponential growth of MOB in native peat samples to determine the specific growth rate [S. N. Dedysh and N. S. Panikov, *Mikrobiologiya* **66**, 563 (1997)].
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20. We performed PCR-mediated amplification using sMMO-targeted primers and reaction conditions as described (*8*). The positive control was DNA of *M. capsulatus* (Bath) ($sMMO^+$). No product was obtained with template DNAs from the negative controls, namely *Methylomonas albus* ($sMMO^-$) and *Methylocystis pyreformis* ($sMMO^-$). Reference strain of *M. capsulatus* (Bath) was obtained from American Type Culture Collection (ATCC, accession number 33009). *Methylocystis pyreformis* strain 44 and *Methylomonas albus* strain 85 were provided by Y. A. Trotsenko (IBPM, Pushchino, Russia).
21. The nucleotide sequence of the *mmoX* gene fragment of acidophilic methanotrophs has been deposited in GenBank under accession number AF004554.
22. The DNA sequence of the gene cluster encoding the sMMO proteins has been determined for three MOB, *M. capsulatus* (Bath), *Methylosinus trichosporium* OB3b, and *Methylocystis* sp. strain M [A. C. Stainthorpe, V. Lees, G. P. C. Salmond, H. Dalton, J. C. Murrell, *Gene* **91**, 27 (1990); D. L. N. Cardy, V. Laidler, G. P. C. Salmond, J. C. Murrell, *Mol. Microbiol.* **5**, 335 (1991); I. R. McDonald, H. Uchiyama, S. Kambe, O. Yagi, J. C. Murrell, *Appl. Environ. Microbiol.* **63**, 1898 (1997)].
23. PCR-mediated amplification of the 16*S* rRNA genes and sequence analyses were done as described [W. Liesack, and K. Finster, *Int. J. Syst. Bacteriol.* **44**, 753 (1994)]. The nucleotide sequence of the 16*S* rRNA gene of strain K has been deposited in the EMBL (European Molecular Biology Laboratory), GenBank, and DDBJ (DNA Data Bank of Japan) databases under accession number Y17144. The phylogenetic position of strains S6, K, and M131 was determined by comparing their 16*S* rRNA gene sequence with α-proteobacterial reference sequences for which at least 1000 nucleotide sequence positions were available. Trees were constructed for a sequence stretch rang-

ing from positions 28 to 1477(*Escherichia coli* 16S rRNA numbering) by using distance matrix and maximum-likelihood methods and the ARB program package [J. Felsenstein, *PHYLIP (phylogeny inference package), version 3.5c* (University of Washington, Seattle, WA, 1993); B. L. Maidak *et al.*, *Nucleic Acids Res.* **24**, 82 (1996); O. Strunk and W. Ludwig, *ARB: A Software Environment for Sequence Data* (Technische Univ., Munich, Germany, 1996)].

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28. We thank C. Flegler (Michigan State University Electron Microscope Center) for assistance with electron microscopy. Supported in part by the Russian Fund of Fundamental Research (grant 96-04-49321), by NSF (grants INT9315089 for Russian collaborative work and DEB9120006), and by the European Community RTD Programme in Biotechnology (grant EV5V-CT94-0499).

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Alterations of the *PPP2R1B* Gene in Human Lung and Colon Cancer

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The *PPP2R1B* gene, which encodes the β isoform of the A subunit of the serine/threonine protein phosphatase 2A (PP2A), was identified as a putative human tumor suppressor gene. Sequencing of the *PPP2R1B* gene, located on human chromosome 11q22-24, revealed somatic alterations in 15% (5 out of 33) of primary lung tumors, 6% (4 out of 70) of lung tumor–derived cell lines, and 15% (2 out of 13) of primary colon tumors. One deletion mutation generated a truncated PP2A-Aβ protein that was unable to bind to the catalytic subunit of the PP2A holoenzyme. The *PP2R1B* gene product may suppress tumor development through its role in cell cycle regulation and cellular growth control.

Carcinomas of the lung and colon account for over 270,000 new cases of cancer each year in the United States (*1*). Many human cancers contain mutations in tumor suppressor genes, which often map to genomic regions that exhibit allelic loss, or loss of heterozygosity (LOH), in tumors. LOH at chromosome 11q22-24 has been associated with lung, colon, breast, cervical, head and neck, and ovarian cancers, as well as melanoma (*2*). Introduction of a normal chromosome 11, or a derivative t(X;11) chromosome containing 11pter-q23, can reverse the tumorigenic potential of lung (*3*), breast (*4*), and cervical carcinoma cells (*5*) and Wilms tumor (*6*) when introduced into nude mice. These studies suggest that one or more tumor suppressor genes are located centromeric to the t(X;11) breakpoint at 11q23.

To identify the tumor suppressor genes on chromosome 11 that are inactivated in lung cancer, we mapped the t(X;11) breakpoint relative to regions of frequent LOH (*7*) and defined a minimum critical region between *D11S1394* and *D11S689* (Fig. 1A). This region of chromosome 11q23 demonstrated high-frequency LOH in a variety of cancers, including lung and colon tumors. Evaluation of LOH in 28 lung cancer and paired normal cell lines revealed allelic loss in 71% of the cancer cell lines. Two polymorphic DNA markers, *D11S1647* and *D11S1987*, showed allelic loss in 42.9 and 46.2% of the cancer cell lines, respectively (*8*), and both markers were lost in 28.6% of the cell lines.

On the basis of these results, the region between *D11S1394* and *D11S689*, and especially that between *D11S1647* and *D11S1987*, was systematically surveyed for candidate tumor suppressor genes. Over 100 candidate genes and expressed sequence tag (EST) markers were identified from a radiation hybrid map of human chromosome 11 (available at http://ftp.well.ox.ac.uk) and from the Human Gene Map (available at http://www.ncbi.nlm.nih.gov/SCIENCE96/ResTools.html). One of the EST sequences (*M65254*) was found to correspond to a subunit of the serine/threonine protein phosphatase 2A (PP2A). PP2A is an important regulatory enzyme that down-regulates the mitogen-activated protein kinase (MAPK) cascade, relays signals for cell proliferation, and has been linked to carcinogenesis (*9*). The PP2A holoenzyme exists in several trimeric forms consisting of a 36-kD core catalytic subunit, PP2A-C; a 65-kD structural/regulatory component, PP2A-A; and a variable regulatory subunit, PP2A-B, which confers distinct properties on the holoenzyme. Each subunit exists as multiple isoforms encoded by different genes, so that there are many forms of the PP2A trimer, differing in expression patterns and specificity. The gene identified at chromosome 11q23, denoted *PPP2R1B* according to standardized nomenclature, encodes the β isoform of the structural/regulatory A subunit PP2A-Aβ. PP2A-Aβ is necessary for interaction of the catalytic PP2A-C and variable PP2A-B subunits and is critical for phosphatase activity (*10*).

The precise physical location of *PPP2R1B* was determined by colocalizing it within P1-derived artificial chromosome (PAC) (*11*) clones that contained sequence-tagged sites (STSs) on chromosome 11q22-23 (Fig. 1A). PAC clone pDJ433L20 contained *PPP2R1B* as well as markers *SHGC9837* and *D11S966E* and was localized to a 15 centiray region between markers *D11S1647* and *D11S1987* on 11q22.2 (*12*). Thus, the *PPP2R1B* gene is located in a region showing high-frequency LOH (Fig. 1A).

The complete sequence of *PPP2R1B* was determined by a combination of EST analysis (*13*), cDNA sequencing (*14*), and 5′ rapid amplification of cDNA ends (RACE) (*15*). The sequence predicts a 601–amino acid protein with extensive homology to the PP2A-Aβ subunits of pig and *Xenopus* and to the human PP2A-Aα isoform (Fig. 1B). Both PP2A-Aα and PP2A-Aβ are composed of 15 internal repeat sequences, each consisting of two amphipathic helices necessary for binding PP2A-B and PP2A-C (*16*).

To determine whether human tumors contain alterations in *PPP2R1B*, we amplified the coding regions by reverse transcriptase–polymerase chain reaction (RT-PCR) (*17*) and carried out direct DNA sequencing of samples from 130 cancer-derived cell lines and 70 primary tumors, including lung, colon, breast, and cervical tumors. Two of the tumors generated altered *PPP2R1B* amplification products. H1450 cells had a 1.8-kb product representing the wild-type *PPP2R1B* and a second 1-kb product (Fig. 2A). Sequencing of the latter revealed an internal in-frame deletion of 867 base pairs (bp), which is predicted to produce a truncated PP2A-Aβ lacking amino acids 230 to 518 (Fig. 2B). The 1.8-kb PCR product contained an A_{1540}→G transition, which changes a highly conserved Asp_{504} to Gly. Thus, both alleles of *PPP2R1B* are altered and possibly inactivated, whereas the matched lymphoblastoid cell line from the same patient, BL7, con-

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Isolation of Acidophilic Methane-Oxidizing Bacteria from Northern Peat Wetlands

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Editor's Summary

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