Spore and Geosmin Production by *Streptomyces tendae* on Several Media

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Geosmin (1,10-*trans*-dimethyl-*trans*-9-decalol) is produced by several microbial taxa and can impart musty/earthy off-flavors to food, potable water, and aquaculture-raised fish. The bacterium *Streptomyces tendae* (ATCC 31160) was grown on Hickey–Tresner (HT), *Streptomyces* (STR), *Actinomyces* (ACT), and American Type Culture Collection sporulation agar (ATCC). The greatest fresh biomass occurred on ATCC and the least on HT media. Sporulation occurred on ATCC and HT but not on STR and ACT media. Cultures produced geosmin on each media. However, sporulating cultures contained more geosmin than nonsporulating cultures. Cultures grown on ACT medium did not produce geosmin prior to 48 h after inoculation, and neither basic, acidic, nor enzymatic hydrolysis released geosmin, indicating that changes in geosmin content were due to de novo biosynthesis rather than release of bound metabolite.

INTRODUCTION

Geosmin (1,10-*trans*-dimethyl-*trans*-9-decalol) (see Figure 1) is produced by several species of blue-green algae, filamentous bacteria, and fungi that inhabit moist soil and surface waters (Aoyama, 1990; Bentley and Meganathan, 1982; Gerber, 1983; Henatsch and Juttner, 1986; Izaguirre, 1982; Juttner, 1984; Naes et al., 1985; Yagi et al., 1981). Geosmin can impart musty/earthy off-flavors to potable water, aquaculture-raised fish, and food (Gerber, 1983; Johnsen and Kuan, 1986; Lovell et al., 1986; Persson, 1980; Maga, 1987), causing economic losses in these and related industries. Geosmin is considered an off-flavor in sugar, sugar beets, Swiss chard, navy beans, sweet corn, and canned mushrooms and a flavor component in table beets (Maga, 1987). Taste and odor thresholds for geosmin in water range from 0.015 to 0.200 $\mu g\ kg^{-1}$ (Maga, 1987), yet surface waters may contain 2 $\mu g\ kg^{-1}$ geosmin (Juttner, 1984).

Existing evidence indicates that geosmin is derived from a sesquiterpene precursor, such as farnesyl pyrophosphate, (Bentley and Meganathan, 1982; Naes et al., 1989) (Figure 1). Geosmin synthesis in *Streptomyces tendae* may involve mixed-function oxidase activity (Dionigi et al., 1990), and L-methionine and folic acid may be involved in geosmin synthesis by *S. griseofuscus* (Aoyama, 1990). Sivonen (1982) qualitatively examined odor production and sugar utilization by several taxa of filamentous bacteria. However, quantitative investigations of spore, biomass, and geosmin production on different media have not been reported. Certain terpene alcohols are bound to glycoside moieties and may be released by hydrolysis (Wu et al., 1990; Yano et al., 1990). Similarly, geosmin may be bound to glycosides and released by hydrolysis. Therefore, it has not been determined whether changes in the geosmin content of cultures are due to de novo biosynthesis or release of bound metabolite.

The objectives of this research were to examine spore, fresh biomass, and geosmin production by the filamentous bacterium *S. tendae* Ettlinger (ATCC 31160) and to determine whether changes in the geosmin content were due to de novo biosynthesis or release of bound metabolite.

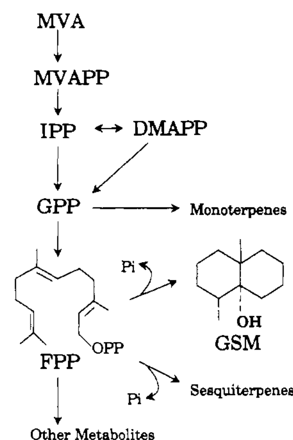

Figure 1. Schematic representation of the initial steps of the terpene pathway showing the possible derivation of the musty/earthy off-flavor microbial metabolite geosmin. Abbreviations: MVA, mevalonic acid; MVAPP, IPP, DMAPP, GPP, and FPP, pyrophosphate esters of mevalonic acid, isopentenol, dimethylallyl alcohol, geraniol, and farnesol, respectively; OPP, pyrophosphate; Pi, inorganic phosphate, GSM, geosmin.

MATERIALS AND METHODS

Bacterial Culture. Cultures of *S. tendae* (ATCC 31160) were grown in 100 × 15 mm polystyrene Petri dishes containing 20 mL of either *Actinomyces* (ACT) (Ajello et al., 1963), American Type Culture Collection sporulation agar (ATCC) (Bridson and Brecker, 1970; Bridson, 1978), Hickey–Tresner (HT) (Hickey and Tresner, 1952), or *Streptomyces* (STR) medium. The ACT and HT media consisted of 5.7% and 3.15% solutions of *Actinomyces* broth medium and Hickey–Tresner agar, respectively (Becton Dickinson Microbiological Systems, Cockeyville, MD). The ATCC medium consisted of 0.1% yeast extract, 0.1% beef extract, 0.2% tryptose, 1.0% glucose, and a trace of $FeSO_4$. The STR medium consisted of 0.2% soybean meal, 0.5% yeast extraction, 0.5% sodium chloride, 10% dextrose, and 0.1% calcium carbonate (w/v) adjusted to pH 7.5. Each medium was solidified with 1.2% (w/v) bacteriological grade agar (Difco Laboratories, Detroit, MI).

Inoculum was prepared by combining spores obtained from a single culture with 1 mL of sterile water. A sterile polycarbonate membrane (90-mm diameter, 0.05-μm pore diameter, Nucleopore Inc., Cambridge, MA) was placed on the surface of the agar prior to inoculation with 100 μL of inoculum. Cultures were incubated in the dark at 28 °C.

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Biomass Determination. The membrane and cells were removed from the medium and weighed. Fresh biomass was determined by subtracting the membrane weight from the total.

Spore Production. Spore production was determined by microscopic examination.

Acidic, Basic, and Enzymatic Hydrolysis. After 48 h of incubation, cells grown on ACT media were separated from a polycarbonate membrane, placed in 4 mL of water, and sonicated for 30 s. Particulate matter was removed by centrifugation. One milliliter of supernatant was placed in a 50-mL glass centrifugation tube with 50 μL of internal standard (25 ppm 2-undecanone in ethanol). Either 1 mL of concentrated hydrochloric acid, 1 mL of 6 N sodium hydroxide, or 1 mL of sodium acetate buffer (pH 6.2) containing β-glucosidase and β-galactosidase (ca. 1 mg/mL each, enzymes obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN) was added to the supernatant. Controls consisted of either buffer containing geosmin, supernatant alone, supernatant plus 6 M *p*-nitrophenyl β-D-galactopyranoside, or supernatant plus 6 M *p*-nitrophenyl β-D-glucopyranoside (both pyranosides were obtained from Sigma Chemical Co., St Louis, MO). Supernatant solutions were covered with 5 mL of hexane to trap any geosmin produced and incubated in glass centrifuge tubes in the dark without shaking for 24 h at 28 °C. Following incubation, the contents of the centrifuge tubes were mixed thoroughly. The nonaqueous fraction was removed and analyzed by gas chromatography.

Geosmin Analysis. Methods for extraction of geosmin from liquid medium and quantification by gas chromatography flame ion detection (Johnsen and Kuan, 1987) were modified (Dionigi et al., 1990) to accommodate samples of agar, cells, and the nonaqueous fraction from the hydrolysis investigations. Additionally, hexane was substituted for the methylene chloride extraction solvent indicated by Dionigi et al. (1990) to avoid chemical incompatibilities with the polycarbonate membranes. In all cases the internal standard was 2-undecanone. Geosmin and internal standard peaks were identified by comparing retention times with those of authentic standards and verified by gas chromatography/mass spectrometry. Geosmin concentrations were calculated from the geosmin and internal standard peak areas and sample weights. Olfaction can be a highly sensitive method for the detection of geosmin (see threshold data of Maga (1987)), and olfactory evaluations of the cultures were also conducted by a trained sensory panelist.

Statistical Analysis. Geosmin and biomass productions on the various media and over time were determined in a series of randomized complete block experiments. Replication was achieved by establishing at least three complete blocks within each experiment and by repeating each experiment. Data were combined and sources of variation analyzed by factorial analysis according to the method of McIntosh (1983).

RESULTS AND DISCUSSION

Sporulation, Biomass, and Geosmin Accumulation. Reports indicate that the production of earthy flavor metabolites by strains of *Streptomyces* is coordinately regulated with aerial mycelium and spore formation (Grafe, 1989). For example, sporulating cultures of *Streptomyces* sp. contained geosmin, whereas nonsporulating mutants did not (Bentley and Meganathan, 1982). Redshaw et al. (1979) also found that mutants of *S. alboniger* simultaneously lost the ability to form aerial mycelium and earthy donors. However, *S. tendae* produced geosmin on media that supported sporulation as well as on media that did not (Table I). For example, geosmin was detected on STR and ACT media, but spores were not observed on STR and ACT media 55 h after inoculation; cultures maintained on these media for several additional days did not sporulate (Table I). The occurrence of geosmin in nonsporulating cultures indicates that geosmin production is not dependent upon sporulation in *S. tendae* (Table I). However, sporulating cultures exhibited greater concentrations of geosmin than nonsporulating cultures (Table I), which agrees with reports that formation of secondary

Table I. Fresh Biomass, Spore Production, and Geosmin Content of *S. tendae* Cultures 55 h after Inoculation[a]

medium	biomass, mg	spores	geosmin, mg/kg
ACT	576.53 ± 10.4	-	0.25 ± 0.08
ATCC	881.53 ± 71.9	+	18.05 ± 0.63
HT	520.35 ± 21.9	+	33.24 ± 2.94
STR	802.57 ● 49.9	-	3.55 ± 0.51

[a] Means are followed by ±2 SE of the mean. Abbreviations: +, present; -, absent; ACT, *Actinomyces*; ATCC, American Type Culture Collection sporulation agar; HT, Hickey-Tresner; STR, *Streptomyces*.

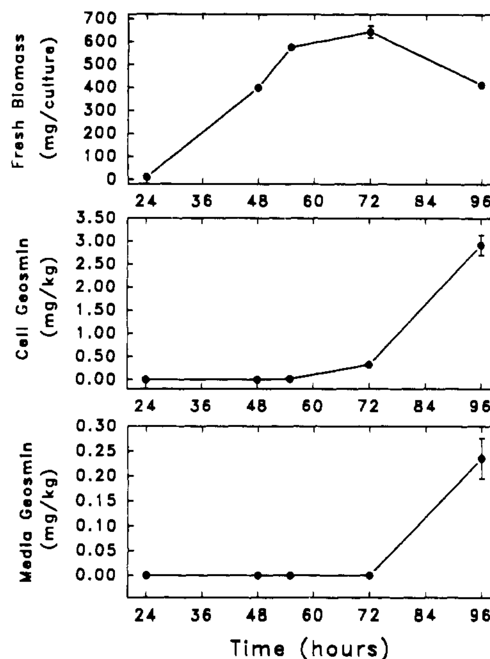

Figure 2. Fresh biomass and geosmin content of cells and medium of *S. tendae* cultures grown on *Actinomyces* agar. Data are expressed ±2 SE of the mean.

metabolites may increase under conditions that support morphological differentiation rather than vegetative growth (Grafe, 1989).

Geosmin and Biomass Accumulation over Time. The point of onset of geosmin biosynthesis by *S. tendae* was affected by media composition. Geosmin was not detected by olfaction or gas chromatography in ACT medium grown cultures before 55 h after inoculation. Late in the time course, these cultures exhibited geosmin production and geosmin release into the medium (Figure 2). In contrast, geosmin was detected in HT medium grown cultures throughout the time course (Figure 3). Cells contained greater concentrations of geosmin than the medium in both the ACT-grown and HT-grown cultures (Figures 2 and 3). This observation agrees with reports indicating that less than 1% of the total geosmin produced by the blue-green algae *Fischerella muscicola* (Wu and Juttner, 1988a) and *Oscillatoria tenuis* (Wu and Juttner, 1988b) was released from the cell. It has been suggested that secondary metabolites may function exogenously to coordinate chemical and morphological differentiation among microbial populations (Luckner and Nover, 1997; Grafe, 1989). The large proportion of geosmin retained within cells suggests that geosmin may have a role within the cell in addition to a possible exogenous role.

De Novo Biosynthesis of Geosmin vs Release of Bound Metabolite. To determine whether changes in the geosmin content of ACT-grown cultures were due to de novo biosynthesis of geosmin or release of bound geosmin, supernatant obtained from ACT-grown cells was

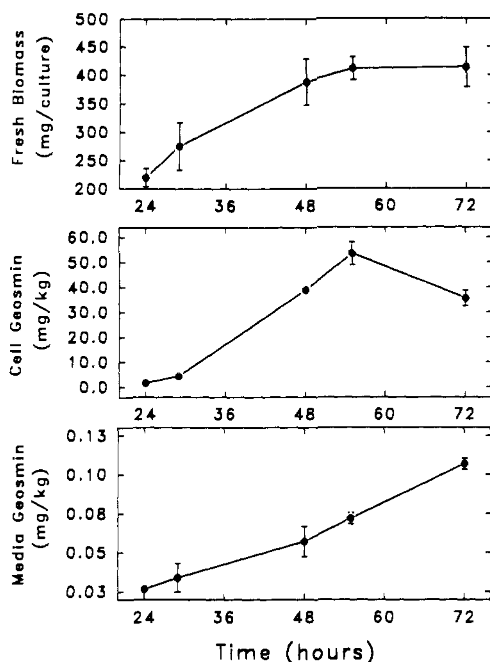

Figure 3. Fresh biomass and geosmin content of cells and medium of *S. tendae* cultures grown on Hickey–Tresner agar. Data are expressed ±2 SE of the mean.

exposed to acid, base, and enzymatic hydrolysis. Positive control solutions containing either *p*-nitrophenyl β-D-galactopyranoside or *p*-nitrophenyl β-D-glucopyranoside became distinctly yellowish after a few minutes of to either acid, base, or enzymatic hydrolysis, indicating hydrolysis of the bond between the glycoside and *p*-nitrophenyl moieties. However, exposure to acid, base, and hydrolytic enzymes for 24 h did not release geosmin from supernatant obtained from ACT-grown cells. Sterile water controls containing geosmin and acid exhibited an additional gas chromatograph peak. This peak was tentatively identified as argosmin C [1,10-dimethyl-1(9)-octalin] by gas chromatography/mass spectral analysis, and Gerber (1983) indicated that geosmin was susceptible to acidic conversion to argosmin C. However, no peak with a retention time similar to that of the tentative argosmin C peak was found in the ACT-grown cell suspending homogenate, indicating that geosmin was not released from a conjugate and then converted to argosmin. The lack of hydrolytic release of geosmin indicates that the occurrence of geosmin after 48 h in ACT-grown cultures represents de novo biosynthesis of geosmin rather than a release of bound metabolite. However, the possible existence of an extremely recalcitrant geosmin conjugate cannot be ruled out.

Investigation of model systems that exhibit differential geosmin expression may facilitate the elucidation of the pathway of geosmin synthesis and the development of strategies to control off-flavor metabolite contamination of food and water resources. This investigation indicates that cultures of *S. tendae* grown on HT and ACT media and cultures obtained from ACT medium at different times exhibit marked differences in their expression of geosmin synthesis. Biochemical and physiological comparisons between these cultures and further investigations with chemical effectors of geosmin synthesis, such as folic acid, L-methionine (Aoyama, 1990), and mixed-function oxidase inhibitors (Dionigi et al., 1990), may provide new information concerning off-flavor metabolite synthesis.

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