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Marine *Vibrio* Species Produce the Volatile Organic Compound Acetone

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While screening aerobic, heterotrophic marine bacteria for production of volatile organic compounds, we found that a group of isolates produced substantial amounts of acetone. Acetone production was confirmed by gas chromatography, gas chromatography-mass spectrometry, and high-performance liquid chromatography. The major acetone producers were identified as nonclinical *Vibrio* species. Acetone production was maximal in the stationary phase of growth and was stimulated by addition of L-leucine but not the other common amino acids, suggesting that leucine degradation leads to acetone formation. Acetone production by marine vibrios may contribute to the dissolved organic carbon associated with phytoplankton, and some of the acetone produced may be volatilized to the atmosphere.

It is now known that the biosphere emits large quantities of volatile organic compounds (VOCs) into the atmosphere. Some VOCs, like methane, isoprene, and monoterpenes, are emitted from terrestrial sources at levels of hundred of millions of metric tons per year on a global basis and have important effects on atmospheric chemistry and global climate (12, 20). Oceans are significant sources of light hydrocarbons, including ethane, ethylene, propane, and propylene (reviewed in reference 26). Presumably, many of these marine hydrocarbons are produced by phytoplankton, which are known to produce a complex array of VOCs (29).

Recently, it was reported that the VOC isoprene is produced in seawater (6, 19, 22). We set out to determine if marine microorganisms could be responsible for this production. During these investigations we detected substantial production of acetone by marine heterotrophic bacteria; our observations concerning this acetone production are reported in this paper.

MATERIALS AND METHODS

Screening marine bacteria for acetone production. Marine heterotrophic bacteria were isolated from seawater by inoculating seawater onto enriched seawater agar containing 80% (vol/vol) seawater, 0.1% (wt/vol) glucose, 0.1% (wt/vol) Bacto Tryptone, and 0.01% (wt/vol) yeast extract. The plates were incubated at 20 to 22°C and, after visible colonies were obtained, were sealed with Parafilm for 4 to 16 h to allow VOCs to accumulate in the headspace above the agar layer. Gas samples were removed with a gas-tight syringe by passing a side port needle though the Parafilm. Usually, 0.25- to 1.0-cm^3 gas samples were removed (approximately 1 to 4% of the available headspace) for analysis by gas chromatography (GC). We used two different instruments and detectors for GC. In our initial experiments we used a Photovac model 10S portable instrument (GC method A); this instrument was equipped with a 6-foot (ca. 183-cm) type CPSil-5 capillary column and a photoionization detector. The carrier gas was hydrocarbon-free air (8 cm^3 min^{-1}), and the column was typically operated at 23°C; under these conditions acetone eluted at 73 ± 2 s. In later experiments, we used a Hewlett-Packard model 5790A instrument equipped with a flame ionization detector and 30-m type DB-5 capillary column (film thickness, 1 μm; J&W Scientific, Rancho Cordova, Calif.) (GC method B). This instrument has been described in detail elsewhere (16). Typically, we performed an isothermal analysis at 50°C, and under these conditions acetone eluted at 1.40 ± 0.02 min.

Verification of acetone by GC-MS and HPLC. Cultures of strain VW2 were grown aerobically in marine broth (marine broth 2216; Difco Laboratories, Detroit, Mich.) and were transferred to vials sealed with Teflon-lined septa, and after incubation for approximately 16 h at 22°C the headspace gases were analyzed by GC-mass spectrometry (GC-MS), using previously described methods (16); an acetone standard was also analyzed by GC-MS at the same time.

Acetone production in marine broth bacterial cultures was analyzed by performing a high-performance liquid chromatography (HPLC) analysis of the liquid phase. Samples of growth medium (after cells were removed by centrifugation) were derivatized with 2,4-dinitrophenylhydrazine, and the resulting dinitrophenylhydrazone (DNP) derivatives were analyzed on a C_{18} reversed-phase column (3.9 by 150 mm; bead diameter, 4 μm; Waters Corp., Milford, Mass.) by the method of Brega et al. (7). Elution with acetonitrile-water (60:40, vol/vol) at a rate of 1 ml/min and detection at 354 nm were used; authentic DNP-acetone eluted at 4.75 ± 0.01 min.

Routine quantitation of acetone formation in bacterial cultures. In most of the experiments described below, headspace acetone content was determined by GC, and the acetone content of the liquid phase was calculated from the liquid-air partitioning value for acetone at 25°C, which was determined by methods similar to those described by Sato and Nakajima (24). In a typical experiment 4.8-ml vials containing liquid samples (0.5 to 1 ml) were sealed with Teflon-lined septa and incubated for 0.5 to 2 h at 25°C, and then headspace samples were analyzed by GC method B. The partitioning of acetone between the culture broth and the headspace was determined after known concentrations of acetone were added to bacterial cultures. To validate the method, the marine broth-air partition coefficient of acetone was measured and was found to be 417 ± 19 (average of eight determinations at 25°C); the previously published blood-air and water-air acetone partition coefficients are 343 and 395, respectively, at 37°C (24).

In some experiments marine *Vibrio* isolates were also grown in a defined marine salts medium (MSM) (medium BM of Baumann and Schubert [5] prepared in half-strength artificial seawater) containing different carbon sources, and the production of acetone was measured by the partition method. Vitamin-free Casamino Acids (Difco) were used for experiments in which casein amino acids were used as carbon sources.

Bacterial identification. Acetone-producing strains VW1, VW2, and VW3, which were isolated from seawater at Newport Beach, Calif. (Table 1), were identified to the genus level by the general procedures described by Austin (1), Austin and Lee (2), Baumann and Schubert (5), and Farmer and Hickman-Brewer (11). TCBS agar (11) was obtained from Difco, and compound 0/129 (2,4-diamino-6,7-diisopropylpteridine) was obtained from Sigma Chemical Co., St. Louis, Mo. Cellobiose-polymyxin B-colistin agar (18) was used to determine that the acetone-producing strains were not *Vibrio vulnificus* strains. An authentic culture of *Vibrio fischeri* ATCC 7744 was obtained from the American Type Culture Collection, Rockville, Md.

RESULTS AND DISCUSSION

Marine heterotrophic bacteria were isolated from seawater by inoculating seawater onto enriched seawater agar. An analysis of the headspace gases (by GC method A) produced by heterotrophic colonies on these plates revealed that there was a large unknown peak at 72 to 74 s, a retention time range identical to the retention time range for authentic acetone

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TABLE 1. Percentages of recovered coastal marine heterotrophic bacteria and marine vibrios that produced acetone

Seawater location	Total no. of heterotroph cells per ml[a]	% of heterotroph samples that produced acetone[b]	Total no. of vibrio-like colonies cells per ml[c]	% of vibrio-like colonies that produced acetone[b]
Huntington Beach Pier, Calif. (surf zone)	4.7×10^5	12 (4/32)[d]	5.2×10^3	93 (28/30)
Newport Beach, Calif. (inlet to Newport Bay)	4.3×10^4	13 (4/31)	3.6×10^3	60 (18/30)
SIO Pier, La Jolla, Calif. (flowing seawater)	1.1×10^3	66 (21/32)	2.2×10^2	100 (32/32)

[a] The numbers of heterotrophs were determined after plating and growth on marine agar; the values are averages of triplicate determinations.
[b] We selected at random 30 to 32 colonies from several plates and tested these colonies for acetone production after growth in marine broth; the GC headspace procedure was used for acetone analysis, and a culture was positive if it contained more than 0.085 mM liquid phase acetone. Typically, the acetone-positive strains produced 0.17 to 0.51 mM acetone. Each sample was analyzed in duplicate.
[c] The numbers of vibrio-like cells were determined after plating and growth on TCBS agar; the values are averages of triplicate determinations.
[d] The values in parentheses are number of positive colonies/number of colonies tested.

(data not shown). Single colonies were isolated by restreaking them on enriched seawater agar and then were analyzed to identify those organisms that produced the putative acetone peak. This resulted in the isolation of numerous acetone-producing bacterial cultures from seawater collected along the California coast. Three pure cultures (strains VW1, VW2, and VW3) were further characterized as described below. These organisms were grown aerobically in marine broth and transferred to vials, and the headspace gases were analyzed by GC method B. This analysis revealed that the major detectable VOC in the culture headspaces was acetone. Headspace samples from strain VW2 were analyzed by GC-MS, and the major detectable VOC was found to be acetone. The major ions detected were *m/e* 58 (parent ion) and *m/e* 43 (CH_3CO^+); this pattern was identical to the authentic acetone pattern.

Acetone production in these bacterial cultures was also verified by HPLC. After growth in marine broth, samples of spent media were derivatized with 2,4-dinitrophenylhydrazine to produce DNP derivatives of carbonyl compounds, and the DNP derivatives were analyzed on a C_{18} reversed-phase column. An analysis of cultures of marine heterotrophs VW1, VW2, and VW3 revealed that these organisms produced similar elution profiles, in which the major peak coeluted with authentic DNP-acetone (retention time, 4.75 ± 0.01 min). In many experiments other putative carbonyl derivative peaks were also observed; one of these peaks coeluted with DNP-acetaldehyde, but we did not confirm that acetaldehyde was present in the culture media.

Acetone-producing strains VW1, VW2, and VW3 were identified to the genus level by using standard taxonomic tests. Briefly, these three strains have the following properties: gram-negative, facultatively anaerobic, motile rods; oxidase positive; positive for indole-phenol; acid, but no gas, is produced from glucose; gelatin positive; urease negative; esculin positive; nonluminescent; sodium chloride is required for growth; growth occurs and a yellow or green pigment is formed on TCBS agar (11); and sensitive to compound 0/129 (2,4-diamino-6,7-diisopropylpteridine). Strains VW1 and VW2, but not VW3, grew at 37°C. None of the strains grew on cellobiose-polymyxin B-colistin agar (18), indicating that they are not *V. vulnificus* strains. On the basis of these characteristics, we identified isolates VW1, VW2, and VW3 as members of a nonluminescent *Vibrio* species; these organisms are most similar to the marine, nonclinical species listed by Farmer and Hickman-Brenner (11). It is noteworthy that a culture collection culture of luminescent, marine isolate *V. fischeri* ATCC 7744 produced acetone at levels similar to the levels produced by strains VW1, VW2, and VW3, suggesting that acetone production may be a widespread trait in marine vibrios.

We performed several experiments to determine the frequency of acetone-producing marine heterotrophs among heterotrophs isolated from coastal waters from southern California. Table 1 shows the results of an experiment in which seawater heterotrophs were recovered by plating them on marine broth agar and were examined at random for acetone production. Separately, the same seawater samples were plated on a vibrio enrichment medium, TCBS agar (11). For each seawater sample the number of heterotroph colonies on marine agar and the number of vibrio-like colonies on TCBS agar were determined, and then 32 colonies were picked at random from each type of plate and tested for acetone production following growth in marine broth. The percentages of recovered marine heterotrophs that produced significant amounts of acetone (>0.085 mM, the detection limit under the conditions used) ranged from 12 to 66% in these experiments. The percentages of acetone-positive colonies recovered on TCBS agar were consistently higher (range, 60 to 100%). These results cannot be extrapolated to total marine heterotroph populations, since plating methods like the one used in this study often underestimate numbers of viable cells (23). Nevertheless, our preliminary results demonstrate that acetone-producing heterotrophs are quite common in coastal waters of southern

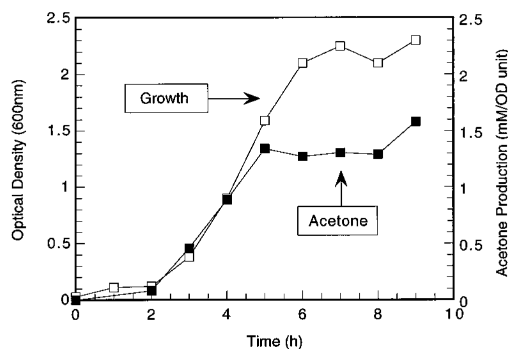

FIG. 1. Production of acetone by *Vibrio* sp. strain VW2 as a function of culture age. Log-phase *Vibrio* sp. strain VW2 was inoculated into fresh marine broth medium and cultured with shaking at 25°C. At intervals, aliquots (1 ml) of the culture were transferred to sealed vials, and the concentration of acetone in the culture medium was determined as described in Materials and Methods. All data are the averages of duplicate determinations; the results of one representative experiment of several replications are shown. OD, optical density.

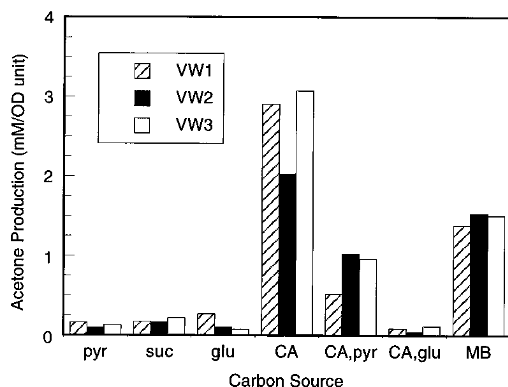

FIG. 2. Production of acetone by three marine *Vibrio* strains as a function of growth medium composition. Strains were inoculated into marine broth (MB) or MSM containing the following carbon sources: 1% (wt/vol) Casamino Acids (CA); 1% (wt/vol) glucose (glu); 1% (wt/vol) sodium pyruvate (pyr); 1% (wt/vol) sodium succinate (suc); 0.5% (wt/vol) Casamino Acids plus 0.5% (wt/vol) glucose (CA, glu); or 0.5% (wt/vol) Casamino Acids plus 0.5% (wt/vol) sodium pyruvate (CA, pyr). Cultures were grown aerobically at 25°C to the stationary phase, and the concentration of acetone in the culture medium was determined as described in Materials and Methods. All data are the averages of duplicate determinations. OD, optical density.

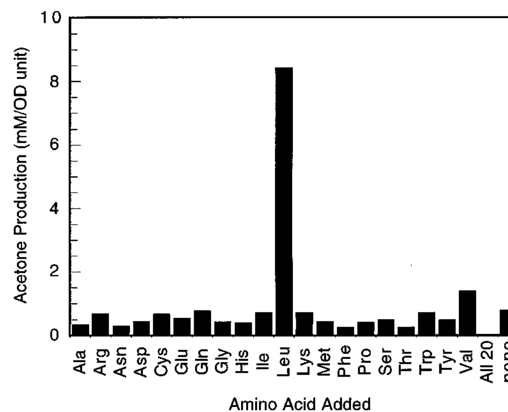

FIG. 3. Addition of L-leucine, but not other amino acids, stimulates acetone production by *Vibrio* sp. strain VW2. Strain VW2 was grown aerobically in MSM preparations containing 0.1% Casamino Acids and 0.2% sodium pyruvate as carbon sources and individual L-amino acids at a concentration of 10 mM. The acetone concentrations in stationary-phase cultures were determined as described in Materials and Methods. The data are the averages of duplicate determinations. OD, optical density.

California and that TCBS agar enriches for acetone-producing vibrios.

We investigated the effects of some growth conditions on acetone production by marine *Vibrio* isolates. Figure 1 shows a growth curve for isolate VW2 in marine broth. The amount of acetone in the culture medium was determined at various growth stages, and we found that acetone was produced primarily in the stationary phase of growth; in this phase of growth the concentration of acetone in the medium was 1.7 to 2.4 mM. Similar results were obtained with strains VW1 and VW3. The three marine *Vibrio* isolates were also grown in MSM containing different carbon sources, and the amount of acetone produced was determined. The results are shown in Fig. 2; the amount of acetone produced after growth in marine broth is shown for comparison. In MSM very little acetone was produced when glucose, pyruvate, or succinate was the carbon source. In contrast, growth on MSM containing 1% (wt/vol) Casamino Acids as a carbon source resulted in production of substantial amounts of acetone; the amount of acetone produced under these conditions was greater than the amount produced in marine broth. The combination of glucose and Casamino Acids or pyruvate and Casamino Acids resulted in production of low levels of acetone, perhaps because of glucose or pyruvate repression of amino acid catabolism.

It seemed likely that one or more amino acids in Casamino Acids or in marine broth stimulated acetone production. To determine whether amino acids could stimulate acetone production, *Vibrio* sp. strain VW2 was grown in MSM containing 0.2% (wt/vol) sodium pyruvate and 0.1% (wt/vol) Casamino Acids as carbon sources; individual medium preparations were supplemented with each of the common L-amino acids (final concentration, 10 mM) or with all 20 amino acids. L-Leucine, but not any of the other L-amino acids, stimulated acetone production (Fig. 3). Interestingly, growth in the presence of all 20 amino acids suppressed acetone formation (Fig. 3). Stimulation of acetone production by L-leucine was also observed with *Vibrio* strains VW1 and VW3.

Acetone is a well-known product of various anaerobic bacteria, including *Clostridium acetobutylicum*, that have been used for commercial production of acetone (14). Other aerobic bacteria are known to produce traces of acetone as a metabolic by-product (4, 13, 17). Harvey (13) found that several strains of *Streptococcus cremoris* and *Streptococcus lactis* produced 0.1 to 1.0 mg of acetone per liter (1.7 to 17 μM) when they were cultured in skim milk. A *Brevibacterium linens* strain was found to produce a variety of volatile carbonyl compounds, including acetone, when it was grown in a casein solution; formation of acetone was stimulated by individual amino acids, including aspartic acid, glutamic acid, and leucine (17). In our study, acetone was a major product in marine vibrio cultures; up to 8.7 mM acetone was detected in a defined medium in the presence of L-leucine.

There is little previously published information concerning the metabolism of amino acids by vibrios. It has been reported in taxonomic studies that a few vibrios, including *Vibrio nereis*, are capable of leucine catabolism (5), but no analysis of the products of leucine catabolism has been reported. Leucine degradation has been detected in a few bacterial genera, and it is thought that bacteria such as *Pseudomonas aeruginosa* use a leucine catabolic pathway similar to the pathway in animal tissues, in which leucine is converted to acetoacetate and acetyl coenzyme A (27). In animal tissues, leucine is considered a ketogenic amino acid since acetoacetate can be further metabolized to the ketone acetone (28). It is not known if the vibrios which we studied use a similar pathway to convert leucine to acetone. In contrast to animal systems, in which lysine and, to a lesser extent, isoleucine, phenylalanine, threonine, tryptophan, and tyrosine are ketogenic, *Vibrio* sp. strain VW2 produced little acetone in the presence of these amino acids (Fig. 3).

The environmental significance of our findings is unclear. It is not known whether marine vibrios produce acetone in situ or whether acetone is found in seawater associated with bacterioplankton. It is known that oceanic bacterial growth is largely supported by organic compounds, including amino acids and proteins, that are released by phytoplankton and zooplankton (reviewed by Ducklow and Carlson [10]). Several workers have described the uptake of free amino acids by marine bacteria (3, 8, 15), including the uptake and metabolism of L-leucine (9). The production of acetone in marine surface films could be an indicator of metabolism of leucine by *Vibrio* species. However, we could find no previous report of the presence of acetone in

seawater. It is interesting that the seawater samples analyzed in this work contained bacteria that are able to grow on acetone as a sole carbon source (21). This suggests that groups of marine bacteria can be either sources or sinks of acetone. Whether marine bacteria are a significant source of atmospheric acetone, one of the major nonmethane VOCs in the troposphere (25), needs to be established.

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