Low Oxygen and Elevated Carbon Dioxide Atmospheres Inhibit Ethylene Biosynthesis in Preclimacteric and Climacteric Apple Fruit

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ABSTRACT. Autocatalytic C₂H₄ biosynthesis in preclimacteric apple fruit (*Malus domestica* Borkh. 'Golden Delicious') was prevented by storage in atmospheres of 20% CO₂-enriched air (17% O₂ + 63% N₂) or 0.25% O₂ (balance N₂). In preclimacteric fruit, both treatments inhibited C₂H₄ biosynthesis by suppressing expression of ACC synthase (ACC-S) at the mRNA level. ACC oxidase (ACC-O) mRNA abundance and in vitro enzyme activity also were impaired by these treatments. However, the conversion of ACC to C₂H₄ never became the rate limiting step in C₂H₄ biosynthesis. C₂H₄ biosynthesis also was effectively inhibited in climacteric apple fruit kept in air + 20% CO₂ or 0.25% O₂. Climacteric apples also exhibited suppressed expression of ACC-S at the mRNA level, while ACC-O transcript abundance, enzyme activity, and protein abundance were reduced only slightly. ACC-S is the key regulatory enzyme of C₂H₄ biosynthesis and is the major site at which elevated CO₂ and reduced O₂ atmospheres inhibit C₂H₄ biosynthesis, irrespective of fruit physiological maturity. Chemical names used: 1-aminocyclopropane-1-carboxylic acid (ACC).

Ethylene plays a paramount role in triggering and coordinating ripening in apple fruit. Suppression of C₂H₄ biosynthesis and action is one of the mechanisms by which atmospheres enriched in CO₂ or reduced in O₂ extend the storage life of climacteric fruit, such as apples. Atmospheres elevated in CO₂ or reduced in O₂ elicit a variety of physiological responses in plant tissue depending on the physiological age of the tissue (Zamponi et al., 1990). Applying atmospheres enriched in CO₂ and reduced in O₂ is most effective in suppressing C₂H₄ biosynthesis and action when these atmospheres are applied to preclimacteric apples (Oetiker and Yang, 1995).

Adams and Yang (1979) determined that C₂H₄ was derived from S-adenosylmethionine (AdoMet) and that ACC was the immediate precursor to C₂H₄. The enzymes ACC synthase (ACC-S) and ACC oxidase (ACC-O), which mediate C₂H₄ biosynthesis in higher plants, have been isolated, purified, and studied extensively. Molecular cloning techniques also have allowed for the isolation of genes that encode for each of these enzymes from apple fruit (Dong et al., 1991, 1992).

The basic physiology of C₂H₄ action, due to its important commercial implications, was extensively studied and defined well before the elucidation of the C₂H₄ biosynthetic pathway. Peacock (1972) established that the induction of C₂H₄ biosynthesis is a feed-forward biochemical mechanism, often referred to as autocatalysis. McMurchie et al. (1972) put forth a generally accepted, unified model that describes two C₂H₄ biosynthesis induction mechanisms designated System I and System II. This model states that immature fruit have a non-autocatalytic System I C₂H₄ biosynthetic capability, and, when the competency to ripen occurs, an autocatalytic System II C₂H₄ biosynthetic capability is induced. Once the competency to ripen occurs, System I C₂H₄ is thought to bind to a receptor site, thus causing the upregulation of ACC-S and ACC-O.

Autocatalytic C₂H₄ biosynthesis requires the constant presence of C₂H₄ (Peiser, 1989). Burg and Burg (1967) demonstrated that elevated CO₂ acts as a competitive inhibitor of C₂H₄ action and postulated that CO₂ displaces C₂H₄ at a receptor site. This result explains why applying elevated CO₂ is less effective in inhibiting C₂H₄ biosynthesis when endogenous C₂H₄ biosynthesis rates are high. Burg and Burg (1967) also demonstrated that the presence of O₂ is a prerequisite for the binding of ethylene to a hypothetical receptor and that lowered O₂ tensions impede the binding of C₂H₄ to a receptor. This blocked perception of C₂H₄ by low O₂ tensions blocks the feed-forward upregulation of C₂H₄ biosynthesis.

More recent studies have demonstrated that CO₂ and O₂ tensions can affect the activity of the enzymes involved in C₂H₄ biosynthesis, particularly ACC-O, which requires CO₂ and O₂ for activation (Poneleit and Dilley, 1993; Yip et al., 1988).

Our objectives were to determine how elevated CO₂ or reduced O₂ atmospheres 1) inhibit the induction of autocatalytic C₂H₄ biosynthesis and 2) inhibit C₂H₄ biosynthesis in apples that have developed autocatalytic C₂H₄ biosynthesis capability. Specifically, we investigated what effects keeping apples in low O₂ or elevated CO₂ atmospheres has on ACC-O protein abundance as well as ACC-S and ACC-O transcript abundance and enzyme activity. We have reported on the effects of long-term controlled atmosphere storage on C₂H₄ biosynthesis of preclimacteric 'Golden Delicious' apples (Gorny and Kader, 1996). This study focused on the effects of fungistatic and/or insecticidal atmospheric treatments (involving 0.25% O₂ and air + 20% CO₂ for a short duration at 20 °C) on C₂H₄ biosynthesis in 'Golden Delicious' apples at two physiological stages.

Materials and Methods

PLANT MATERIALS AND STORAGE CONDITIONS. 'Golden Delicious' apple fruit were harvested from a commercial orchard near Sebastapol (Sonoma County), Calif. Ethylene production, internal C₂H₄ concentration, soluble solids content, titratable acidity, and firmness were assessed on the day of harvest to determine the physiological maturity of the apple fruit. After preliminary sorting and cooling, apple fruit were stored at 0 °C in airtight stainless steel tanks under a continuous flow of humidified air for 5 or 40 d to

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obtain preclimacteric and climacteric fruit, respectively. Following storage at 0 °C, apple fruit were placed at 20 °C in a continuous flow (100 mL·min⁻¹) of humidified air, air + 20% CO₂ (17% O₂ + 63% N₂), or 0.25% O₂ + 99.75% N₂. In subsequent analysis, three preclimacteric fruit were considered a replicate, whereas individual climacteric fruit were considered replicates. All data points represent the mean and SE of three replicates.

Ethylene production rates of apple(s) in each replicate were determined by quantifying the amount of C₂H₄ present in the exit stream of gas leaving each jar, using a Carle gas chromatograph (Hach Carle, Loveland, Colo.) equipped with a flame ionization detector. Internal C₂H₄ concentrations of apples were determined by the method of Saltveit (1982). Tissue samples used in Northern and Western blot analyses were frozen in liquid N₂ and stored at -80 °C until analyzed.

IN VITRO ACC-S ENZYME ACTIVITY. On removal from storage treatments, apple cortical and skin tissues were homogenized in a chilled Waring blender with 1 mL·g⁻¹ of 4 °C extraction media [400 mmol potassium-phosphate (pH 8.5), 1 mmol EDTA, 5 mL·L⁻¹ 2-mercaptoethanol, 0.01 mmol pyridoxal phosphate]. The homogenate was filtered through four layers of cheese cloth and then centrifuged at 28,000× g_n for 30 min. The supernatant was discarded, and the pellet was resuspended in extraction media. The resuspended pellet then was centrifuged at 28,000× g_n for 20 min, and the supernatant was discarded. The resulting pellet was solubilized, and ACC-S activity was assayed as described by Yip et al. (1991).

IN VITRO ACC-O ENZYME ACTIVITY. On removal from storage treatments, apple cortical and skin tissues were frozen in liquid N₂ and homogenized by mortar and pestle in 2 mL·g⁻¹ tissue of extraction medium containing 400 mmol 3-[N-Morpholino] propane-sulfonic acid (MOPS) buffer (pH 7.2), 100 mL·L⁻¹ glycerol, 30 mmol ascorbate, and 500 mg·g⁻¹ PVPP (Dong et al., 1992; Fernandez-Maculet and Yang, 1992). After the slurry melted, it was filtered through four layers of cheesecloth and centrifuged at

28,000× g_n for 20 min. The supernatant was used for enzyme assays. The standard reaction mixture contained 50 mmol MOPS (pH 7.2), 100 mL·L⁻¹ glycerol, 1 mmol ACC, 0.02 mmol FeSO₄, 5 mmol ascorbate, 1 mmol dithiothreitol (Poncelit and Dilley, 1993), and 20 mmol sodium bicarbonate (Smith and John, 1993). The enzyme assay reaction was initiated by adding 0.2 mL of enzyme preparation to a 15-mL test tube that contained the standard reaction mixture (1.8 mL). The test tube then was sealed, and, after a 1-h incubation with shaking at 30 °C, a 1-mL gas sample was withdrawn with a syringe from the headspace for C₂H₄ determination.

PROTEIN ASSAY. Protein content of enzyme extracts used to determine in vitro ACC-S and ACC-O activity, as well as protein extracts for ACC-O Western blot analysis, was determined by the method of Bradford (1976) using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, Calif.) and bovine serum albumin (BSA) as a standard.

ACC-O WESTERN BLOTS. Total proteins were extracted from apple flesh and skin tissue by the method of Paull and Chen (1990) with the following modifications. Ten grams of apple tissue were extracted in a buffer containing 500 mmol Tris (pH 7.4), 500 mmol sucrose, 100 mmol KCl, 50 mmol EDTA, 2 mL·L⁻¹ 2-mercaptoethanol, 2 mL·L⁻¹ Triton X-100, and 50 g·L⁻¹ PVPP.

GEL ELECTROPHORESIS AND IMMUNOBLOTTING. Thirty micrograms of total protein per lane were separated by SDS-PAGE as described by Laemmli (1970). Two gels 150 g·L⁻¹ (15%) were run in replicate and stained with Coomassie blue or electroblotted onto supported nitrocellulose membranes (Bio-Rad) and was probed with a rabbit polyclonal antibody specific to ACC-O (Dong et al., 1992), a gift from the laboratory of S.F. Yang. Immunodetection with the alkaline phosphatase reaction was performed as described by Birkett et al. (1985). The following proteins (biotinylated for Western blots) were used to determine the molecular mass of the ACC-O band: phosphorylase b (97.4 ku), BSA (66 ku), ovalbumin (43 ku), carbonic anhydrase (31 ku), and soybean trypsin inhibitor (21.5 ku).

RNA ISOLATION AND ANALYSIS. Total RNA was isolated from apple fruit as described by Manning (1991), and 25 mg from each sample were separated by electrophoresis on a 12-g·L⁻¹ (1.2%) agarose, 2.2-mol formaldehyde denaturing gel (Sambrook et al., 1989), transferred to nylon membrane (Hybond-N, Amersham), and ultraviolet crosslinked. The blots then were stained and destained by the method of Herrin and Schmidt (1988) to visualize the 18S and 28S ribosomal bands. Staining was done to visually determine if equal lane loading and transfer had occurred before blot probing was initiated. RNA molecular weight markers cut from the formaldehyde gel and stained with ethidium bromide were used to determine the M_r of the ACC-S and ACC-O bands.

PROBE PREPARATION AND NORTHERN BLOTTING. The resulting blots were hybridized with ³²P-labeled probes derived from gel-purified inserts of pAAS2 for ACC-S (Dong et al., 1991) and pAE12 for ACC-O (Dong et al., 1992). Both cDNA inserts used as probes were originally derived from 'Golden Delicious' apple fruit and were generous gifts from the laboratory of S.F. Yang. Autoradiography was performed at -80 °C using Kodak XAR-5 film and intensifying screens (Cronex, DuPont, Newtown, Conn.).

Results

INITIAL APPLE PHYSIOLOGICAL MATURITY AND C₂H₄ BIOSYNTHESIS RATE. At harvest, the apple fruit used in this study had an internal C₂H₄ content of 0.04 ± 0.01 mL·L⁻¹ and were preclimacteric with regard to C₂H₄ biosynthesis but near commercial harvest maturity

Fig. 1. Ethylene biosynthesis rates of 'Golden Delicious' apple fruit of different physiological maturities (I = preclimacteric and II = climacteric) held in atmospheres of air, air + 20% CO₂, or 0.25% O₂ at 20 °C, after being held in air at 0 °C for 5 or 40 d. Data points are the means of three replications. Pooled least significant difference at the 5% level is shown for each experiment.

by the indices tested (titratable acidity, soluble solids content, and flesh firmness). On removal from 0 °C storage, apple fruit that were designated preclimacteric were unchanged in their C_2H_4 biosynthesis rate. As fruit advanced in physiological maturity while being held at 0 °C, the capacity for autocatalytic C_2H_4 biosynthesis also advanced, as demonstrated by higher initial C_2H_4 biosynthesis rates. Climacteric fruit had an initial mean C_2H_4 biosynthesis rate at 20 °C of $94.9 \pm 16.3 \text{ nL} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$. Irrespective of apple fruit physiological maturity, atmospheres of air + 20% CO_2 or 0.25% O_2 prevented a further increase in their C_2H_4 biosynthesis capacity (Fig. 1).

IN VITRO ACC-S ACTIVITY. There was no significant difference in the ACC-S activity of preclimacteric apples among the three treatments during the first 7 d at 20 °C (Fig. 2). After 14 d, air-treated fruit exhibited significantly higher levels of ACC-S activity than either the air + 20% CO_2 or 0.25% O_2 treatments, with the latter two being comparable in ACC-S activity.

Climacteric apples had much greater initial ACC-S activity than preclimacteric apples. ACC-S activity of climacteric apple fruit held in air was always significantly greater than that of fruit held in either air + 20% CO_2 or 0.25% O_2 .

IN VITRO ACC-O ACTIVITY. ACC-O activity of preclimacteric apple fruit increased with time in all three treatments during the first 10 d at 20 °C (Fig. 3). After 14 d at 20 °C, the air-treated preclimacteric apple fruit had a significantly higher ACC-O activity level than either the air + 20% CO_2 or 0.25% O_2 treated fruit.

Initial ACC-O activity was 10-fold higher in climacteric apple fruit than in preclimacteric apple fruit. The air + 20% CO_2 or 0.25% O_2 treatments had little effect on slowing the induction rate of ACC-O activity in climacteric apple fruit. In general ACC-O activity increased over time in all treatments.

ACC-O WESTERN BLOT ANALYSIS. The ACC-O protein could only be detected in preclimacteric apple fruit that were held for 14 d in air (Fig. 4). The elevated CO_2 and reduced O_2 atmosphere treatments slowed the accumulation of ACC-O protein in fruit that produced $<0.2 \text{ nL} \cdot \text{g}^{-1} \cdot \text{h}^{-1} C_2H_4$. ACC-O protein abundance in climacteric apple fruit was lowest in fruit held in 0.25% O_2 . Neither elevated CO_2 nor low O_2 atmospheres reduced the abundance of ACC-O protein in climacteric fruit, which had a well-developed capacity for C_2H_4 biosynthesis.

ACC-S NORTHERN BLOT ANALYSIS. The ACC-S transcript was not evident in preclimacteric apple fruit until 14 d in air (Fig. 5), when concomitantly C_2H_4 biosynthesis significantly increased. The elevated CO_2 and reduced O_2 atmosphere treatments prevented accumulation of ACC-S transcripts in fruit that produced $<0.2 \text{ nL} \cdot \text{g}^{-1} \cdot \text{h}^{-1} C_2H_4$.

Atmospheres of air + 20% CO_2 or 0.25% O_2 prevented mRNA accumulation of ACC-S transcripts in climacteric apple fruit. Transcript levels of ACC-S in climacteric apples correlated well with in vitro ACC-S activity and C_2H_4 production rates in all treatments. The abundance of ACC-S transcripts in climacteric apple fruit increased significantly after 1 and 3 d in air at 20 °C (Fig. 5). Air + 20% CO_2 -treated climacteric fruit had significantly lower levels of ACC-S transcript after 1 and 3 d at 20 °C than fruit held in air at 20 °C. However, ACC-S transcripts continued to accumulate in climacteric fruit held in air + 20% CO_2 , while ACC-S transcript levels in climacteric fruit held in 0.25% O_2 dropped below detectable limits after 3 d.

ACC-O NORTHERN BLOT ANALYSIS. ACC-O transcripts in preclimacteric apple fruit held in air reached detectable levels after 7 d and became abundant after 14 d (Fig. 6). Preclimacteric apple fruit held in 0.25% O_2 had no detectable ACC-O transcripts after 7 or 14 d of storage at 20 °C, which indicates that ACC-O mRNA

synthesis requires atmospheric O_2 levels $>0.25\%$. Preclimacteric apple fruit held in air + 20% CO_2 for 14 d had detectable but significantly reduced levels of ACC-O transcripts compared to air-treated fruit. The air + 20% CO_2 treatment also had the effect of decreasing the abundance of ACC-O mRNA between days 7 and 14 in preclimacteric fruit.

Fig. 2. ACC synthase (ACC-S) activity of 'Golden Delicious' apple fruit of different physiological maturities (I = preclimacteric and II = climacteric) held in atmospheres of air, air + 20% CO_2 , or 0.25% O_2 at 20 °C, after being held in air at 0 °C for 5 or 40 d. Data points are the means of three replications. Pooled least significant difference at the 5% level is shown for each experiment.

Fig. 3. ACC-oxidase (ACC-O) activity of 'Golden Delicious' apple fruit of various physiological maturities (I = preclimacteric or II = climacteric) held in atmospheres of air, air + 20% CO_2 , or 0.25% O_2 at 20 °C after being held in air at 0 °C for 5 or 40 d. Data points are the means of three replications. Pooled least significant difference at the 5% level is shown for each experiment.

Climacteric apples had much greater initial levels of ACC-O mRNA transcripts than preclimacteric apples. Irrespective of storage atmosphere, climacteric apple fruit exhibited an increase in ACC-O mRNA abundance after 1 d at 20 °C when compared to initial levels. ACC-O mRNA abundance continued to increase over time and after 3 d at 20 °C; there was no significant difference in the ACC-O transcript abundance of climacteric apple fruit held in air or in air + 20% CO₂. However, climacteric apple fruit held in 0.25% O₂ after 3 d had slightly lower abundance of ACC-O transcripts than climacteric apple fruit that were held for 3 d in either air or air + 20% CO₂.

Discussion

Atmospheres of air + 20% CO₂ or 0.25% O₂ effectively prevented development of autocatalytic C₂H₄ biosynthesis in preclimacteric apple fruit that produced <0.2 nL·g⁻¹·h⁻¹ C₂H₄. These treatments also were effective in reducing the C₂H₄ biosynthesis rate of fruit that had already attained the capacity for autocatalytic C₂H₄ production. However, once autocatalytic C₂H₄ biosynthesis began, elevated CO₂ and reduced O₂ atmospheres could not reduce C₂H₄ biosynthesis rates to preclimacteric production levels.

Elevated CO₂ and reduced O₂ atmospheres suppressed the accumulation of ACC-S mRNA transcripts in apple fruit at both stages of physiological maturity. Development of ACC-S activity was completely suppressed by the air + 20% CO₂ or 0.25% O₂ treatments in preclimacteric fruit. Treatment of climacteric fruit

with atmospheres of air + 20% CO₂ or 0.25% O₂ reduced the ACC-S activity of these fruit but not to preclimacteric ACC-S activity levels. ACC-S mRNA abundance was low in all treatments, and autoradiography was performed for 10 to 14 d to visualize the transcripts seen in Fig. 5. The ability of fruit tissue to convert AdoMet to ACC is tightly regulated by low transcript abundance and rapid protein turnover rate (Kim and Yang, 1992). Therefore, the significant reductions in ACC-S transcript abundance due to exposure to atmospheres elevated in CO₂ or reduced in O₂ would be expected to have a large impact on the amount of ACC-S protein present, which may explain the reduced rate of C₂H₄ production (Fig. 1). Our data are consistent with the observations of Bufler (1984), namely that elevated levels of CO₂ inhibit C₂H₄ biosynthesis by reducing the ACC-S activity of apple tissue.

Elevated CO₂ or reduced O₂ levels also slow the development of ACC-O activity in preclimacteric apples with only System I C₂H₄ biosynthetic capability. This result is consistent with previous observations regarding elevated CO₂ and reduced O₂ effects on apple fruit C₂H₄ biosynthesis (Chaves and Tomas, 1984; Levin et al., 1993; Li et al., 1983). However, once autocatalytic C₂H₄ biosynthesis begins, the ACC-O transcripts and protein are abundant and placing apple fruit in an atmosphere of air + 20% CO₂ or 0.25% O₂ had little impact on reducing further development of ACC-O activity or the accumulation of ACC-O transcripts and protein. Reductions in ACC-O transcript abundance of climacteric fruit stored in atmospheres elevated in CO₂ or reduced in O₂ did not sufficiently limit the abundance of ACC-O protein (Fig. 4) or the amount of catalytically active enzyme (Fig. 3) to levels that would be rate limiting for C₂H₄ biosynthesis.

Our data demonstrated the occurrence of a concomitant increase in C₂H₄ biosynthesis (Fig. 1) and ACC-S activity (Fig. 2) in preclimacteric apples held in air. Preclimacteric apple fruit held in air, air + 20% CO₂, or 0.25% O₂ did not differ significantly in ACC-O activity (Fig. 3), while C₂H₄ biosynthesis rates (Fig. 1) steadily increased after 4 d in preclimacteric air-treated fruit but not in fruit held in air + 20% CO₂ or 0.25% O₂. From this result, we conclude that the rate of C₂H₄ biosynthesis in apple fruit held in elevated CO₂ or reduced O₂ atmospheres is due to reduced in vivo ACC-S activity. However, in vivo ACC-O activity may be reduced by atmospheres enriched in CO₂ or reduced in O₂ due to direct effects on the enzyme (Adams and Yang, 1979; Mizutani et al., 1995).

Exogenous application of C₂H₄ analogs has reduced the time needed to induce autocatalytic C₂H₄ biosynthesis (Eaks, 1980; Peacock, 1972). Therefore, it has been hypothesized that System I C₂H₄ biosynthesis and its perception play a

Fig. 4. ACC oxidase (ACC-O) Western blot analysis of proteins from 'Golden Delicious' apple fruit held in atmospheres of air, air + 20% CO₂, or 0.25% O₂ at 20 °C. (I) preclimacteric: 0, 7, or 14 d and (II) climacteric: 0, 1, or 3 d. Proteins (30 mg/lane) were separated by SDS-PAGE and reacted with the antibody. The ACC-O bands had a molecular mass of 39 ku as determined by biotinylated molecular mass markers.

Fig. 5. Northern blot analysis of 'Golden Delicious' apple fruit tissue held in atmospheres of air, air + 20% CO₂, or 0.25% O₂ at 20 °C. (I) preclimacteric: 0, 7, or 14 d and (II) climacteric: 0, 1, or 3 d. Total RNA (25 mg/lane) was separated by formaldehyde agarose gel electrophoresis, and blots were analyzed for hybridization to the [32P]dCTP-labeled pAAS2, ACC synthase (ACC-S) probe. The ACC-S bands had an M_r of 1.9 kb as determined by RNA molecular mass markers; 18S rRNA was used as an internal control.

role in triggering autocatalytic C_2H_4 biosynthesis (Peacock, 1972; Yang, 1985). When atmospheres elevated in CO_2 or reduced in O_2 are applied to apple fruit with only System I capacity, these atmospheres may effectively reduce C_2H_4 production and prevent activation of an C_2H_4 receptor(s) that slows induction of autocatalytic C_2H_4 biosynthesis by suppressing the upregulation of the genes that encode ACC-S and ACC-O (Bufler, 1984; Yang, 1987). Our data support these hypotheses, by demonstrating a reduced rate of accumulation of transcripts for ACC-S and ACC-O in preclimacteric apple fruit held in atmospheres of air + 20% CO_2 or 0.25% O_2 . In particular, these atmospheres slow the accumulation rate of ACC-S transcripts in preclimacteric apple fruit.

How elevated CO_2 and reduced O_2 atmospheres may potentially mediate the expression of various ACC-S and ACC-O genes and isozymes warrants further investigation, as does the role these atmospheres may play in mediating the expression and functionality of C_2H_4 receptor(s).

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Fig. 6. Northern blot analysis of 'Golden Delicious' apple fruit tissue held in atmospheres of air, air + 20% CO_2 , or 0.25% O_2 at 20 °C. (I) preclimacteric: 0, 7, or 14 d and (II) climacteric: 0, 1, or 3 d. Total RNA (25 mg/lane) was separated by formaldehyde agarose gel electrophoresis and blots were analyzed for hybridization to the [32P]dCTP-labeled pAE12, ACC oxidase (ACC-O) probe. The ACC-O bands had an M_r of 1.3 kb as determined by RNA molecular mass markers; 18S rRNA was used as an internal control.