Mutagenesis by ethidium bromide and N-methyl-N′-nitro-N-nitrosoguanidine in off-flavour compound producing strains of *Streptomyces*

ISMAIL SAADOUN*, AHMED ELBETIEHA and WILLARD T BLEVINS†

Department of Biological Sciences, Jordan University of Science and Technology, Irbid 22110, Jordan
†Suncrest Laboratories, LLC, 9500 AL Hwy 14 W, Notasulga, AL 36866, USA

**Corresponding author (Fax, 962-2-295123; Email, isaadoun@just.edu.jo).*

Various species of actinomycetes and cyanobacteria can impart earthy/musty off-flavours to drinking water supplies and to pond-raised fish and other aquatic food animals. The genetic determinants for production of the most common off-flavour compounds [geosmin and 2-methylisoborneol (MIB)] have not been extensively studied. An attempt has been made to study the genetics of production of these compounds was demonstrated by DNA-curing analysis. The effects of two curing agents [ethidium bromide (EB) and N-methyl-N′-nitro-N-nitrosoguanidine (NTG)] on the loss of linear plasmid DNA and generation of bald mutants (no aerial mycelia) in *Streptomyces halstedii* and *Stereptomyces violaceusniger* which produce geosmin and MIB, respectively, were observed. Production of earthy/musty odour was not eliminated, but was reduced by 55–95% in the plasmid cured strain. Data suggested that off-flavour production is likely chromosomally-encoded in these *Streptomyces* isolates.

Introduction

Geosmin (*trans*-1,10-dimethyl-*trans*-9-decalol) and methyl-isoborneol (1,2,7,7-tetramethyl-*exo*-bicyclo-[2,2,1]-heptan-2-ol or MIB) are secondary metabolites produced by cyanobacteria (Tabachek and Yurkowski 1976; Isaguirre *et al* 1982) as well as actinomycetes (Gerber 1979; Bentley and Meganathan 1981), and these compounds can impart earthy/musty off-flavours (or tastes and odours) to the aquatic environment. There are few studies regarding the genetic basis for the biosynthesis of these off-flavour compounds or the regulation of their synthesis. Some reports suggest that the genetic determinants for off-flavour compounds in actionomycetes may be carried on plasmids (Redshaw *et al* 1979; Ishibashi 1992). Plasmids are abundant in the genus *Streptomyces*, and usually exist as covalently closed circular (CCC) DNA molecules. Some species harbour linear DNA plasmids, with streptomycetes being the only prokaryotic organisms reported to contain linear plasmids (Hayakawa *et al* 1979; Hirochika *et al* 1982, 1984; Chadron-Loriaux *et al* 1986; Kinashi and Shimaji 1987; Keen *et al* 1988; Ishibashi 1992; Saadoun and Blevins 1997). Pulsed-field gel electrophoresis (PFGE) has been used to detect a giant (500 kb) linear plasmid proposed to encode for geosmin production in *Streptomyces lasaliensis* (Ishibashi 1992). Treatment of *Streptomyces* species with ethidium bromide (EB), acriflavine (AF), or acridine orange (AO) can generate "bald" mutants which have lost the ability to sporulate and may also lack the ability to synthesize off-flavour compounds (Redshaw *et al* 1976, 1979). Transposable elements, which are also mutagenic, could contribute to loss of odour producing ability due to chromosomal mutations induced by these agents (Sermonti *et al* 1980).

In this communication, the effects of two curing agents [EB and N-methyl-N′-nitro-N-nitrosoguanidine (NTG)] on production of off-flavour compounds as well as loss of linear plasmid DNA and aerial mycelia by *Streptomyces halstedii* and *Stereptomyces violaceusniger* is reported.

Keywords. *Streptomyces*; geosmin; MIB; curing agent; aerial mycelium; bald mutant; linear plasmid

Therefore, the purpose of this work is to ascertain whether the genes for earthy/musty compounds production are either plasmid-borne or encoded on the chromosome.

2. Materials and methods

2. *Microbial cultures*

The following microbial cultures were used in this study:

(i) *S. halstedii*: (A-1 strain) a geosmin-producing actinomycete isolated from a catfish production pond, Auburn, AL, USA.
(ii) *S. violaceusniger*: (C_4-S strain) a MIB-producing actinomycete isolated from sediment of a local stream, Auburn, AL, USA.

2.2 *Recovery of plasmid DNA from actinomycetes*

High molecular weight plasmid DNA from protoplasts of *S. halstedii* and *S. violaceusniger* was detected by PFGE according to the protocol of Kinashi and Shimaji (1987) and Saadoun and Blevins (1997). Electrophoresis was conducted with 1% PFGE agarose (Biorad, Hercules, CA, USA) in 0·5 × TBE at 180 V for 18 h, with a switching time of 30–60 s at 12°C using a contour-clamped homogeneous electric field (CHEF-DR-II) (Bio-rad, USA) system. All gels were stained with a solution of 0·5 mg/ml ethidium bromide for 20 min at room temperature. Gels were destained by rinsing with distilled water and viewed using an ultraviolet (302 nm) trans-illuminator Model TM-36 (Ultra Violet Products Inc., San Gabriel, CA, USA). The gels were then photographed using a Polaroid DS-34 Direct Screen Instant Camera (Polaroid Corporation, USA) fitted with a Tiffen 40·5 mm deep yellow 15 filter (Tiffen Manufacturing Co., USA).

2.3 *Plasmid DNA curing*

Spore suspensions of *S. halstedii* and *S. violaceusniger* were freshly prepared or frozen in 20% glycerol according to Hopwood (1985). Cultures of both strains were grown on Hickey–Tresner (HT) (Hickey and Tresner 1952) agar (pH 7·3) for 10 days at 28°C. The spores were harvested by scraping, suspended in 0·1% Tween 80, then vortexed for 9 min at room temperature. The suspension was filtered through glass wool and Whatman 1 filter paper, and the spore pellet was suspended in 20% glycerol. Curing of the plasmid was attempted by inoculating the spore suspension into HT broth containing 10 mM EB or 2·5–3·0 mg/l NTG. Broth cultures were incubated at 28°C/200 rpm for 3 weeks. After growth, serial dilutions were plated onto HT agar medium, and plates were incubated for 48 h at 28°C. Presumptive aerial mycelium-negative (amy$^-$) colonies were picked aseptically and re-plated onto HT agar medium under the same conditions.

2.4 *Gas chromatography analysis*

The amy$^-$ colonies were scored for geosmin and MIB production by absorption of the metabolites with dibutyl-phthalate (DBP) (Eastman Kodak, USA). Each colony was separately streaked onto YD agar plates to obtain confluent growth, and a single depression slide containing 0·1 ml of DBP was placed in the lid of each Petri dish. A standard of geosmin was prepared by mixing 1 ml of 200 ppm geosmin in 0·1 ml DBP, and the GC retention time of this standard was recorded. At 5, 10, and 14 days incubation at 28°C, 0·1 ml of DBP was injected into a Perkin-Elmer model 8500 GC (Perkin-Elmer Corp., USA). The absorption of geosmin and MIB by DBP permits a qualitative detection of off-flavour compounds produced by microbial isolates (Durham 1975). A Stabilwax fused silica capillary column (30 m × 0·25 mm i.d. × 0·25 mm film thickness, Perkin-Elmer) was used with a flame ionization detector (FID). Helium served as carrier gas at a linear velocity of 20 cm/s and split ratio of 5 : 1. The column temperature was set at 70°C for 1 min, then programmed successively to 150°C at 10°C/min and iso-time of 2 min, and finally, to 250°C/min and iso-time of 19 min. The injector and detector were set at 300°C, and the gauge pressure was set at 20 psi (1 psi = 6·895 Kpa). Borneol and different concentrations of authentic standards of geosmin and MIB were used to make cubic fit standard curves from PE Nelson Omega System Hardware (Perkin-Elmer). These curves were used to identify and quantify geosmin and MIB in culture samples. The GC peaks corresponding to geosmin and MIB were confirmed by GC-mass spectrometry using a model VG70E mass spectrometer (VG Analytical, Manchester, England) at Auburn University Mass Spectrometry Center. The chromatograms of bald mutants were compared with two controls, negative (pure DBP) and positive (A-1 or C_4-S with DBP). To confirm that the amy$^-$ colonies were negative for geosmin and MIB production, the amy$^-$ cultures were scaled up by growth in RS medium (per liter: 20 g glucose, 12 g $NaNO_3$, 2 g K_2HPO_4, 1 g $MgSO_4 \cdot 7H_2O$, 0·2 g KCl, and 0·01 g $FeSO_4 \cdot 7H_2O$, pH 7·5) (Romano and Safferman 1963) at 28°C and 200 rpm for 5 days. Cultures were distilled to 20% of culture volume, and distillates were extracted successively with 20% and 10% volumes of methylene chloride. Combined extracts were concentrated by air to 0·1 ml for further analysis by GC. A stabilwax fused silica capillary column (30 m × 0·25 mm i.d. × 0·25 mm film thickness, Supelco, Inc., USA) was used. For analysis of extracts, GC analysis was as stated above, using different column conditions (Schrader and Blevins 1993).

3. Results and discussion

Redshaw *et al* (1979) suggested that treatment of *Streptomyces* species with mutagenic agents (also curing agents) such as EB, AF, and AO may cause strains to lose the ability to synthesize the earthy odours. These agents are also responsible for the production of bald mutants (loss of formation of aerial mycelia). If curing were to result in the loss of synthesis of odour compounds, this could suggest that odour production in actinomycetes could be encoded for by plasmid genes. This study showed the formation of bald mutants and loss of aerial mycelia (amy^-) after treatment of *Streptomyces* species with EB and NTG (figures 1 and 2). The giant linear plasmid (PIS1) that was detected previously by Saadoun and Blevins (1997) was not found in bald isolates of

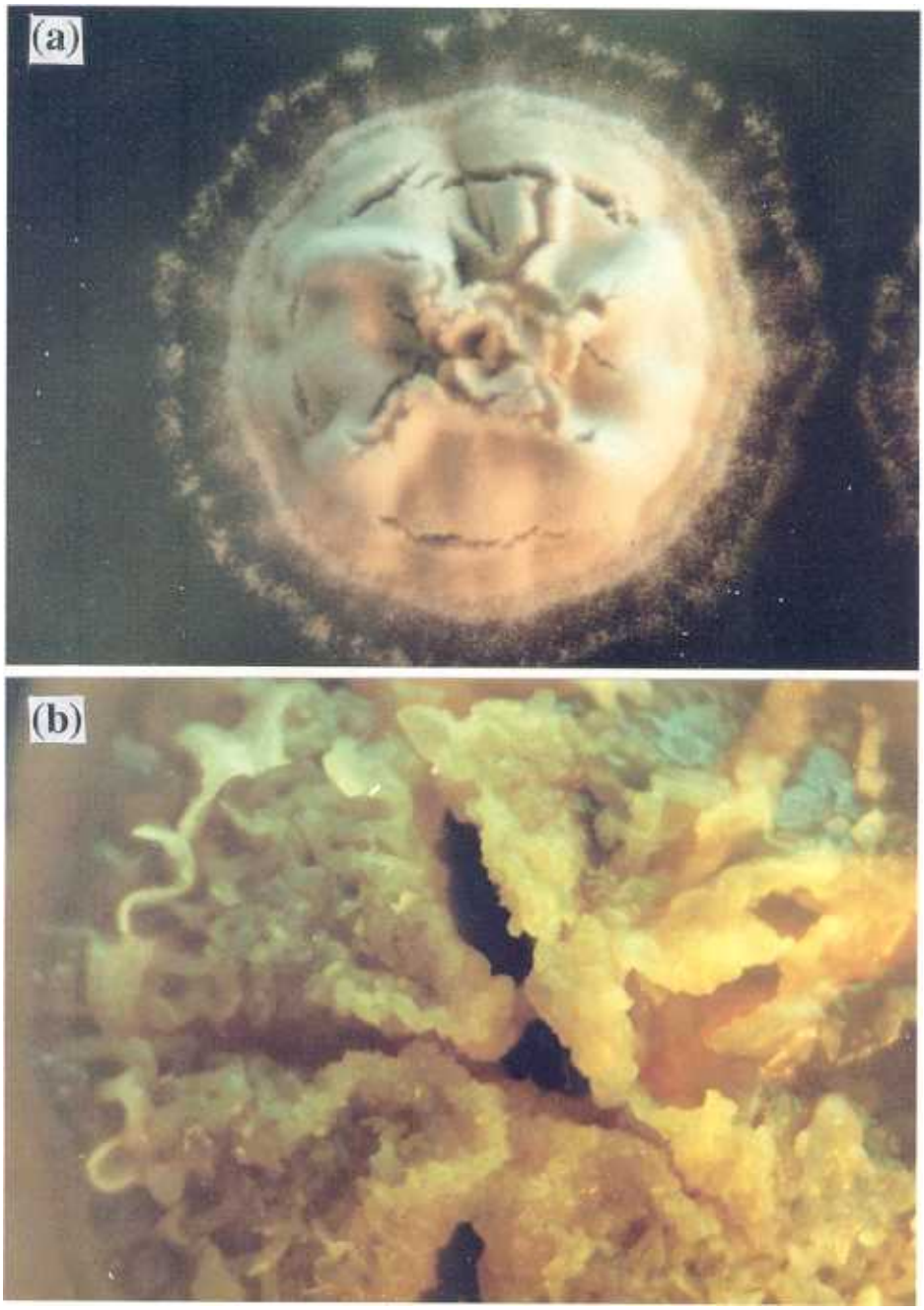

Figure 1. Treatment of *S. halstedii* (A-1) with 10 mM EB. **(a)** Wild type. **(b)** Bald isolate.

S. halstedii (Figure 3a, lanes 3, 9, and figure 3b, lane 3), or PIS2 in bald isolates of *S. violaceusniger* (figure 3a, lanes 2 and 8). Bald isolates of *S. halstedii* (figure 1b) were screened for geosmin production by absorption by DBP. Table 1 shows that the bald isolates still produced geosmin, and did so consistently even after 14 days of incubation. Geosmin production by these bald strains was confirmed by scaled-up growth in RS medium. The ability of amy$^-$ mutants (obtained by EB treatment) to produce geosmin is shown in table 2. Geosmin production was reduced by 55–95% and geosmin/biomass (G/B) by 80–97%; however, biomass dry weight increased by 22–40% (table 2). MIB was produced by the bald strain of *S. violaceusniger* (figure 2b); however, a 60–92%

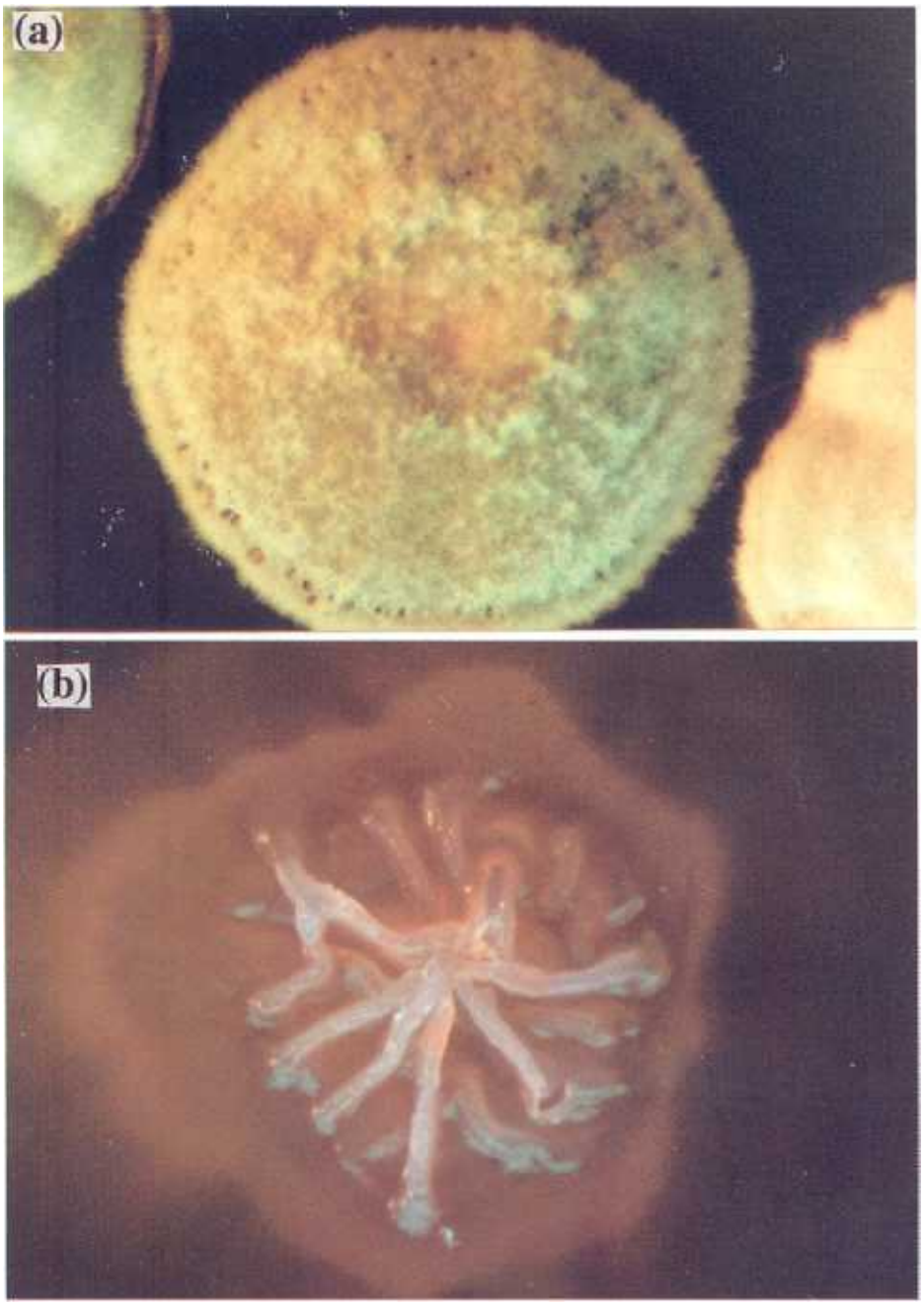

Figure 2. Treatment of *S. violaceusniger* (C_4-S) with 0·25 mg/ml NTG. (**a**) Wild type. (**b**) Bald isolate.

reduction in MIB was revealed, with MIB/biomass dry weight reduced by 62–94% and biomass dry weight increased by 61% (table 3).

Attempts were made to cure these plasmids by treatment of cultures with dyes. Colonies derived by treatment with EB and NTG appeared soft and did not form characteristic aerial mycelia. This loss of aerial mycelia after dye treatment has often been reported in *Streptomyces* species (Ikeda *et al* 1981; Hopwood *et al* 1973). Bald strains generated by treatment with EB, AF or AO may lose the ability to synthesize earthy odours (Redshaw *et al* 1979). Linear PIS1 and PIS2 plasmids that were previously recovered from untreated *S. halstedii* and *S. violaceusniger* (Saadoun and Blevins 1997) were not detected in our bald isolates, but geosmin and MIB production was not completely lost. This would suggest that off-flavour production is likely chromosomally-encoded in these *Streptomyces* isolates used in this study. However, the possible presence of a low copy number plasmid can not be ignored, and mutagenesis of a chromosomal gene could be responsible for this loss, since the dyes used could cause chromosomal aberrations as well as the elimination of plasmids.

The processes of secondary metabolism (antibiotic and odour compound production) and morphological differentiation (aerial mycelia formation and subsequent spore formation) in streptomycetes have been "linked" because both phenomena apparently occur simultaneously during the life cycle of these organisms. The coincidence of

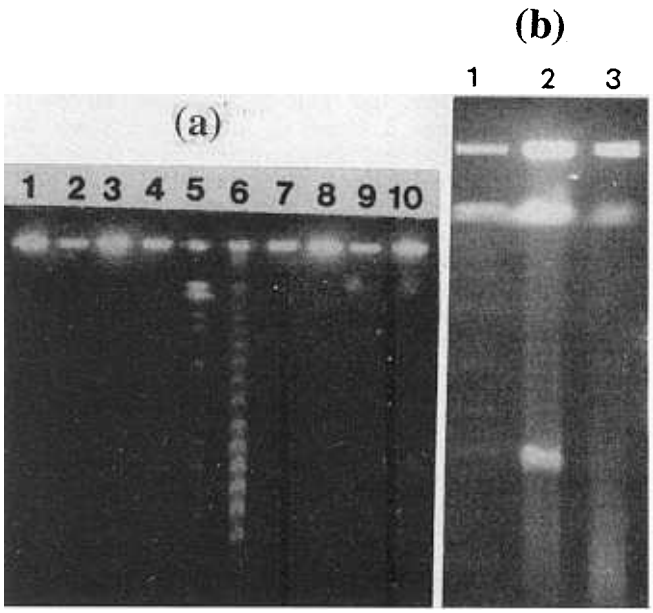

Figure 3. (**a**) Curing of PIS1 plasmid DNA with 10 mM EB and PIS2 plasmid DNA with 0·25 mg/ml NTG. (0·5 × TBE, 1% pulsed-field agarose, 12°C, 200 V, 24 h, A-time × 30 s, final A-time × 60 s), *S. violaceusniger* (lanes 1 and 10); *S. violaceusniger*/0·25 mg/ml NTG (lanes 2 and 9); *S. halstedii* (A-1) (lanes 4 and 7); *S. halstedii*/10 mM EB (lanes 3 and 8); *S. cerevisiae:* 225–2200 kb (lane 5); and 1 DNA ladder: 50–1000 kb (lane 6). (**b**) Curing of PIS1 plasmid DNA with 10 mM EB. (0·5 × TBE, 1% pulsed-field agarose, 12°C, 200 V, 24 h, A-time = 30 s, final A-time = 60 s), *S. cerevisiae*: 225–2000 kb (lane 1); *S. halstedii* (lane 2) and *S. halstedii*/10 mM EB (lane 3).

Table 1. Screening of bald isolates of *S. halstedii* for production of geosmin

	Geosmin production* Strain**							
	A-1		2	3	13	15	16	17
5 Days	+	+	+	–	+	+	+	+
10 Days	+	+	+	+	+	+	+	+
14 Days	+	+	+	+	+	+	+	+

*Geosmin standard retention time is 14·8–14·94 min.
**Strains 1, 2 and 3, and 13, 15, 16 and 17 were generated using 10 and 20 mM EB respectively.

Table 2. Geosmin production in wild-type and amy⁻ mutants of *S. halstedii.*

	Geosmin (mg/l)	Biomass (mg/ml)	Geosmin/biomass (ng/mg)
A	22·57 ± 2·80 (0·00)	2·92 ± 0·34	13·10 ± 2·63 (0·00)
B	10·10 ± 1·23 (55·20)	3·56 ± 0·11	2·64 ± 0·16 (79·85)
C	1·22 ± 1·30 (96·60)	4·08 ± 0·79	0·37 ± 0·34 (97·20)

A, *S. halstedii*; B, *S. halstedii*/10 mM EB (strain 2); C, *S. halstedii*/20 mM EB (strain 17).
Values in parentheses represent the % of geosmin and % of geosmin/biomass reduction.

Table 3. MIB production in wild-type and amy⁻ mutants of *S. violaceusniger.*

	MIB (mg/l)	Biomass (mg/ml)	MIB/biomass (ng/mg)
A	55·26 ± 2·54 (0·00)	5·13 ± 1·13	11·97 ± 2·16 (0·00)
B	4·58 ± 0·73 (91·71)	6·18 ± 1·33	0·75 ± 0·16 (793·70)
C	22·53 ± 2·77 (59·23)	5·06 ± 0·58	4·50 ± 0·04 (62·40)

A, *S. violaceusniger*; B, *S. violaceusniger*/10 mM EB (strain 1); C, *S. violaceusniger*/0·25 mg/ml NTG (strain 2).
Values in parentheses represent the % of geosmin and % of geosmin/biomass reduction.

sporulation with the onset of secondary metabolite production has been described by some as correlative relationship but not an interdependent one (Bu'lock 1961; Schaeffer 1969). Production of earthy/musty compounds during secondary metabolism has been described (Gerber 1979; Bentley and Meganathan 1981), and Redshaw *et al* (1979) reported the loss of odour production coincident with the loss of aerial mycelia production when spores of some *Streptomyces* species were germinated in the presence of intercalating dyes. However, the lack of detection of the earthy odour in any of the amy$^-$ isolates of *S. alboniger, S. scabies* and *S. violaceuruber* was based on sniffing trials and not on GC analysis. Decreased production of geosmin or MIB by the amy$^-$ bald isolates in this study was highly observed; however, biomass production increased, indicating a re-directing of cell energy toward primary metabolism.

References

Bentley R and Meganathan R 1981 Geosmin and methylisoborneol biosynthesis in streptomycetes; *FEBS Lett.* **125** 220–222

Bu'lock J D 1961 Intermediary metabolism and antibiotic synthesis; *Adv. Appl. Microbiol.* **3** 293–342

Chardon-Loriax I, Charpentier M and Percherson F 1986 Isolation and characterization of a linear plasmid from *Streptomyces rimosus*; *FEMS Microbiol. Lett.* **35** 151–155

Durham D R 1975 *Environmental factors affecting the production of geosmin, an earthy-musty odorous metabolite produced by aquatic actinomycetes* MS thesis, Auburn University, Auburn, Alabama, USA

Gerber N N 1979 Volatile substances from actinomycetes: their role in odour pollution of water; *Crit. Rev. Microbiol.*, **7** 91–214

Hayakawa T, Kanaka T, Sakaguchi N, Otake N and Yonchara H 1979 A linear plasmid-like DNA in *Streptomyces* sp. producing lankacidin group antibiotics; *J. Gen. Appl. Microbiol.* **25** 255–260

Hickey R J and Tresner H D 1952 A cobalt-containing medium for sporulation of *Streptomyces* species; *J. Bacteriol.* **84** 891–892

Hirochika H K, Nakamura K and Sakaguchi 1982 Analysis of linear plasmids isolated from *Streptomyces*: association of protein with the ends of the plasmid DNA; *Plasmid* **7** 59–65

Hirochika H K, Nakamura K and Sakaguchi 1984 A linear DNA plasmid from *Streptomyces rochei* with an inverted terminal repetition of 614 base pairs; *EMBO J.* **3** 761–766

Hopwood D A, Bibb M J, Chater K F, Kieser T, Burton C J, Kieser H M, Lydrate D J, Smith C P, Ward J M and Schrempf H 1985 *Genetic manipulation of Streptomyces. A laboratory manual* (Norwich: The John Innes Foundation)

Hopwood D A, Chater K F, Dowding J E and Vivian A 1973 Advances in *Streptomyces coelicolor* genetics; *Bacteriol. Rev.* **37** 371–405

Ikeda H, Tanaka H and Omura S 1981 Genetic and biochemical features of spiromycin biosynthesis in *Streptomyces ambofaciens* RM 265. J6; *J. Antibiot.* **35** 507–516

Ishibashi Y 1992 Genetic studies into musty odor production by actinomycetes; *Water Sci. Tech.* **25** 171–176

Izaguirre G, Huang C J, Krasner S W and Means E G 1982 Geosmin and 2-methylisoborneol from cyanobacteria in three water supply systems; *Appl. Environ. Microbiol.*, **43** 708–716

Keen C L, Mendeloritz S, Cohen G, Aharonowitz Y and Roy K L 1988 Isolation and characterization of a linear DNA plasmid from *Streptomyces clavuligereus*; *Mol. Gen. Genet.* **212** 172–176

Kinashi H and Shimaji M 1987 Detection of giant linear plasmid in antibiotic producing strains of *Streptomyces* by the OFAGE technique; *J. Antibiot.* **40** 913–916

Redshaw P A, Mccann P A, Pentella M A and Pogell B M 1979 Simultaneous loss of multiple differentiation in aerial mycelium-negative isolates of *Streptomyces*; *J. Bacteriol.* **137** 891–899

Redshaw P A, Mccann P A, Sankaran L and Pogell B M 1976 Control of differentiation in streptomycetes: Involvement of extrachromosomal deoxyribonucleic acid and glucose repression in aerial mycelium development; *J. Bacteriol.* **125** 698–705

Romano A H and Safferman R S 1963 Studies on actinomycetes and their odours; *J. AWWA* **55** 169–176

Saadoun I and Blevins W T 1997 Detection of giant linear plasmids in off-flavor compound-producing strains of *Streptomyces* by PFGE; *Actinomycetes* **8** 58–65

Schaeffer P 1969 Sporulation and the production of antibiotics, exoenzymes, and exotoxins; *Bacteriol. Rev.* **33** 48–71

Schrader K K and Blevins W T 1993 Geosmin-producing species of *Streptomyces* and *Lyngbya* from aquaculture ponds; *Can. J. Microbiol.* **39** 834–840

Sermonti G, Lanfaloni L and Micheli M R 1980 A jumping gene in *Streptomyces coelicolor* A3(2); *Mol. Gen. Genet.* **177** 453–458

Tabachek J L and Yorkowski M 1976 Isolation and identification of blue-green algae producing mud dor metabolites, geosmin, and 2-methylisoborneol, in saline lakes in Manitoba; *J. Fish. Res. Board Can.* **33** 25–35

MS received 25 May 1998; accepted 9 September 1998

Corresponding editor: DURGADAS P KASBEKAR