Streptomyces sanyensis sp. nov., isolated from mangrove sediment

Jin-Lei Sui,^{1,2} Xiao-Xiong Xu,¹ Zhi Qu,¹ Hai-Long Wang,¹ Hai-Peng Lin,¹ Qing-Yi Xie,¹ Ji-Sheng Ruan¹ and Kui Hong¹

Correspondence
Kui Hong
k1022@163.net

¹Lab of Tropical Microbial Resource, Hainan Province; Key Lab of Tropical Crop Biotechnology, Ministry of Agriculture; Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agriculture Sciences, Haikou 571101, PR China

²College of Environment and Plant Protection, Hainan University, Danzhou 571737, PR China

A novel strain, 219820^T, whose metabolites were found to be active against tumour cells, was isolated and characterized. The isolate belonged to the genus *Streptomyces* and had white to grey aerial mycelium and long chains of smooth spores in the aerial mycelium. A phylogenetic tree based on 16S rRNA gene sequences showed that strain 219820^T had highest similarity to members of the genus *Streptomyces* and was most closely, albeit loosely, associated with *Streptomyces crystallinus* NBRC 15401^T (98.624 % similarity), *Streptomyces melanogenes* NBRC 12890^T (98.565 %) and *Streptomyces noboritoensis* NBRC 13065^T (98.564 %). However, DNA–DNA relatedness and phenotypic data readily distinguished strain 219820^T from these phylogenetically related type strains. It is evident from the combination of genotypic and phenotypic data that strain 219820^T represents a novel species of the genus *Streptomyces*, for which the name *Streptomyces sanyensis* sp. nov. is proposed; the type strain is 219820^T (=CGMCC 4.5626^T =DSM 42014^T).

The genus *Streptomyces*, proposed by Waksman & Henrici (1943), currently encompasses more than 500 species, the largest genus in the domain ‘Bacteria’ (Hain *et al.*, 1997). Members of the family *Streptomycetaceae* have attracted great attention because of their ability to produce various natural products of considerable commercial value (Bérdy, 2005).

During a search for novel antibiotic producers, strain 219820^T, which showed activity against the human colon tumour cell line HCT-116, was isolated from a composite mangrove soil sample collected in Sanya, Hainan Province, China (Hong *et al.*, 2009). The soil sample was heated in an oven at 120 °C for 60 min, treated with a solution of chloramine-T (1.0 %, w/v) (Hayakawa *et al.*, 1997) and then incubated for 20 days at 28 °C on isolation plates of glucose-aspartic acid-vitamin medium (Hayakawa *et al.*, 1991). The purified strain was maintained on yeast extract-malt extract agar [International *Streptomyces* Project (ISP) medium 2] (Shirling & Gottlieb, 1966) slants at 4 °C for short-term storage and as glycerol suspensions (20 %, v/v)

at –20 °C for long-term preservation (Wellington & Williams, 1978).

Morphology was observed by light (80i; Nikon) and scanning electron (FEI QUANTA) microscopy of ISP 2 agar cultures incubated at 28 °C for 15 days. Cultural characteristics were determined after 2 weeks at 28 °C using methods described by Shirling & Gottlieb (1966). Colony colour was determined by using ISCC–NBS colour charts (Kelly, 1964). Physiological and biochemical characteristics of strain 219820^T and its phylogenetically most closely related neighbours were tested together using the well-established procedures of Williams *et al.* (1983) and Kämpfer *et al.* (1991). Tolerance to temperature and pH was tested on plates of modified Bennett’s agar (Jones, 1949) incubated for 7–14 days. NaCl tolerance was tested in liquid modified Bennett’s medium incubated at 28 °C for 7–14 days.

Biomass for chemical and molecular systematic studies was obtained after incubation at 28 °C for 4–7 days in shake flasks (about 180 r.p.m.) of ISP 2 broth. Amino acids and sugars of whole-cell hydrolysates were analysed by TLC as described previously (Hasegawa *et al.*, 1983; Lechevalier & Lechevalier, 1980). Cellular fatty acids were extracted by the method of Sasser (1990) and the composition was determined by GC (Oliver & Colwell, 1973). Menaquinones were extracted from freeze-dried biomass, purified according to Minnikin *et al.* (1984) and analysed

Abbreviations: DAP, diaminopimelic acid; ISP, International *Streptomyces* Project.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Streptomyces sanyensis* 219820^T is FJ261968.

A supplementary table is available with the online version of this paper.

by HPLC with an ODS-BP C_{18} column (4.6×250 mm). Phospholipids were analysed by TLC using the technique described by Minnikin *et al.* (1984).

Genomic DNA preparation, and PCR amplification and sequencing of the 16S rRNA gene, were carried out using the procedure described by Goodfellow *et al.* (2007). The 16S rRNA gene sequence of strain 219820^T was multiply aligned with representative sequences of related streptomycetes obtained from GenBank/EMBL/DBJ using CLUSTAL X software. The alignment was verified manually and adjusted prior to the reconstruction of a phylogenetic tree. The unrooted phylogenetic tree was generated via the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) tree-making algorithms in MEGA version 4.0 (Tamura *et al.*, 2007); evolutionary distances for the neighbour-joining algorithm were calculated with the Kimura two-parameter model (Kimura, 1980). Topologies of the resultant trees were evaluated in a bootstrap analysis (Felsenstein, 1985) based on 1000 replicates. Values for pairwise 16S rRNA gene sequence similarities among the closest strains were determined using the EzTaxon server (<http://www.Eztaxon.org>) (Chun *et al.*, 2007). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). It was apparent from the initial neighbour-joining analysis, which included more than 500 related sequences of *Streptomyces* type strains, that isolate 219820^T represented a bona fide member of the genus *Streptomyces* (data not shown).

Chromosomal DNA was extracted as described by Pospiech & Neumann (1995). The DNA G+C content of strain 219820^T was determined by the HPLC method (Mesbah *et al.*, 1989). DNA–DNA relatedness studies were performed between strain 219820^T and related type strains using a modified nylon membrane hybridization method based on PCR-mediated amplification. Chromosomal DNA of the strains was digested with *Sau*3AI and fragments of 200 bp to 2 kb were collected. *Sau*3AI-digested DNA was ligated with the appropriate linker applying T4 DNA ligase (TaKaRa) using a method modified from Wassill *et al.* (1998). The linker-modified target DNA was labelled using a PCR DIG Probe Synthesis kit (Roche). The unlabelled PCR products were spotted onto Hybond-N+ nylon membranes (Amersham) for hybridization. After a minimum of 1 h of prehybridization, hybridization was carried out overnight at 65 °C in rotating glass tubes. One hundred nanograms of denatured DNA probe labelled with digoxigenin was added to the hybridization solution. Washing was performed as follows: 2×5 min with 20 ml $2 \times$ SSC, 0.1 % SDS, then 2×15 min with 20 ml $0.1 \times$ SSC, 0.1 % SDS at 65 °C. The filter was then processed with phosphatase-labelled anti-Dig antibody and signals were produced with the chromogenic substrate NBT/BCIP as described in the manufacturer's protocol (Roche). Signals were quantified densitometrically.

Strain 219820^T grew well on most of the media tested (Supplementary Table S1, available in IJSEM Online). Pale yellow to dark yellowish-brown colonies that bore white to

grey aerial spore mass were formed on ISP media 1, 2, 3, 4 and 6 (Shirling & Gottlieb, 1966), modified Bennett's agar, Czapek's agar and nutrient agar (Dong & Cai, 2001). Strain 219820^T exhibited poor growth on ISP 5 and ISP 7 agar media. No diffusible pigment was detected on any of the media tested. Morphological observation of a 15-day-old culture of strain 219820^T grown on ISP 2 agar revealed that both aerial and vegetative hyphae were abundant, well-developed and not fragmented. Strain 219820^T showed typical characteristics of the genus *Streptomyces*. Spore chains were *Rectiflexibiles* in nature. Spores were elliptical or shaped like short rods ($0.3\text{--}0.6 \times 0.7\text{--}1.1$ µm). The spore surfaces were smooth (Fig. 1).

Strain 219820^T was positive in tests for gelatin liquefaction, milk coagulation and peptonization, melanin production, H_2S production, urease production, catalase production, degradation of Tweens 20 and 80, growth on cellulose, and the oxidase test; it was negative for starch hydrolysis, nitrate reduction, L-tyrosine degradation, aesculin degradation and carboxymethyl-cellulose degradation (Table 1). Growth occurred at pH 5.0–9.0 (optimum, pH 7.0), at 10–40 °C (optimum, 28 °C) and with 0–10 % (w/v) NaCl (optimum, 0 % NaCl). Dextrin, glucose, mannose, maltose, glycerol, D-ribose and trehalose were utilized as sole carbon and energy sources, but L-arabinose, D-fructose, D-galactose, *myo*-inositol, inulin, lactose, D-mannitol, raffinose, L-rhamnose, salicin, sucrose and D-xylose were not. L-Alanine, D-arginine, L-asparagine, glycine, L-histidine, L-serine and L-ornithine were metabolized as sole nitrogen sources, but not L-cysteine, L-hydroxyproline, L-methionine, L-phenylalanine, L-proline or L-valine.

Polar lipid analysis revealed the presence of phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol monomannoside, indicating that the phospholipid was of type II *sensu* Lechevalier *et al.* (1977). The menaquinones were MK-9(H_8) (58.5 %), MK-9(H_6)

Fig. 1. Scanning electron micrograph of spore chains of strain 219820^T grown on yeast extract-malt extract agar (ISP 2) at 28 °C for 15 days. Bar, 2.0 µm.

Table 1. Morphological and physiological characteristics of strain 219820^T and related species of the genus *Streptomyces*

Taxa: 1, *S. sanyensis* sp. nov. 219820^T; 2, *S. crystallinus* CGMCC 4.1600^T (=NBRC 15401^T); 3, *S. melanogenes* CGMCC 4.1979^T (=NBRC 12890^T); 4, *S. noboritoensis* CGMCC 4.1457^T (=NBRC 13065^T). +, Positive; ±, weakly positive; –, absent, negative; v, variable. All data were determined in this study.

Characteristic	1	2	3	4
Pigmentation on ISP 3				
Colour of colony	Pale yellow	White	Pinkish-white	White to grey
Reverse side of colony	Pale yellow	Dark brown	Yellow brown	Dark brown
Growth on Czapek's agar	+	±	±	±
Growth at/with:				
10 °C	±	+	+	+
NaCl (7 %, w/v)	+	–	–	–
pH 5	±	+	+	+
Nitrate reduction	–	+	+	+
Diffusible pigment	–	+	+	+
Carbon source utilization				
L-Arabinose	–	+	+	+
D-Xylose	–	–	+	+
myo-Inositol	–	–	+	v
D-Mannitol	–	–	+	+
D-Fructose	–	–	+	+
Raffinose	–	+	+	+
D-Galactose	–	+	+	+
Lactose	–	+	+	+
Mannose	+	–	+	+
Nitrogen source utilization				
D-Arginine	+	–	+	+
L-Hydroxyproline	–	+	+	+
L-Phenylalanine	–	+	+	+
Degradation of:				
L-Tyrosine	–	+	+	+
CM-cellulose	–	+	+	+
Aesculin	–	+	+	+
Starch	–	–	+	±
Gelatin	+	±	–	–

(38.9 %), MK-9(H₄) (1.9 %) and MK-8(H₄) (0.7 %). The major cellular fatty acids of the isolate were iso-C_{16:0} (21.86 %), anteiso-C_{15:0} (17.94 %), iso-C_{15:0} (11.04 %), iso-C_{14:0} (9.90 %), 10-methyl C_{16:0} (8.39 %), C_{16:1}ω₉c (8.21 %) and summed feature 3 (C_{16:1}ω₇c and/or iso-C_{15:0} 2-OH; 5.94 %). In addition, C_{17:1}ω₈c (3.96 %), anteiso-C_{17:0} (3.54 %), C_{17:0} (2.38 %), iso-C_{17:0} (1.95 %), C_{15:0} (1.38 %), C_{17:1}ω₆c (1.19 %) and C_{19:0} (1.02 %) were detected. The G + C content of the genomic DNA of strain 219820^T was 73.24 mol%. These characteristics are consistent with those of the genus *Streptomyces*. According to Lechevalier & Lechevalier (1980), the diagnostic amino acids of the genus *Streptomyces* are LL-diaminopimelic acid (DAP) and glycine, corresponding to cell-wall chemotype I. Strain 219820^T contained both LL- and meso-DAP, which is similar to the type strain of the most closely related species, *Streptomyces crystallinus* NBRC 15401^T (data from this study). Whole-cell hydrolysates of strain 219820^T contained galactose and glucose.

The almost-complete 16S rRNA gene sequence (1489 nt) of strain 219820^T was aligned manually with the corresponding partial 16S rRNA gene sequences of the type strains of representative members of the genus *Streptomyces* retrieved from GenBank/EMBL/DBJ. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain 219820^T fell into a distinct subclade with the type strains of *S. crystallinus*, *Streptomyces melanogenes* and *Streptomyces noboritoensis*, an association that was supported by both of the tree-making algorithms employed. Strain 219820^T shared highest 16S rRNA gene sequence similarity with *S. crystallinus* NBRC 15401^T (98.624 %), which corresponds to 20 nt differences at 1489 locations with gaps, and lower values with *S. melanogenes* NBRC 12890^T (98.565 %) and *S. noboritoensis* NBRC 13065^T (98.564 %). The phylogenetic tree based on the 16S rRNA gene sequences of strain 219820^T and the most closely related type strains of species of the genus *Streptomyces* is shown in Fig. 2.

Fig. 2. Neighbour-joining phylogenetic tree, based on nearly complete 16S rRNA gene sequences (1447 nt), showing the relationships between strain 219820^T and strains of related species of the genus *Streptomyces*. Numbers at branching points indicate bootstrap percentages (based on 1000 replications); only values more than 50% are shown. Asterisks indicate branches of the tree that were also recovered using the maximum-parsimony tree-making algorithm. Bar, 0.002 substitutions per nucleotide position.

DNA–DNA hybridization tests were carried out between strain 219820^T and the phylogenetically most closely related type strains. DNA–DNA relatedness values between strain 219820^T and *S. crystallinus* NBRC 15401^T ($31.0 \pm 0.7\%$), *S. melanogenes* NBRC 12890^T ($27.8 \pm 0.9\%$) and *S. noboritoensis* NBRC 13065^T ($17.5 \pm 0.5\%$) were all significantly lower than 70%, the threshold value for the delineation of genomic species (Stackebrandt & Goebel, 1994), thus suggesting that the novel strain should be considered as a representative of a separate species. Comparison of the phenotypic characteristics of strain 219820^T and its closest phylogenetic neighbours also

revealed significant differences (Table 1). *S. crystallinus* was selected for a detailed cultural characteristic comparison with strain 219820^T on the basis of phylogenetic distance and DNA–DNA relatedness data and its close position to the novel isolate in the phylogenetic tree. The two strains could be easily differentiated based on cultural characteristics (Supplementary Table S1).

A combination of genotypic and phenotypic data show that strain 219820^T merits recognition as the type strain of a novel species in the genus *Streptomyces*, for which the name *Streptomyces sanyensis* sp. nov. is proposed.

Description of *Streptomyces sanyensis* sp. nov.

Streptomyces sanyensis (sa.ny.en'sis. N.L. masc. adj. *sanyensis* pertaining to Sanya, a coastal city in Hainan Province, where the mangrove sediment was collected from which the type strain was isolated).

Aerobic, Gram-positive actinomycete. Forms extensively branched substrate mycelium and aerial hyphae that differentiate into long *Rectiflexibiles* spore chains of up to 20 spores. Spores are elliptical or short rods with a smooth surface. Pale yellow to dark yellowish-brown colonies that carry white to grey aerial spore mass are formed on ISP media 1, 2, 3, 4 and 6, modified Bennett's agar, Czapek's agar and nutrient agar. No diffusible pigment is produced on any of the media tested. Melanin is produced on peptone-yeast extract-iron agar and tyrosine agar. Negative in tests for starch hydrolysis, nitrate reduction, L-tyrosine degradation, aesculin degradation and carboxymethyl-cellulose degradation; positive for gelatin liquefaction, milk coagulation and peptonization, H₂S production, urease production, catalase production, degradation of Tweens 20 and 80, growth on cellulose, and the oxidase test. Dextrin, glucose, mannose, maltose, glycerol, D-ribose and trehalose are utilized as sole carbon sources, but L-arabinose, D-fructose, D-galactose, myo-inositol, inulin, lactose, D-mannitol, raffinose, L-rhamnose, salicin, sucrose and D-xylose are not. L-Alanine, D-arginine, L-asparagine, glycine, L-histidine, L-serine and L-ornithine are metabolized as sole nitrogen sources, but not L-cysteine, L-hydroxyproline, L-methionine, L-phenylalanine, L-proline or L-valine. Growth occurs at pH 5.0–9.0 (optimum, pH 7.0), at 10–40 °C (optimum, 28 °C) and with 0–10 % (w/v) NaCl (optimum, 0 % NaCl). The cell wall contains LL-DAP with nearly the same amount of meso-DAP. Whole-cell hydrolysates contain galactose and glucose. Predominant menaquinones are MK-9(H₈) and MK-9(H₆). The diagnostic phospholipid is phosphatidylethanolamine. Major cellular fatty acids are iso-C_{16:0}, anteiso-C_{15:0}, iso-C_{15:0}, iso-C_{14:0}, 10-methyl C_{16:0}, C_{16:1}ω_{7c} and summed feature 3 (C_{16:1}ω_{7c} and/or iso-C_{15:0} 2-OH).

The type strain is 219820^T (=CGMCC 4.5626^T =DSM 42014^T), isolated from a composite mangrove sediment sample collected in Sanya, Hainan Province, China, and exhibits cytotoxic activity against the human colon tumour cell line HCT-116. The G+C content of the genomic DNA of the type strain is 73.24 mol%.

Acknowledgements

This research was supported by grants of the National High Technology Research and Development Program of China (863 Program) (no. 2007AA09Z415), National Natural Science Foundation of China (no. U0633008), and National Nonprofit Institute Research Grant (ITBBZD0753).

References

- Bérdy, J. (2005). Bioactive microbial metabolites. *J Antibiot (Tokyo)* **58**, 1–26.
- Chun, J., Lee, J. H., Jung, Y., Kim, M., Kim, S., Kim, B. K. & Lim, Y. W. (2007). EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *Int J Syst Evol Microbiol* **57**, 2259–2261.
- Dong, X.-Z. & Cai, M.-Y. (editors) (2001). Determination of biochemical properties. In *Manual for the Systematic Identification of General Bacteria*, pp. 370–398. Beijing: Science Press (in Chinese).
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Fitch, W. M. (1971). Toward defining the course of evolution: minimum change for a specific tree topology. *Syst Zool* **20**, 406–416.
- Goodfellow, M., Kumar, Y., Labeda, D. P. & Sembiring, L. (2007). The *Streptomyces violaceusniger* clade: a home for streptomycetes with rugose ornamented spores. *Antonie van Leeuwenhoek* **92**, 173–199.
- Hain, T., Ward-Rainey, N., Kroppenstedt, R. M., Stackebrandt, E. & Rainey, F. A. (1997). Discrimination of *Streptomyces albidoflavus* strains based on the size and number of 16S–23S ribosomal DNA intergenic spacers. *Int J Syst Bacteriol* **47**, 202–206.
- Hasegawa, T., Takizawa, M. & Tanida, S. (1983). A rapid analysis for chemical grouping of aerobic actinomycetes. *J Gen Appl Microbiol* **29**, 319–322.
- Hayakawa, M., Sadakata, T., Kajiura, T. & Nonomura, H. (1991). New methods for the highly selective isolation of *Micromonospora* and *Microbispora* from soil. *J Ferment Bioeng* **72**, 320–326.
- Hayakawa, M., Iino, H., Takeuchi, S. & Yamazaki, T. (1997). Application of a method incorporating treatment with chloramine-T for the selective isolation of *Streptosporangiaceae* from soil. *J Ferment Bioeng* **84**, 599–602.
- Hong, K., Gao, A. H., Xie, Q. Y., Gao, H., Zhuang, L., Lin, H. P., Yu, H. P., Li, J., Yao, X. S. & other authors (2009). Actinomycetes for marine drug discovery isolated from mangrove soils and plants in China. *Mar Drugs* **7**, 24–44.
- Jones, K. L. (1949). Fresh isolates of actinomycetes in which the presence of sporogenous aerial mycelia is a fluctuating characteristic. *J Bacteriol* **57**, 141–145.
- Kämpfer, P., Kroppenstedt, R. M. & Dott, W. (1991). A numerical classification of the genera *Streptomyces* and *Streptoverticillium* using miniaturized physiological tests. *J Gen Microbiol* **137**, 1831–1891.
- Kelly, K. L. (1964). *Inter-Society Color Council – National Bureau of Standards Color Name Charts Illustrated with Centroid Colors*. Washington, DC: US Government Printing Office.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.
- Lechevalier, M. P. & Lechevalier, H. A. (1980). The chemotaxonomy of actinomycetes. In *Actinomycete Taxonomy*, Special Publication no. 6, pp. 227–291. Edited by A. Dietz & D. W. Thayer. Arlington, VA: Society for Industrial Microbiology.
- Lechevalier, M. P., De Bièvre, C. & Lechevalier, H. A. (1977). Chemotaxonomy of aerobic actinomycetes: phospholipid composition. *Biochem Syst Ecol* **5**, 249–260.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Minnikin, D. E., O'Donnell, A. G., Goodfellow, M., Alderson, G., Athalye, M., Schaal, A. & Parlett, J. K. (1984). An integrated procedure for the extraction of bacterial isoprenoid quinones and polar lipids. *J Microbiol Methods* **2**, 233–241.
- Oliver, J. D. & Colwell, R. R. (1973). Extractable lipids of Gram-negative marine bacteria: fatty-acid composition. *Int J Syst Bacteriol* **23**, 442–458.

- Pospiech, A. & Neumann, B. (1995).** A versatile quick-prep of genomic DNA from gram-positive bacteria. *Trends Genet* **11**, 217–218.
- Saitou, N. & Nei, M. (1987).** The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sasser, M. (1990).** *Identification of bacteria by gas chromatography of cellular fatty acids*, MIDI Technical Note 101. Newark, DE: MIDI Inc.
- Shirling, E. B. & Gottlieb, D. (1966).** Methods for characterization of *Streptomyces* species. *Int J Syst Bacteriol* **16**, 313–340.
- Stackebrandt, E. & Goebel, B. M. (1994).** Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.
- Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007).** MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.
- Waksman, S. A. & Henrici, A. T. (1943).** The nomenclature and classification of the actinomycetes. *J Bacteriol* **46**, 337–341.
- Wassill, L., Ludwig, W. & Schleifer, K. H. (1998).** Development of a modified subtraction hybridization technique and its application for the design of strain specific PCR systems for lactococci. *FEMS Microbiol Lett* **166**, 63–70.
- Wellington, E. M. H. & Williams, S. T. (1978).** Preservation of actinomycete inoculum in frozen glycerol. *Microbios Lett* **6**, 151–157.
- Williams, S. T., Goodfellow, M., Alderson, G., Wellington, E. M. H., Sneath, P. H. A. & Sackin, M. J. (1983).** Numerical classification of *Streptomyces* and related genera. *J Gen Microbiol* **129**, 1743–1813.