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Short Peptide Induces an "Uncultivable" Microorganism To Grow In Vitro▽

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Microorganisms comprise the bulk of biodiversity, but only a small fraction of this diversity grows on artificial media. This phenomenon was noticed almost a century ago, repeatedly confirmed, and termed the "great plate count anomaly." Advances in microbial cultivation improved microbial recovery but failed to explain why most microbial species do not grow in vitro. Here we show that at least some of such species can form domesticated variants capable of growth on artificial media. We also present evidence that small signaling molecules, such as short peptides, may be essential factors in initiating growth of nongrowing cells. We identified one 5-amino-acid peptide, LQPEV, that at 3.5 nM induces the otherwise "uncultivable" strain *Psychrobacter* sp. strain MSC33 to grow on standard media. This demonstrates that the restriction preventing microbial in vitro growth may be different from those offered to date to explain the "great plate count anomaly," such as deficiencies in nutrient composition and concentrations in standard media, medium toxicity, and inappropriate incubation time. Growth induction of MSC33 illustrates that some microorganisms do not grow in vitro because they are removed from their native communities and the signals produced therein. "Uncultivable" species represent the largest source of unexplored biodiversity, and provide remarkable opportunities for both basic and applied research. Access to cultures of some of these species should be possible through identification of the signaling compounds necessary for growth, their addition to standard medium formulations, and eventual domestication.

An overwhelming majority of environmental microorganisms do not grow in vitro (we use the term "in vitro" in reference to standard laboratory cultivation conditions), and the nature of this phenomenon remains an enigma. The disparity between the total and cultivable microbial counts was first noticed almost a century ago (1, 30), confirmed in more recent studies (19, 45, 50), and termed the "great plate count anomaly" (39). Uncultivated species comprise the bulk of microbial diversity, and a majority of phyla have few or no cultivated representatives (35, 37). While culture-independent approaches opened a window into the biology of these yet-"uncultivable" species (13, 46), the "great plate count anomaly" remains one of the most important unresolved phenomena in microbiology (18). (The term "uncultivable" is widely used but is to some extent misleading. Since "uncultivable" species are able to grow in nature, the term should be expanded to "presently uncultivable in the laboratory using standard culture conditions." It is in this sense that we will use the term "uncultivable," choosing this word for the sake of brevity.)

Research over last decade demonstrated a renewed interest in microbial cultivation. Several new approaches have been designed and have produced a wealth of new, ecologically important isolates (3, 9–11, 23, 34, 40, 48). The majority of the new cultivation techniques seek to circumvent growth restrictions by altering medium composition, incubation time, cell density, and other factors. For example, the high concentrations of nutrients used in many cultivation attempts may inhibit a large portion of microorganisms, as many natural microbial communities flourish in oligotrophic conditions. Indeed, the use of low concentrations of such nutrients increased microbial recovery (10, 20, 48). Environmental microorganisms may also have longer generation times than observed for laboratory models, and using increased incubation times did yield novel isolates (36, 40). Standard media may not meet the metabolic needs of a syntrophic bacterium without the addition of the partner's by-products. Absent syntrophic growth factors could include siderophores (15, 42), vitamins (14), specific carbon sources (5), or essential nutrients (43). Amending growth media with known signal molecules has been used as a strategy to increase microbial recovery from environmental samples (6, 7). However, the above-described modifications to cultivation conditions have not yet resulted in a dramatic increase in microbial recovery, and the overall rate of such recovery remains low, typically a few percentage points or less.

In one more radical innovation in cultivation, cells are placed into a diffusion chamber and returned to their natural environment during incubation (23). Growth components diffuse into the chamber and provide the contained microorgan-

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TABLE 1. "Uncultivable" microbial isolates and their helper strains

"Uncultivable" isolate and closest cultured relative (% 16S rRNA gene sequence similarity)	Helper strain(s) and closest cultured relative(s) (% 16S rRNA gene sequence similarity)
MSC1 (accession no. AY062176), *Lewinella persicus* (95)	MSC4, undetermined identity; MSC5, *Maribacter dokdonensis* (99); MSC6, *Pseudoalteromonas* sp. (98); MSC7, *Pseudoalteromonas* sp. (100)
MSC16, *Janthinobacterium* sp. (99)	MSC107, *Psychrobacter pulmonis* (99); MSC108, *Psychrobacter pulmonis* (99)
MSC33, *Psychrobacter* sp. (95)	MSC102, *Psychrobacter* sp. (99); MSC105, *Cellulophaga lytica* (99)
MSC101, *Pseudoalteromonas atlantica* (89)	MSC102, *Psychrobacter* sp. (99); MSC109, *Psychrobacter* sp. (99); MSC110, *Psychrobacter* sp. (99)
MSC104, *Thalassomonas ganghwensis* (96)	MSC103, *Zobellia russellii* (99)
MSC106, *Pantoea agglomerans* (99)	MSC105, *Cellulophaga lytica* (99)

isms with the necessary growth conditions. This approach increased microbial recovery by several hundredfold (23) and allowed us to grow and isolate into pure cultures microorganisms that, in isolation, were unable to grow in vitro. Further experimentation with these isolates showed that at least some of them could in fact grow on synthetic media if paired with another species from the same environment (23). Growth in coculture (but not alone) occurred on poor as well as rich media, suggesting that the basis of growth induction is not cross-feeding. We suggested that growth of uncultivable bacteria might depend on growth-promoting signals (23). The ability to grow on synthetic media in vitro could result from a regulatory restriction, which could be overcome through selection or by addition of growth factors. Here we address both possibilities, and we report that an uncultivable microorganism can produce a cultivable variant and that the growth of the uncultivable species can be induced by a small peptide.

MATERIALS AND METHODS

Cultivation and isolation. Environmental microorganisms were obtained from a marine intertidal sand flat in Massachusetts Bay, close to the Marine Science Center of Northeastern University, Nahant, MA (42°26′ N, 70°56′ W). The uppermost 2-mm-thick oxygenated sediment layer was used as a source of microorganisms. Sediments were mixed with autoclaved, locally collected seawater and shaken vigorously for 120 seconds. The sand grains were allowed to settle for 60 seconds before aliquots of supernatant were used as inocula in microbial growth experiments in diffusion growth chambers as described previously (23). Colonies grown in the diffusion chamber and targeted for isolation into pure culture were collected into aliquots of autoclaved seawater, disrupted by vortexing and passaging through 25 gauge PrecisionGlide needles (Becton Dickinson, Franklin Lakes, NJ), mixed with warm agar supplemented with 0.01% technical-grade casein (Sigma Aldrich, St. Louis, MO), and poured into new chambers. A series of these reinoculations resulted in pure cultures. Parallel inoculation into petri dishes was used to verify their inability to grow in vitro on the same medium. Isolates unable to form colonies in agar were then subcultured by passaging them through series of diffusion chambers (22). Diffusion chambers were incubated in marine aquaria containing approximately 20 liters of undisturbed tidal flat sediment freshly collected from the intertidal flat described above. The sediments were covered with 10 to 20 cm of freshly collected unaltered seawater. The temperature in the aquaria was maintained at 16°C, which is representative of the ambient level at the time of experimentation. Petri dishes were incubated in the lab at 16°C. Microbial cultures were checked for purity by examination at a magnification of ×1,000 using a Zeiss Axioskop 50 compound microscope equipped with differential interference contrast (DIC), phase contrast, and epifluorescence. Additionally, the purity of one strain (MSC33) was verified using scanning electron microscopy (SEM) and periodically by 16S rRNA gene amplification (see below).

Cultivable isolates were obtained from standard pour plates and isolated into pure culture using standard microbiological techniques. The source of microorganisms was identical to that used in the diffusion chamber experiments. Microorganisms were grown in 0.7% agar (Becton Dickinson, Franklin Lakes, NJ) supplemented with 0.01% (wt/vol) technical-grade casein, 0.01% (wt/vol) Casamino Acids (Becton Dickinson, Franklin Lakes, NJ), and 4% (wt/vol) sea salts (Sigma Aldrich, St. Louis, MO) (hereafter called CA medium).

Stock cultures of cultivable isolates were prepared from a 1:20 dilution of stationary-phase cultures grown in 2.5% Luria broth in 4% (wt/vol) sea salts (LBSW) at 25°C, aliquoted in 19% (vol/vol) glycerol (final concentration), and kept frozen at −80°C. Stock cultures of MSC33 were prepared from cells grown on CA in coculture with MSC105 (see below). Colonies were disrupted by vortexing and passaging through 25-gauge PrecisionGlide needles in 500 μl of 4% (wt/vol) sea salts and then supplemented with glycerol, aliquoted, and frozen as described above.

Cultivation of MSC33. Diffusion chamber-raised MSC33 was cocultivated in a pairwise manner with isolates obtained from standard petri dishes (Table 1). The cocultures were established using tissue culture inserts (Nalgene Nunc International, Naperville, IL) incubated on top of CA agar plates as follows (Fig. 1A). MSC33 cells were diluted and mixed with CA medium, and 500-μl aliquots were poured into 24-well plates (Corning, Lowell, MA). Tissue culture inserts were inoculated with 100 μl CA medium containing 1:33 dilutions of frozen stock from each cultivable isolate and placed in the middles of individual wells of the plates with MSC33. The 0.03-μm-pore-size membranes at the bottoms of the inserts separated the two microbial cultures. Wells that received no inserts or inserts containing uninoculated CA served as controls. Cocultures were incubated for 14 days at 25°C. Agar cores totaling approximately 5 to 10 μl per well were then sampled using Pasteur pipettes and examined for microcolonies at a magnification of ×1,000 using DIC. Groups containing more than 10 cells were counted as colonies, and their total number was used to estimate the number of microcolonies per assay well. In some trials, 60-mm petri dishes were used in place of 24-well plates. We note that in several trials MSC33 growth additionally occurred in the (macro) form of a biofilm-like lawn around the insert (Fig. 1B) We observed this form of growth only as additional to microcolonies and exclusively in the presence of helper species. Spent media, their extracts, and their individual components (see below) induced only microcolony formation in MSC33, and consequently we used microcolonies to quantify induction of growth throughout the study.

Repetitive cultivation of MSC33 in coculture with MSC105 led to the appearance of the domesticated variant MSC33c, which was capable of growth on the same synthetic medium in pure culture. MSC33c was grown in CA and both solid and liquid LBSW, as well as 0.5% yeast extract with 4% (wt/vol) sea salts.

Isolation and identification of an MSC33 growth-inducing factor. We performed bioassay-directed fractionation and analysis of MSC33c-conditioned LBSW in search of a factor responsible for MSC33 growth induction.

(i) Natural product chemical analyses. Five 200-ml batches of LBSW stationary-phase cultures of MSC33c were prepared by growing a 1:1,000 dilution of stationary-phase MSC33c cultures for 12 h with shaking at 300 rpm at 25°C. The grown cells were removed via centrifugation for 10 min at 4,000 rpm at 20°C. The resulting supernatant was recentrifuged three to five times for further cell removal and sterilized with 0.22-μm-pore-size filters (Nalgene Nunc International, Naperville, IL). The filtrate was stored at −80°C until processing.

A total of 400 ml of MSC33c-conditioned medium was freeze-dried and

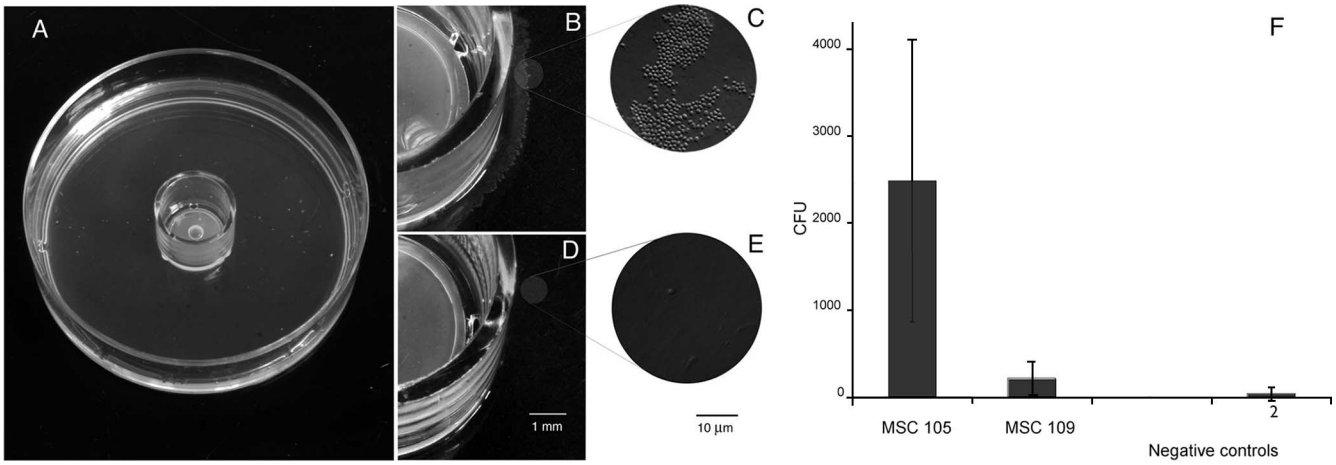

FIG. 1. Growth induction of MSC33 by the helper strain MSC105. (A) General view of the two-compartment chamber for cocultivation of two microorganisms. A tissue culture insert with a porous (0.02-μm) bottom is placed on the surface of a petri dish, providing the means to grow two pure microbial cultures in chemical contact with each other. (B and C) Inoculation of the insert with the helper strain MSC105 leads to the induction of growth of MSC33 inoculated into the petri dish. (D and E) An empty insert produces no effect, and MSC33 remains in the form of single cells. In all cases, microorganisms were grown for 14 days on casein medium supplemented with Casamino Acids. (F) The microcolony counts of MSC33 in the presence of MSC105 are statistically significantly higher ($P < 0.05$) than the counts of MSC33 in the presence of the "helper" strain MSC109, which in separate experiments was found to be a "helper" strain to MSC101 (Table 1). Three replicate wells were observed for each treatment. Error bars indicate standard deviations. (Note that the growth of MSC33 with the insert containing no microbial culture and with no insert present [negative controls 1 and 2, respectively] is only apparent. CFU in the negative controls corresponded throughout the study to the number of small clumps of MSC33 cells present in the cells' stock suspension [data not shown]. In order to show the relative scale of such "noise" CFU, we chose not to subtract these counts from the counts of colonies that indeed grew during the experiments.)

extracted with a 1:1 dichloromethane-methanol mixture (Fisher Scientific, Hampton, NH). The organic extract was dried using a Speed-Vac, solubilized in 2 ml distilled water (VWR International, West Chester, PA), and applied to a reversed-phase flash column (Fisher Scientific, Hampton, NH).

The column was eluted using a binary solvent system consisting of distilled water (solvent A) and methanol (solvent B) in a stepwise manner with steps of 0, 25, 50, 75, and 100% solvent B. The fractions were tested for their ability to induce MSC33 growth (see below), and the active fraction (75% methanol in water) was further purified on a reversed-phase high-pressure liquid chromatography (HPLC) column (10 mm by 250 mm by 10 μm [Alltech Applied Sciences, Breda, Netherlands]; Agilent 1100 series HPLC system [Agilent Technologies, Santa Clara, CA]) using a linear gradient of 15 to 45% acetonitrile (Fisher Scientific, Hampton, NH) in water containing 0.02% trifluoroacetic acid and 0.1% acetic acid (EMD Biosciences, San Diego, CA) for 20 min (4 ml/min; UV detection at 250 nm). A total of 16 fractions were obtained based on the UV absorbance. Two HPLC fractions, fractions 5 (retention time, 5.8 min) and 14 (retention time, 16.9 min), induced MSC33 microcolony formation (Fig. 2B). We chose fraction 14 for a detailed investigation in this study because its UV spectrum suggested that it consisted of two individual compounds, whereas fraction 5 appeared in UV to be significantly more complex. Fraction 14 was further purified by HPLC-UV spectrometry (C_8, 4.6 mm by 250 mm by 5 μm; Alltech Applied Sciences, Breda, Netherlands) using a linear gradient of 40 to 70% acetonitrile in water (15 min at 1 ml/min with UV detection at 210 nm) to obtain a pure compound capable of MSC33 induction. HPLC-diode array detector-UV spectrometry, ^{1}H nuclear magnetic resonance spectroscopy, and liquid chromatography-mass spectrometry confirmed purity and established that the compound was a peptide.

(ii) Mass spectrometry instrumentation. A custom-built qQq Fourier transform mass spectrometer was utilized in this study (21, 32). The instrument was designed to employ several fragmentation methods, including Q2 collision-activated dissociation (Q2 CAD) (21, 32), electron capture dissociation (ECD) (27, 51, 52), and sustained off-resonance irradiation CAD (12, 29). For the CAD experiments, the parent ions were isolated by Q1 and were fragmented by low-energy (15- to 30-eV) collisions with N_2 gas in Q2. For the ECD experiments, the parent ions were isolated in Q1 and externally accumulated in Q2. After being transmitted and trapped into the cylindrical ion cyclotron resonance cell, ions were irradiated with ~0.2-eV electrons from a 1.2-A heated dispenser cathode (44) for 15 to 300 ms. The total pulse duration for CAD and ECD experiments varied between 1 and 2 s, and all data were analyzed without apodization and with two zero fills to improve mass accuracy. Other experimental parameters were reported previously (21, 32, 49).

(iii) Peptide sequencing. Amino acid analysis was performed on a Beckman model 6300 automated amino acid analyzer according to the manufacturer's protocol (Beckman Coulter, Fullerton, CA). N-terminal amino acid sequencing was performed on Applied Biosystems model 474A and 494A sequencers ac-

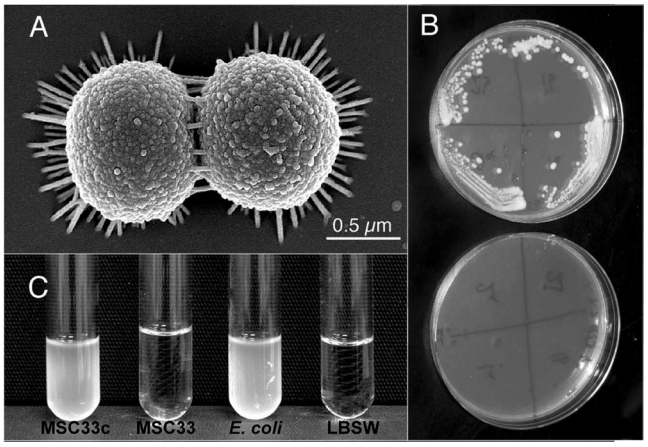

FIG. 2. MSC33 and growth properties of its cultivable variant MSC33c. (A) SEM view of MSC33c cells. (B) MSC33c shows substantial growth at 4°C after 23 days of incubation on LB medium. In parallel incubation, there was no proliferation of *Escherichia coli*. (C) MSC33 exhibits no growth after 23 h of incubation in LBSW with shaking at 240 rpm and 30°C. The cultivable variant MSC33c grew under the same conditions as *E. coli* (the latter was incubated at 37°C). For MSC33 and MSC33c, 1,000 cells were used as inocula. LBSW served as a sterile control. All the experiments were conducted in triplicate.

cording to the manufacturer's protocols (Applied Biosystems, Foster City, CA). All natural product analyses were performed using HPLC-grade reagents.

(iv) Bioassays. The effect of MSC33c-conditioned and unconditioned media, their extracts, and HPLC fractions of conditioned medium (hereafter collectively referred to as probes) on MSC33 growth was tested in uniformly designed bioassay experiments. The experiments utilized standard 24-well culture plates. Freeze-dried probes were reconstituted in deionized water to 5×, 1×, and 1/3× their respective concentrations in the MCS33c-conditoned spent medium, assuming no losses during extraction/fractionation. To each well, 5 μl of the probe was added, followed by pour plating of 500 μl of CA-MSC33 mix (as described above for the establishment of MSC33 cocultures) containing approximately 1,000 cells. Each bioassay included positive controls, in which induction of MSC33 growth was verified by using tissue culture inserts with MSC33c (see above), and negative controls, in which the probe either was absent or was replaced by an equivalent volume of LBSW or deionized water. Plates were incubated for 14 days at 25°C before microcolonies were counted microscopically as described above. A single researcher performed the counts, periodically in a double-blind manner, to control for a possible bias. To confirm results, each bioassay was replicated two to four times. Bioassay treatments were compared within an experiment using a two-sample *t* test that assumed homoscedasticity.

In an equivalent manner, we performed bioassays with synthetic peptides commercially synthesized at the Molecular Biology Core Facility (Dana-Farber Cancer Institute, Boston, MA) and the Biopolymers Laboratory (Harvard Medical School, Boston, MA). MSC33 cells were grown in CA medium amended with the synthesized 24-amino-acid peptide SLPQNIPPLTQTPVVVPPFLQPEV, the 16-amino-acid peptide LTQTPVVVPPFLQPEV, and the 8-amino-acid peptide PPFLQPEV and tested alongside solutions of their component amino acids (Sigma Aldrich, St. Louis, MO) in deionized water. Different parts of the 8-amino-acid peptide (PFLQPEV, FLQPEV, LQPEV, QPEV, and PEV, as well as LQPEV at concentrations varying from 0.35 nM to 35 mM) were tested similarly. We also used an alternative, casein-free (CF) medium consisting of 0.7% agar (Becton Dickinson, Franklin Lakes, NJ) supplemented with 0.02% (wt/vol) yeast extract and 4% (wt/vol) sea salts (Sigma Aldrich, St. Louis, MO).

Microscopy. Light microscopy observations were made using a Zeiss Stemi 2000C dissecting microscope equipped for dark-field microscopy and a Zeiss Axioskop 50 compound microscope equipped for DIC microscopy.

SEM was performed on MSC33c cells grown in LBSW overnight with shaking at 300 rpm at 25°C. A drop of this culture was incubated for 5 min on a methanol-washed coverslip treated with 0.1% poly-L-lysine. Excess cells and medium were removed by washing with deionized water. The coverslip was then fixed in electron microscopy-grade osmium in phosphate buffer and dried before being attached to a specimen stub with a carbon adhesive tab, sputter coated with 15-nm gold-palladium, and examined using an Amray AMR-1000 SEM.

Microbial identification. Colony PCR was used to amplify the 16S rRNA gene from each isolate with Platinum PCR SuperMix (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol using eubacterial primers 27F (AGA GTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) (26). PCR products were purified with a MinElute kit (Qiagen, Valencia, CA), cloned using a TA cloning kit with TOP10 cells (Invitrogen, Carlsbad, CA), and commercially sequenced at the University of Maine Sequencing Facility (Orono, ME). Sequences were compared to the GenBank database using BLAST and aligned to the ARB database (28).

Nucleotide sequence accession numbers. The sequences of isolates MSC5, MSC6, MSC7, MSC16, MSC33, MSC101, MSC102, MSC103, MSC104, MSC105, MSC106, MSC107, MSC108, MSC109, MSC110, MSC8, MSC22, MSC23, MSC44, MSC32, MSC34, MSC39, MSC9, MSC19, MSC33R, MSC35, MSC38, MSC10, MSC24, and MSC31 have been deposited in the GenBank database under accession numbers EU753120 to EU753149, respectively.

RESULTS

In order to identify a good model organism to study the nature of uncultivability, we isolated, using the diffusion chamber-based approach (23), a variety of uncultivable bacteria from a marine sediment community from the U.S. Northeast Atlantic coastal environment. Six isolates that did not grow in vitro in pure culture were identified (Table 1). We then paired them with a panel of cultivable marine isolates from the same environment and identified appropriate "helper" species for all six strains. We term a species a "helper" if at least one uncultivable isolate can grow on agar medium in its presence. Cocultures were established in a two-chamber apparatus where the insert containing the helper is placed on top of a petri dish seeded with the uncultured organism. A semipermeable membrane separates the insert compartment from the petri dish (Fig. 1A). Typically, each isolate would respond to more than one (but not all) of the helpers, and a single helper would induce growth of more than one (but not all) of the isolates tested. Further experimentation focused on one of the isolates, MSC33, which shared 95% 16S rRNA gene sequence identity with *Psychrobacter* spp. (Fig. 2). MSC33 did not grow on synthetic media in isolation but could be cocultured with MSC105, an isolate sharing 99% 16S rRNA gene similarity to *Cellulophaga lytica*.

In order to learn whether MSC33 could be selected for growth in the absence of a helper species, the culture was incubated repeatedly in two-compartment dishes with MSC105 as a helper. This produced a domesticated variant, MSC33c, with growth properties resembling those of conventional cultivable organisms. MSC33c produced macrocolonies (Fig. 2B) and formed a dense culture overnight on LB medium (Fig. 2C). MSC33c grows well at low temperature (Fig. 2B), which is typical for psychrophylic species from the North Atlantic, and has a 16S rRNA gene sequence identical to that of MSC33. To check whether this domesticated variant could act as a helper to the uncultivable parental strain MSC33, we paired them in the two-chamber dishes and observed the induction of growth (Fig. 3A). Furthermore, two additional rounds of cocultivation led to the appearance of cultivable cells in the MSC33 population, and the newly domesticated cells proved to be helpers to other populations of MSC33 (data not shown). The pattern of the initial lack of growth on casein-based medium, induction of growth by the presence of the helper strain, and eventual transformation into domesticated cells could be reproduced on a rich medium (LBSW) thus appeared to be growth medium independent.

We were interested to know whether the ability of uncultivable isolates to undergo domestication is a general phenomenon, and we tested a panel of 23 isolates that combined strains obtained in this and prior studies (22). Most isolates (74%) became amenable to colony formation on agar following just one to four transfers in diffusion chambers (Table 2). Apparently, the diffusion chamber presents an intermediate between natural and laboratory conditions and selects for domesticated variants. These findings support a hypothesis that uncultivability is a regulatory restriction that can be overcome by selection. Further empirical evidence has been provided by Bollmann et al. (3). As this restriction can also be relieved by the presence of a helper strain (Fig. 1B, C, and F and 3A), we proceeded with isolation and identification of the growth factor produced by such strains.

We obtained a 75% methanol fraction of the MSC33c-conditioned medium and found that it induced microcolony formation of MSC33 when added to agar-based media. Further bioassay-guided fractionation lead to identification of two active HPLC regions of this extract (Fig. 3B). We focused on the region that appeared to have a single active peak (fraction 14 in Fig. 3B) and identified this compound using mass spectrometry and N-terminal Edman degradation analysis.

Initial CAD experiments on a triple-quadrupole mass spec-

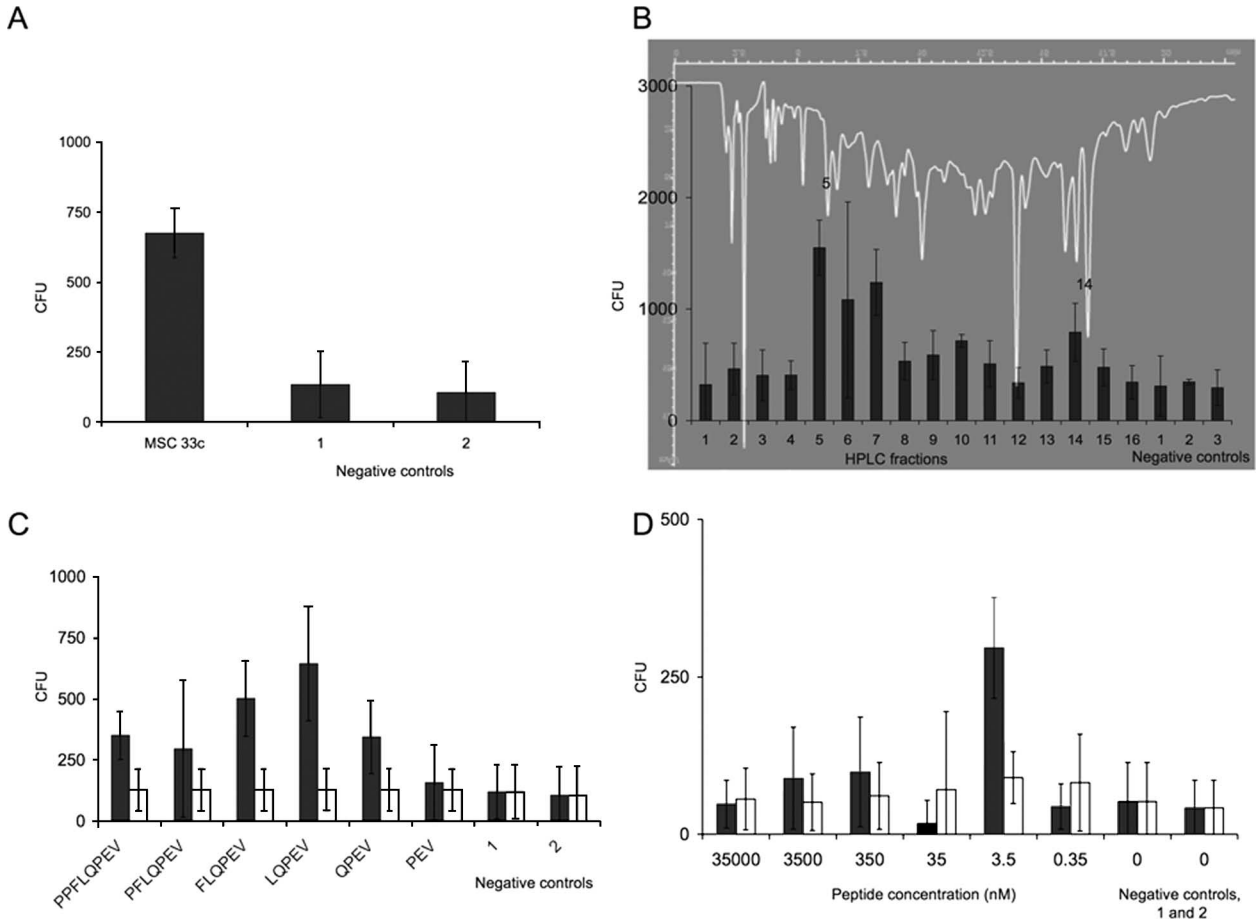

FIG. 3. Growth induction of MSC33 by its cultivable variant MSC33c and peptides produced in the presence of MSC33c. (A) The microcolony counts of MSC33 in the presence of MSC33c are statistically significantly higher ($P < 0.002$) than the background counts (negative control 1, insert not inoculated; negative control 2, no insert present). (B) Inverted HPLC trace of the 75% methanol extract of MSC33c-conditioned medium (LBSW). Out of the 16 HPLC fractions tested, three fractions (5, 7, and 14) induced statistically significant ($P < 0.05$) MSC33 growth. LBSW medium, deionized water, and no amendment (negative controls 1, 2, and 3, respectively) had no effect on MSC33 growth. (C) MSC33 growth in media amended with 2 nM of 3- to 8-amino-acid fragments of the peptide isolated from HPLC fraction 14 (solid bars). The 5-amino-acid peptide was the strongest inducer of MSC33 growth. Individual amino acids produced no effect on MSC33 growth (open bars), as did deionized water and no amendment (negative controls 1 and 2, respectively). (D) Effect of the 5-amino-acid peptide concentration on MSC33 growth, as for panel C. Six replicate wells were observed for each treatment. Error bars indicate standard deviations. See the parenthetical material at the end of the Fig. 1 legend on the nature of CFU in negative controls.

trometer were unable to distinguish between two similar sequences, SLPQNIPPLTQTPVVVPPFLQPEV and SLPQPIP PLTQTPVVVPPFLQNEV, which differ only in swapping two amino acid residues. Furthermore, the predicted fragment masses overlap heavily between the two species. Use of the qQq Fourier transform mass spectrometry system described in Materials and Methods provided both high mass accuracy and the ability to use combined CAD and ECD to determine the

TABLE 2. Numbers of chamber-to-chamber transfers of "uncultivable" isolates before emergence of a cultivable variant

Isolates, closest cultured relatives (% similarity)	No. of transfers
MSC4, undetermined identity; MSC6, *Colwellia psychroerythraea* (99); MSC8, *Vibrio* sp. strain R-3884 (97); MSC22, *Colwellia psychroerythraea* (94); MSC23, *Colwellia psychroerythraea* (96); MSC44, *Colwellia*-like sp. strain IC169 (95)	1
MSC32, *Pseudomonas trivialis* (100); MSC34, *Pseudomonas fluorescens* (99); MSC39, *Colwellia*-like sp. strain IC169 (95)	2
MSC9, *Colwellia* sp. strain IE1-3 (97); MSC19, *Acinetobacter junii* (97); MSC33R, *Pseudomonas fluorescens* (97); MSC35, *Microbulbifer degradans* (97); MSC38, *Colwellia psychroerythraea* (97)	3
MSC10, *Methylobacterium* sp. strain (97); MSC14, *Pseudomonas gessardii* (99); MSC17, *Pseudomonas gessardii* (100)	4
MSC3, undetermined identity; MSC16, *Janthinobacterium* sp. strain (99); MSC33, *Psychrobacter* sp. strain (95); MSC101, *Pseudoalteromonas atlantica* (89)	≥5
MSC24, *Aeromonas bestiarum* (97); MSC31, *Pseudomonas mephitica* (100)	Unreliable growth

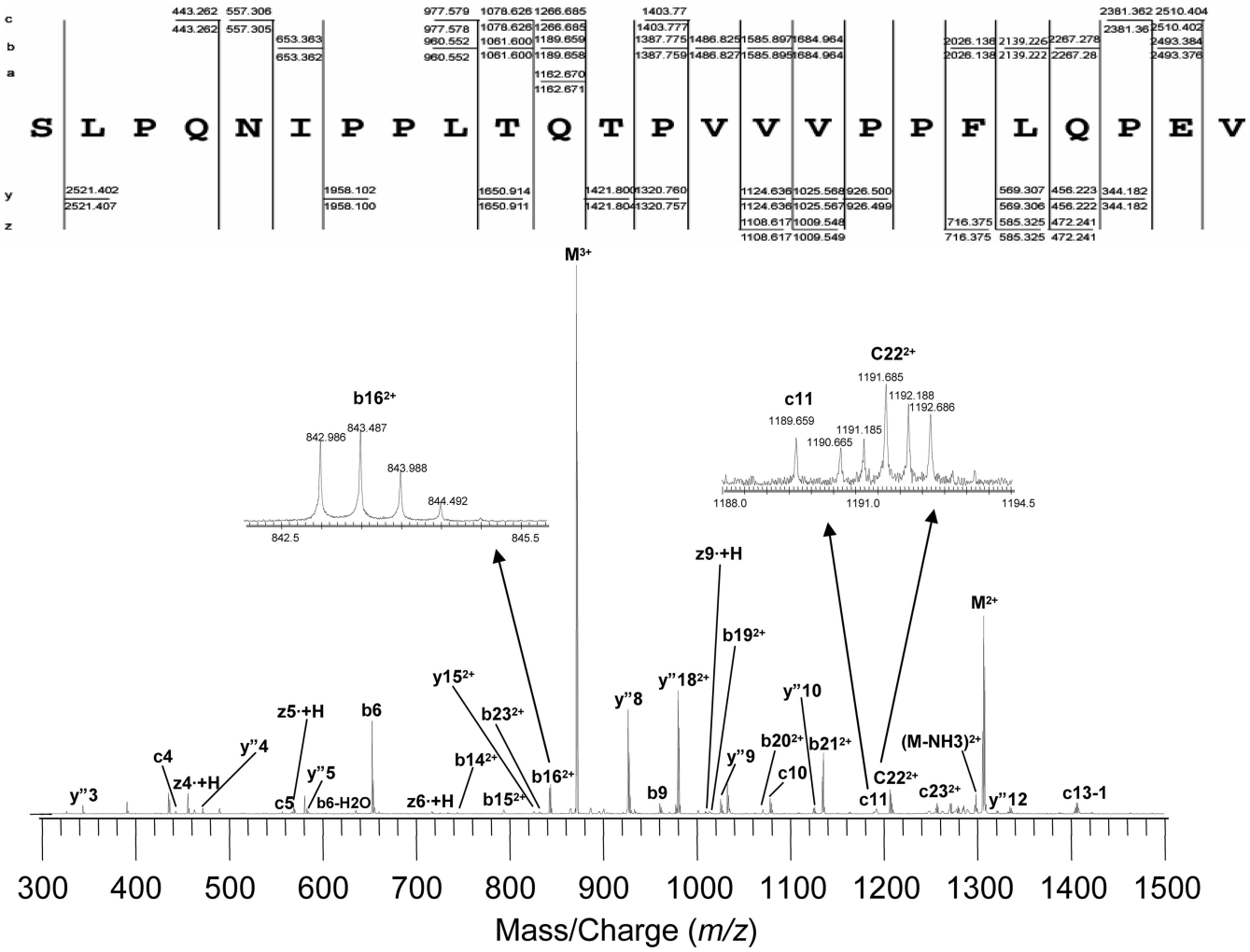

FIG. 4. Results of CAD/ECD experiment showing the characteristic fragmentation pattern of the peptide (bottom spectrum). The insets show an expanded view of the indicated ions illustrating the resolution of the mass spectrometric measurements. The sequence at the top is marked with observed cleavages, including the exact masses. For each pair of masses, the upper one represents the measured mass, while the lower is the mass of the fragment as calculated from the given sequence.

directionality of the cleavages (16). Thus, the amino acid sequence of the purified peptide was established as SLPQNIPP LTQTPVVVPPFLQPEV with a standard deviation of 2 ppm in the errors, while the opposite sequence yielded mass errors with a standard deviation of ~37 ppm. A typical spectrum is provided in Fig. 4. The sequence was further verified by N-terminal Edman degradation analysis, which confirmed the active compound to be a 24-amino-acid peptide, SLPQNIPPL TQTPVVVPPFLQPEV.

The sequence of the peptide matched that of β-casein. Casein is a component of the growth medium, which seemed to suggest that MSC33c was promoting growth of MSC33 by providing nutrients, products of extracellular casein digestion. We tested this possibility by modifying the medium in the growth induction experiments in two ways: (i) by adding mixtures of the 24-mers component amino acids and (ii) by using a CF growth medium. Neither affected the pattern of MSC33 growth: the first medium alone did not support growth (Fig. 3C and D), whereas in the second growth could be induced (Fig. 5). We then checked whether different fragments of the peptide exhibited relevant activity and discovered that the 16-mer fragment (LTQTPVVVPPFLQPEV) had no effect on MSC33 but the 8-mer fragment (PPFLQPEV) was active (data not shown). The activity of the 8-amino-acid fragment was higher than that of the 24-mer, and we conducted a detailed investigation of different peptides from 3 to 8 amino acids long. Most of these peptides showed a degree of activity, whereas the addition of the respective mixes of individual amino acids had no effect (Fig. 3C). The 5-amino-acid peptide LQPEV proved to be the strongest inducer of MSC33 growth (Fig. 3C), being particularly active at 3.5 nM (Fig. 3D).

DISCUSSION

It is unclear why a majority of microorganisms are uncultivable using standard methods (2, 23, 31). In recent years, mimicking natural growth conditions (11, 40), and especially their direct simulation (3, 9, 10, 23, 34, 48), has been successful

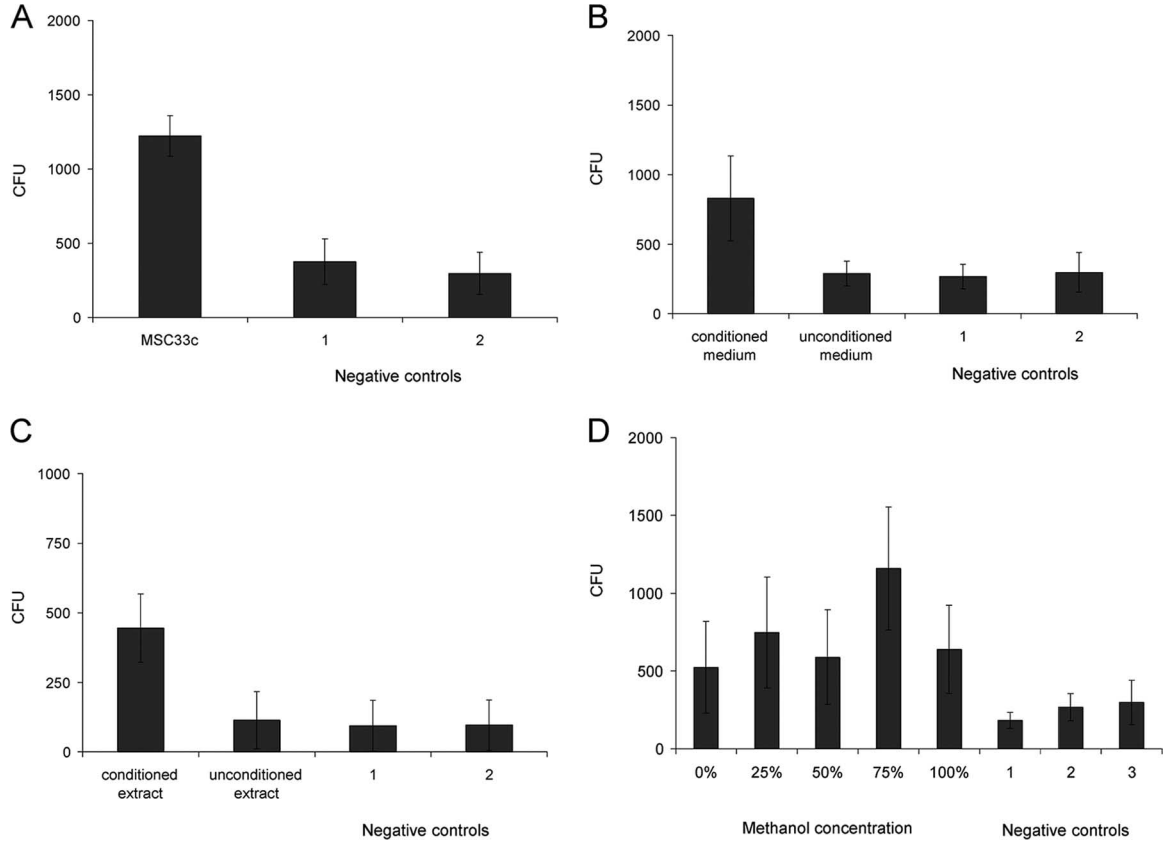

FIG. 5. Growth induction of MSC33 in the absence of casein. (A) The microcolony counts of MSC33 in the presence of MSC33c are statistically significantly higher ($P < 0.02$) than the background counts in CF medium (negative control 1, insert not inoculated; negative control 2, no insert present). (B) The microcolony counts of MSC33 in CF medium amended with MSC33c-conditioned medium (artificial seawater-based yeast extract) are statistically significantly higher ($P < 0.02$) than control counts. Deionized water and unconditioned yeast extract medium (negative controls 1 and 2) had no effect on MSC33 growth. (C) The microcolony counts of MSC33 in CF medium amended with an extract of MSC33c-conditioned medium (artificial seawater-based yeast extract) are statistically significantly higher ($P < 0.02$) than control counts. Unconditioned medium extract, deionized water, and unconditioned yeast extract medium (negative controls 1, 2, and 3, respectively) had no effect on MSC33 growth. (D) CFU counts of MSC33 in CF medium amended with methanol extracts of MSC33c-conditioned medium (artificial seawater-based yeast extract) are statistically significantly higher ($P < 0.05$) than control counts. Negative controls are as for panel C. Six replicate wells were observed for each treatment. Error bars indicate standard deviations. See the parenthetical material at the end of the Fig. 1 legend on the nature of CFU in negative controls.

in gaining additional isolates and increasing recovery and diversity of cultivated species. This is consistent with the idea that positive microbial interactions, rather than simple deficiencies in medium composition, are key to cultivating the microbial majority.

In this study, we focused on the interactions between *Psychrobacter* sp. strain MSC33 and its "helper" species as models to describe the nature of uncultivability. The genus *Psychrobacter* belongs to the order *Pseudomonales*, and representative organisms include marine, freshwater, and soil species and possible opportunistic pathogens (4). The genomes of two *Psychrobacter* species have been sequenced, and they indicate the ability of bacteria from this group to use a broad variety of nutrients. It therefore seemed unlikely that the inability of our *Psychrobacter* isolate to grow in vitro was due to the lack of a specific nutrient.

We isolated a 24-amino-acid peptide from helper-conditioned medium capable of MSC33 growth induction in agar. Our observations suggest that this peptide derived from casein acts as a signaling molecule and not as a nutrient. Specifically, the 5-amino-acid segment LQPEV was particularly active at 3.5 nM (Fig. 3C). This indicates that MSC33c did not induce MSC33 colony formation by providing additional nutritional support. Syntrophy is especially unlikely because LQPEV is active at a concentration three orders of magnitude below what is typically regarded as oligotrophic conditions (25). Further, previously reported signal peptides are active at similar (nanomolar) concentrations (38). A more probable explanation is that MSC33c produces a short peptide acting upon MSC33 as a growth-inducing signal. Accordingly, the 24-amino-acid peptide that we isolated must have simply mimicked this signal. The activity of the 5-amino-acid fragment suggests that it may either be identical to or closely match the naturally occurring molecule. Irrespective of the origin of the peptides investigated here, the discovered pattern of their activity points strongly toward the signal-based growth regulation of MSC33 and likely of other uncultivable strains studied. We hypothesize that the difference between the cultivable and uncultivable variants of

the same organism may be in the amount of the signaling molecule produced, with the former being self-sufficient in building up the signal's concentration necessary for growth. The "uncultivable" strain responds to the signal produced by the helper strain and starts growing. This eventually leads to the selection of cells overproducing the signaling compound and thus to domestication. This explanation is consistent with the recently reported (3) pattern of domestication of freshwater bacterial isolates.

First suggested by the interdependence of growth of two marine strains, MSC1 and MSC2 (23), a signaling hypothesis explains that at least some "uncultivable" microorganisms do not grow in vitro because the standard petri dish does not provide the signaling molecules and communication network of natural communities. The efficiency of cultivation of aquatic microorganisms has also been improved by the addition of the signal molecules cyclic AMP and *N*-acyl homoserine lactone (6). Many microbial processes are regulated via cell-cell communication (8, 47), with some utilizing short peptides (24, 41), and our findings indicate that growth may be one such process. We observed that MSC33 could be induced to grow in synthetic medium not only by MSC33c but by unrelated species as well (e.g., MSC105 [Fig. 1B, C, F] and *Salmonella enterica* serovar Typhimurium [data not shown]), indicating the importance of both intra- and interspecies communication in regulating microbial growth. In nature, such regulation would ensure that the cell commits to division not only in the environment with a sufficient nutrient supply but additionally when and where its kin and/or some other specific strains are already growing successfully in close proximity to the cell. The selective advantage of such a strategy is that the cell divides only when a specific set of favorable conditions has existed in the habitat for a period of time and therefore is not ephemeral. In the lab, the use of standard media to isolate pure cultures of microbes with such growth regulation would naturally lead to the cells' growth arrest. We suggest that this arrest contributes to the "great plate count anomaly." Accordingly, the resolution of the phenomenon and domestication of uncultivated species in the lab should be possible through identification of the signaling compounds regulating microbial growth and their addition to standard medium formulations, as exemplified in this study.

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