APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1992, p. 237–242
0099-2240/92/010237-06$02.00/0

Vol. 58, No. 1

# Regulatory Influences on the Production of Gamma-Aminobutyric Acid by a Marine Pseudomonad

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Received 30 July 1991/Accepted 30 October 1991

**A pseudomonad capable of producing γ-aminobutyric acid (GABA) was isolated from seawater via an enrichment in which glutamate was the sole carbon and nitrogen source. The organism grew optimally at pH 7.3 and at 25°C. Putrescine, alanine, and glucose-nitrate also served as effective growth substrates. The isolate grew poorly on GABA. Cell suspensions of the organism in 0.02 M phosphate buffer (pH 7.6) containing NaCl (19.4 g liter$^{-1}$) and $MgCl_2 \cdot 6H_2O$(3 g liter$^{-1}$) produced GABA from succinic semialdehyde in combination with glutamate or alanine but not from any substrate alone. Little or no GABA was produced with putrescine or glucose-nitrate as substrates. GABA production in the amino acid cosubstrate systems was transitory with optimum levels occurring in the suspension fluid after 3 h of incubation (0.3 and 0.03 mM for glutamate and alanine cosubstrates, respectively). However, yields of GABA in the cell suspension fluid were low, and quantities near that predicted from stoichiometry could be obtained only by extracting cell suspensions with methanol. GABA release in the suspension fluid was increased with higher pH or by decreasing NaCl. Substitution of the salt by the equivalent Tris-HCl or KCl likewise resulted in increased GABA release. When nigericin (10 μg ml$^{-1}$) was added to cell suspensions in which NaCl was not decreased, GABA release increased in a way similar to that observed in suspensions with decreased NaCl. The ionophore also decreased GABA uptake by cell suspensions of GABA-grown cells, and the effect was duplicated by lowering NaCl in cell suspensions. The results indicate a role for an $Na^+$-dependent transport system in GABA release.**

Gamma-aminobutyric acid (GABA) and related compounds inhibit velar movement in planktonic larvae of certain marine molluscs, inducing their settlement (13–18, 22). However, it is not clear whether these substances result entirely from algal metabolism or whether microorganisms on algal surfaces are also involved in their production. Microorganisms are known to produce GABA from several substrates (8, 11, 12, 20, 23), and they also utilize the compound (1, 2, 4, 5, 21, 26). Thus, it is conceivable that they could affect the settlement of invertebrate larvae by producing or consuming the settlement inducer. Recently we reported that marine microorganisms can utilize GABA (7). In this communication we report on microorganisms from marine environments that produce GABA from amino acids. We also describe the effects of several factors likely to be encountered in these environments which influence GABA release.

## MATERIALS AND METHODS

**Organism.** *Pseudomonas* sp. strain G-17, isolated from seawater, was the organism used in this study. The isolate was identified by P. Short (New Zealand Communicable Disease Center, Wellington, New Zealand), and deposited in the New Zealand Reference Culture Collection (NZRM no. 3260) located at the Communicable Disease Center.

**Culture medium.** Culture medium duplicated the mineral composition of seawater and was prepared according to the formula of Zobell (27) but with peptone and yeast extract omitted. Vitamins (9) were added (0.5% vol/vol), and the medium was dispensed into conical culture flasks in 10- or 50-ml aliquots. Flasks were plugged with cotton wool and autoclaved at 15 lb/in$^2$ for 15 min. After cooling and prior to inoculation, sterile 0.2 M phosphate buffer (pH 7.6) was added to flasks (2% vol/vol) followed by addition of sterile substrate to give a final concentration of 10 to 20 mM.

**Culture techniques.** Cultures were maintained on agar slants in McConkey bottles in which the basal medium contained 10 mM sodium glutamate and 2% (wt/vol) agar. Cultures were transferred every month after incubation at 25°C. Transfer of cultures to liquid medium was achieved by inoculation from a slant into 10 ml of medium by using a loop and subsequent inoculation into 50 ml of medium with a 5% (vol/vol) inoculum of the liquid culture at full growth. All incubations were conducted at 25°C, and liquid cultures were shaken on a gyro-rotatory shaker at 60 oscillations min$^{-1}$.

**Culture purity.** Purity of the organism was established by routinely streaking onto agar plates containing marine broth (Difco Laboratories) no. 2216 at 37.4 g liter$^{-1}$ and examining colonies after 1 week of growth at 25°C. Cultures were also routinely checked by phase-contrast microscopy for purity.

**Cell suspension experiments.** Cells grown in two 50-ml cultures with glutamate as the substrate unless otherwise stated were harvested by centrifugation at 6,000 × *g* for 15 min at 2°C, at full growth ($A_{600}$ = 1.2). The supernatant was discarded, and the pellet was resuspended in 50 ml of 0.02 M phosphate buffer (pH 7.6) containing NaCl (19.4 g liter$^{-1}$) and $MgCl_2 \cdot 6H_2O$ (3 g liter$^{-1}$) (PSM buffer) to give a cell concentration of between 1 and 2 mg (dry weight) per ml. Aliquots (6 to 8 ml) of cell suspension were then dispensed into centrifuge tubes and centrifuged at 6,000 × *g* for 15 min at 2°C. Pellets were resuspended in the same volume of PSM buffer containing the pre-added substrates (unlabelled or with [U-$^{14}$C]GABA at the appropriate concentrations). Where required, inhibitors or ionophores were added to PSM buffer at the time of substrate addition and, in cases where they were sparingly soluble in water, were predis-

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solved in *N,N*-dimethylformamide prior to addition to PSM. The total volumes of each cell suspension were transferred to 50-ml conical flasks, the tops of which were covered with aluminum foil, and incubated with shaking at 25°C. At appropriate intervals during the course of incubation, samples (0.5 or 1 ml) were removed and either treated with the equivalent volume of methanol (to give total GABA content of sample) or centrifuged. Methanol-treated samples or supernatants were stored at −18°C for high-performance liquid chromatography (HPLC) analysis. Where radioactive measurements were required, 0.2-ml aliquots of supernatant were transferred to liquid scintillation vials.

**Analytical techniques.** Substrates and products in cell suspensions were determined by HPLC after derivatization with phenylisothiocyanate (7) and were routinely quantified by comparison of peak heights with known standards; the detection limit for amino acids, including GABA, was 1 μM. GABA was also quantified by measuring the formation of NADPH from NADP in the presence of GABase (γ-aminobutyric glutamic transaminase, succinic semialdehyde dehydrogenase) and α-ketoglutarate (25).

For determination of GABA utilization by radioactivity measurements, 0.1 ml of 15% $H_2SO_4$ was added to samples in scintillation vials followed by 0.5 ml of methanol. Samples were evaporated to near dryness under $N_2$ and received 0.2 ml of $H_2O$ and 0.5 ml of methanol. Samples received 10 ml of toluene-based scintillant mixed with Triton X-100 (2:1, vol/vol) (19) and were counted by liquid scintillation procedures.

Analysis of inorganic cations was carried out by atomic absorption spectrometry.

**Growth and dry weight measurements.** Growth was determined by measurement of the $A_{600}$; to estimate dry weight, suspensions of cells were washed twice with distilled water and dried at 60°C to a constant weight.

**Microscopy.** To establish whether or not cell damage or lysis occurred, cell suspensions were examined by phase-contrast microscopy.

**Chemicals.** GABA and GABase were obtained from Sigma Chemical Co., St. Louis, Mo. [U-$^{14}$C]GABA (specific activity, 220 μCi μmol$^{-1}$) was obtained from the Radiochemical Centre, Amersham, England. All other chemicals were of reagent grade and were obtained from commercial sources.

## RESULTS

**Enrichment and isolation of GABA-producing strains.** Flasks containing 50 ml of culture medium with 20 mM glutamate as the carbon and nitrogen source showed growth 10 days after inoculation with 10 ml of seawater. At full growth ($A_{600}$, 1.5), 10% of the volume of enrichment was transferred to new medium, and the enrichment was maintained by transfer every 72 h. After the enrichment was maintained for 1 month, serial dilutions (1 to $10^{-8}$ ml) of a culture at full growth were streaked onto agar plates containing 20 mM glutamate or no substrate. After incubation of the plates for 72 h, colonies appeared on the glutamate plates, and these were picked after 2 weeks. On the basis of colony type and morphology, 17 isolates which were capable of growth on glutamate were obtained from the enrichment. The cultures were further purified by plating and were maintained on glutamate agar slants.

When the isolates were grown on 20 mM glutamate in liquid culture, only eight grew well on the substrate (μ, ≥0.06 h$^{-1}$; $A_{600}$ at full growth, 0.9). The remaining isolates grew poorly on the substrate (μ, <0.03 h$^{-1}$). Examination of growth of the eight strains on 20 mM GABA in liquid culture revealed that with the exception of strain G-17 all of the isolates grew well on this substrate (μ, ≥0.1 h$^{-1}$). Strain G-17 and two other strains which grew rapidly on GABA (strains G-3 and G-20) were then examined for their ability to produce GABA in cell suspension either in the presence of glutamate alone, with glutamate plus succinic semialdehyde, or with succinic semialdehyde alone. None of the strains produced GABA in the presence of either substrate alone (detection limit, <0.001 mM). In the presence of the combined substrates, strain G-17 produced 0.3 mM GABA after 3 h of incubation while the other two strains produced less than 0.02 mM.

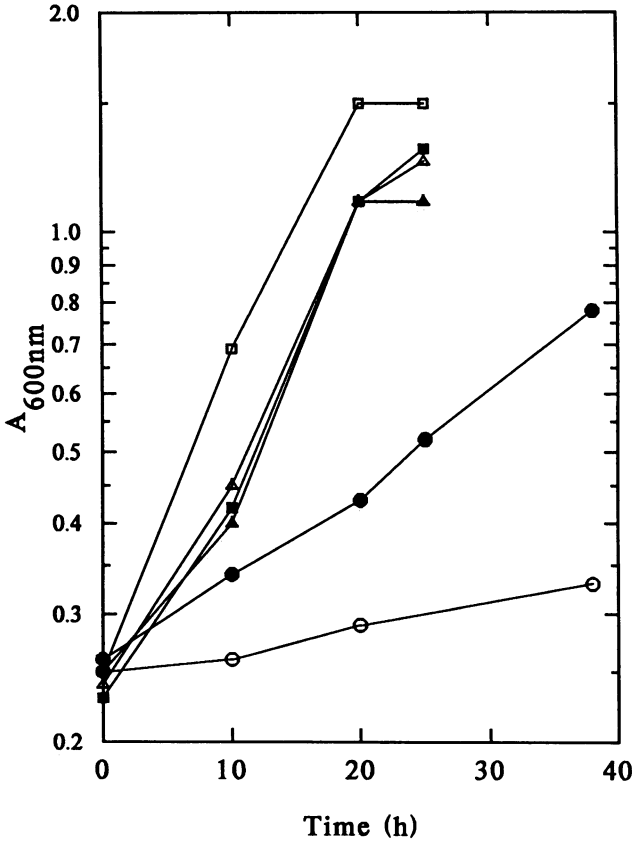


FIG. 1. Growth of *Pseudomonas* strain G-17 on various substrates. Media (50 ml) containing 20 mM substrate were inoculated with 5 ml of culture at full growth and incubated as described in the text. Symbols: ■, glucose-$NH_4NO_3$; ▲, putrescine; △, alanine; □, glutamate; ●, GABA; ○, no substrate.

**Optimal conditions for growth of *Pseudomonas* strain G-17 and growth on different substrates.** The optimum temperature for growth of strain G-17 was 25°C, and the optimum pH was in the range 7.3 to 7.6. The most favorable salinity for growth was 32‰, and decreased growth rates were obtained when NaCl was omitted from the culture medium. A shaking speed of 60 rpm on the gyro-rotatory shaker gave optimal growth, and relatively poor growth was obtained in unshaken cultures.

Strain G-17 grew well on glucose-$NH_4NO_3$, putrescine, alanine, and glutamate, but relatively slow growth was obtained on GABA (Fig. 1). Growth rates on those substrates which actively supported growth were similar (μ, ~0.09 h$^{-1}$), although slightly faster growth was obtained with glutamate. The $A_{600}$ at full growth ranged from 1.2 to

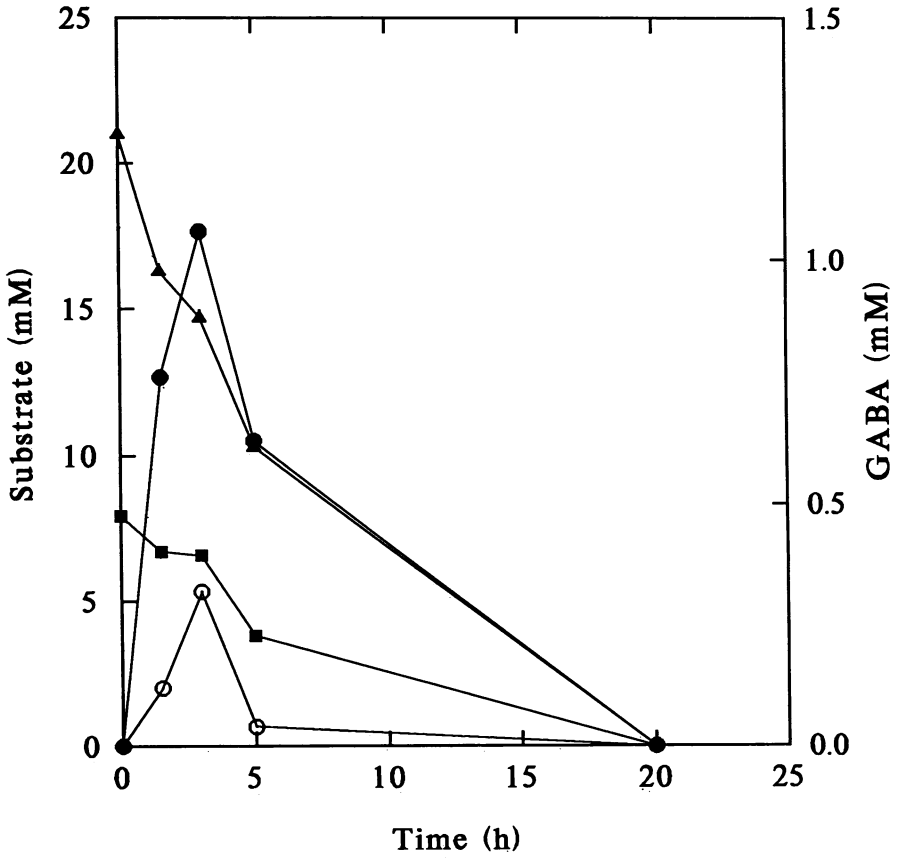


FIG. 2. Production of GABA from glutamate and succinic semialdehyde by cell suspensions of *Pseudomonas* strain G-17 grown on glutamate. Cell suspensions (6 ml) contained cells at a concentration of 1.2 mg (dry weight) $ml^{-1}$ and were incubated as described in the text. Symbols: ■, succinic semialdehyde; ▲, glutamate; ○, GABA (suspension fluid only); ●, GABA (cells plus suspension fluid). Values are means of duplicate determinations.

1.5. During the time course of growth on these substrates, little or no GABA was detected in the culture fluid (≤0.02 mM).

**GABA production by *Pseudomonas* strain G-17 in cell suspension.** When cell suspensions of glutamate-grown *Pseudomonas* strain G-17 were incubated with 20 mM glutamate and 7.5 mM succinic semialdehyde in PSM buffer, GABA was produced. Total GABA (cells plus suspension fluid) or GABA in suspension fluid alone peaked after about 3 h incubation (Fig. 2), after which levels declined. At no stage in the incubation did GABA in the suspension fluid comprise more than 30% of the total GABA pool. After 20 h of incubation, GABA that had been produced was completely utilized, as were the starting substrates, succinic semialdehyde and glutamate. At peak GABA production near stoichiometric quantities of the compound was produced (approximately 0.7 mol/mol of succinic semialdehyde). No GABA was produced by incubation of cell suspensions with either starting substrate alone, and increasing the starting concentrations of either substrate did not lead to an increase in GABA production over that observed in Fig. 2. Decreased GABA levels were obtained by decreasing starting substrate concentrations, and addition of aminooxyacetic acid (an aminotransferase inhibitor) at 100 μg $ml^{-1}$ inhibited GABA production by more than 90%.

Suspensions of cells grown on glucose-$NH_4NO_3$ or putrescine produced little or no GABA during incubation with these substrates at 20 mM each (≤0.01 mM GABA in cells plus suspension fluid). No GABA was produced by suspensions of alanine-grown cells in the presence of 20 mM alanine alone, but in combination with succinic semialdehyde (7.5 mM) GABA production reached 0.1 mM in cells plus suspension fluid but was less than 0.03 mM in the suspension fluid only.

**Influence of pH on GABA production in cell suspensions.** When cell suspensions of strain G-17 were incubated with glutamate and succinic semialdehyde at various pHs, the ratio of GABA released to the total GABA pool increased with increased pH (Fig. 3). Thus, at pH 8.8 released GABA accounted for almost all of the total GABA pool, compared to <30% at pH 7.6. The increased release at elevated pH did not appear to result from cell damage, as microscopic examination of cells showed no lysis or change in their appearance compared with those incubated in the pH range 7.0 to 8.0. The optimum pH for total GABA production was 7.8.

**Effect of decreasing sodium on GABA production and release in cell suspensions.** When cell suspensions of strain G-17 were incubated with glutamate and succinic semialdehyde in PSM buffer with decreased NaCl, the ratio of GABA released to the total GABA pool increased but total GABA production was little affected (Fig. 4). Rates of glutamate and succinic semialdehyde disappearance were not markedly affected by the decreased sodium levels (data not presented).

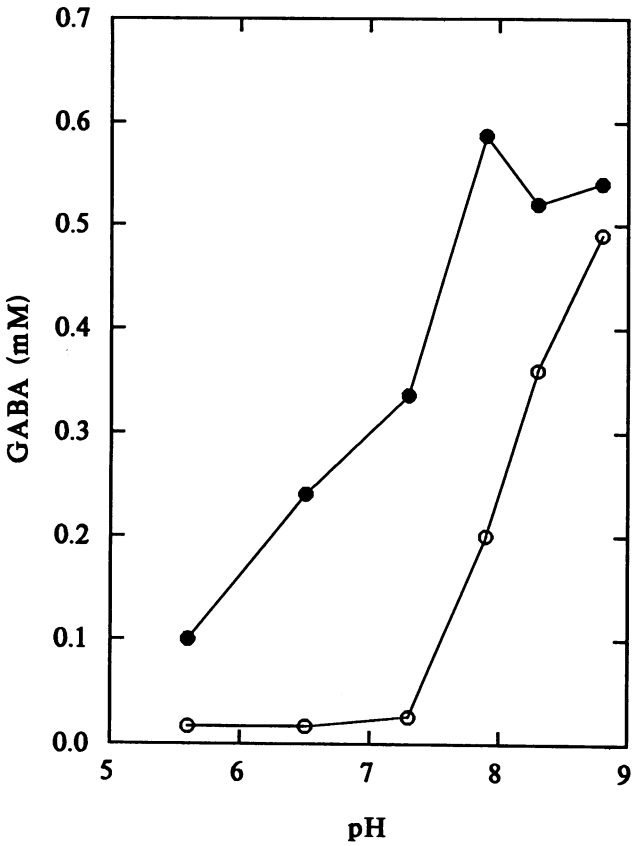


FIG. 3. Influence of pH on GABA production by cell suspensions of *Pseudomonas* strain G-17. Cells (1.6 mg [dry weight] $ml^{-1}$) were incubated at various pHs in the presence of succinic semialdehyde (7.5 mM) and glutamate (20 mM), and GABA was determined after 3 h of incubation. Symbols: ●, GABA (cells plus suspension fluid); ○, GABA (suspension fluid only). Values are means of duplicate determinations.

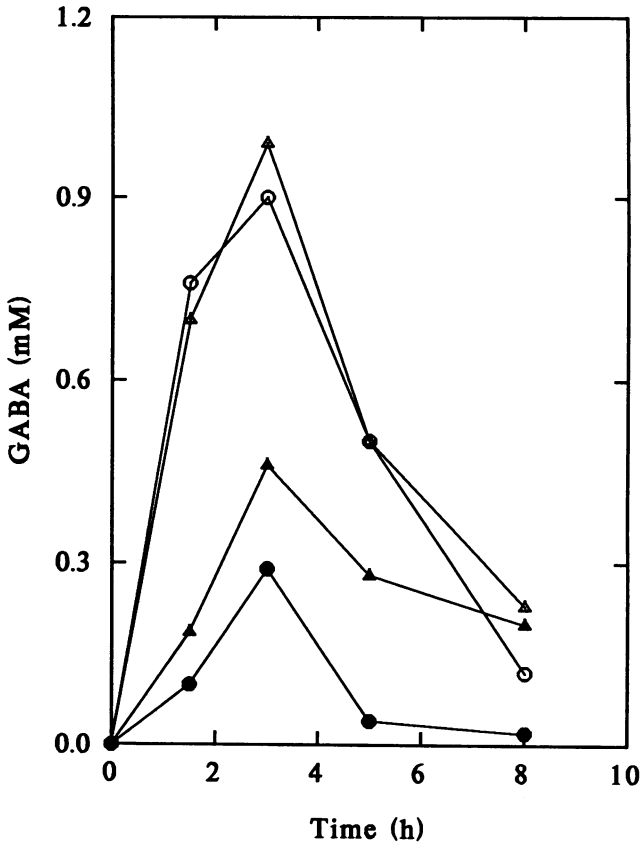


FIG. 4. Effect of sodium depletion on GABA release by cell suspensions of *Pseudomonas* strain G-17. Cells (1.2 mg [dry weight] per ml) were incubated in the presence of succinic semialdehyde (7.5 mM) and glutamate (20 mM). ○ and △, GABA (cells plus suspension fluid); ● and ▲, suspension fluid only; for incubations containing 0.33 and 0.03 M NaCl, respectively. Values are means of duplicate determinations.

At peak GABA production, the amount released in the suspension with decreased NaCl was equivalent to that retained in cells, whereas in the system in which the salt was not decreased it accounted for less than 50% of the cell-associated GABA (Table 1; Fig. 4). A similar effect was obtained by replacing NaCl with the equivalent KCl or Tris-HCl, although slightly more GABA was released than retained by the cells. Examination of the $Na^+$-depleted suspensions by phase-contrast microscopy revealed no obvious damage or lysis of cells.

**Evidence for Na-dependent GABA transport system in strain G-17.** An effect similar to that of removing $Na^+$ from cell suspensions could be produced by the addition of nigericin (an ionophore which collapses the sodium gradient) to cells in PSM buffer in which NaCl was not decreased (Fig. 5). Of interest was the fact that in the nigericin treated system there was no uptake of released GABA in contrast to control incubations with no ionophore present. Thus after 8 h of incubation the level of GABA in suspension fluid with nigericin present was 0.13 ± 0.005 mM compared to 0.045 ± 0.005 mM in the control (values were significantly different; $P < 0.05$). These results indicate that nigericin may be an inhibitor of the transport system for GABA uptake. To confirm this, cell suspensions of GABA-grown cultures were incubated with various concentrations of GABA in the range 0.01 to 0.2 mM. Aminooxyacetic acid was also added to prevent metabolism of GABA taken up by the cells; measurement of GABA uptake and total GABA in cells revealed that less than 10% of the GABA utilized was metabolized in the presence of the inhibitor. Nigericin was found to de-

TABLE 1. Ion dependence of GABA release by cell suspensions of *Pseudomonas* strain G-17[a]

| Addition | GABA (mM) | | |
|---|---|---|---|
| | Cells plus suspension fluid | Suspension fluid only | Cells only[b] |
| 0.33 M NaCl | 0.9 ± 0.02 | 0.28 ± 0.03 | 0.62 ± 0.04 |
| 0.03 M NaCl | 0.9 ± 0.11 | 0.46 ± 0.06 | 0.44 ± 0.12 |
| 0.03 M NaCl–0.3 M KCl | 1.2 ± 0.10 | 0.72 ± 0.10 | 0.48 ± 0.14 |
| 0.03 M NaCl–0.3 M Tris-HCl | 1.3 ± 0.4 | 0.77 ± 0.30 | 0.53 ± 0.25 |

[a] Cells (1.2 mg [dry weight] per ml) were incubated in 6 ml of PSM buffer containing salts as indicated together with glutamate (20 mM) and succinic semialdehyde (7.5 mM). GABA was determined after 3 h of incubation. Values are means of at least duplicate determinations ± 1 standard deviation.

[b] Determined as the difference between GABA in cells plus suspension fluid and GABA in suspension fluid only.

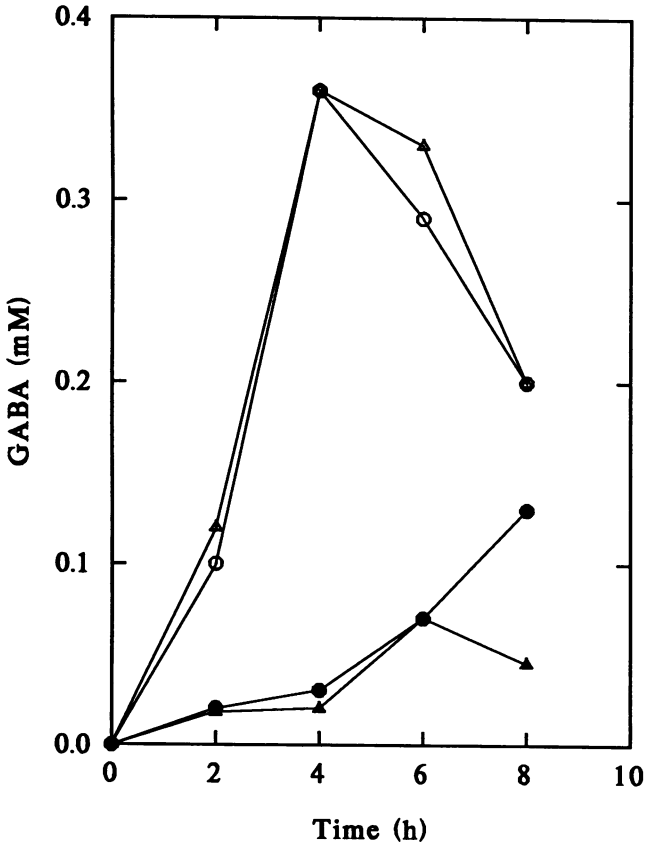


FIG. 5. Effect of nigericin on GABA release by cell suspensions of *Pseudomonas* strain G-17. Cells (1.6 mg [dry weight] per ml) were incubated in the presence of succinic semialdehyde (7.5 mM) and glutamate (20 mM). ○ and △, GABA (cells plus suspension fluid); ● and ▲, suspension fluid only; for incubations with and without ionophore, respectively. The concentration of nigericin in cell suspension fluid was 10 μg $ml^{-1}$. Values are means of duplicate determinations.

crease GABA uptake, and from double reciprocal plots of uptake rate versus substrate concentration the $K_m$ value was found to be unaffected by the inhibitor while $V_{max}$ declined (Table 2). A similar effect was observed by depleting NaCl from cell suspensions.

TABLE 2. Effect of nigericin addition to and sodium depletion of cell suspension fluid on GABA utilization by *Pseudomonas* strain G-17[a]

| Addition | $K_m$ (μM) | $V_{max}$ (nmol of GABA mg [dry weight]$^{-1}$ $h^{-1}$) |
|---|---|---|
| 0.33 M NaCl | 73 ± 5 | 95 ± 4 |
| 0.33 M NaCl–30 μg of nigericin $ml^{-1}$ | 70 ± 4 | 39 ± 5 |
| 0.03 M NaCl | 75 ± 5 | 49 ± 13 |

[a] Cells (1 to 2 mg [dry weight] per ml) were incubated in PSM buffer containing GABA in the range 10 to 200 μM and aminooxyacetic acid (100 μg $ml^{-1}$). GABA was determined at various intervals in the suspension fluid over a 3-h time course by either HPLC or by measurement of the disappearance of radioactivity by using [U-$^{14}$C] GABA. Values for $K_m$ and $V_{max}$ were determined from double reciprocal plots of rate (nanomoles of GABA milligram [dry weight]$^{-1}$ $hour^{-1}$) versus substrate concentration (micromolar) and are means of at least duplicate determinations ± 1 standard deviation.

## DISCUSSION

In this study we provide the first report of GABA production in marine isolates of bacteria, principally *Pseudomonas* spp., thus demonstrating the potential role bacteria have in the production of a settlement inducer. In a series of studies, Morse and coworkers demonstrated that fractions of crustose red algae induce settlement of abalone larvae (13–15); however, the inducer molecule or molecules have not been identified and it is not known whether they are produced solely by the crustose red algae. The demonstration here of bacterial production of GABA raises the possibility for bacterial involvement in the settlement process. However, further studies will be required to determine whether or not such involvement results from the association of bacteria with crustose red algae.

The mechanism for GABA production in the strain of *Pseudomonas* described here was most likely by transamination facilitated by a GABA-glutamate aminotransferase similar to that described for other species of *Pseudomonas* (24). The enzyme system in whole cells was inhibited by aminooxyacetic acid, an inhibitor specific for aminotransferase, resulting in decreased GABA production from glutamate and succinic semialdehyde. Other substrates such as glucose or putrescine which supported growth of the bacterium were ineffective as GABA precursors. Currently we are investigating whether other marine isolates can produce GABA from these substrates.

Most of the GABA that was produced by strain G-17 was cell associated, and only a small fraction was released into the surrounding medium. Increased release of the inducer was achieved by decreasing $Na^+$ or increasing the pH of the external medium. These findings may be important in providing insight into environmental factors which could trigger or influence abalone larval settlement. These could include freshwater influence in hatchery situations or in sheltered bays where settlement occurs or changes in pH due to changes in metabolism of bacterial populations or of crustose red algae. Such changes might be expected to occur in response to light, levels of $HCO_3^-$, oxygen tension, and nutrient availability. Any one or a combination of these factors may be expected to influence GABA release, and there is scope for further study on the influence of such factors on the production of the settlement inducer.

The increased release of GABA by strain G-17 in response to low levels of $Na^+$ was similar to that obtained by addition of nigericin, which collapses the $Na^+$ gradient across the cell membrane. These results indicate that GABA release was influenced by an $Na^+$-dependent transport system which controls GABA uptake. Similar mechanisms have been documented in other microorganisms to account for GABA uptake (6, 10), and permeases have been identified as mediators of uptake in yeasts (3). The increase in GABA release with pH (Fig. 3) indicates that a proton gradient may also be important to the functioning of the transport system in strain G-17. Further studies demonstrating the changes in GABA uptake with pH and the effects of protonophores will be required before this second influence can be confirmed. However, such studies, together with those on the effects of $Na^+$ depletion, highlight the importance of studying transport mechanisms and their response to metabolites and environmental factors so that a clearer understanding of the regulatory processes in the production of settlement inducers such as GABA can be obtained.

Although this investigation suggests a potential role for

bacteria in the production of a settlement inducer for abalone larvae, bacterial involvement in the production of inducers on surfaces such as those of crustose red algae is yet to be defined. Furthermore, the importance of GABA as a natural settlement inducer in relation to fractions from crustose red algae which have also been ascribed a settlement function remains to be determined. Resolution of these fractions into the component or components which induce settlement remains a major objective before such comparisons can be made, and the possibility remains that not one but several substances could be involved.

## ACKNOWLEDGMENTS

We gratefully acknowledge technical assistance from Rod Asher, Jan Holland, and David White. We also thank Henry Kaspar for his helpful criticisms during the preparation of the manuscript and Yvonne Graham for typing.

This work was supported by the New Zealand Government.

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