***Amycolatopsis sacchari* sp. nov., a moderately thermophilic actinomycete isolated from vegetable matter**

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The taxonomic position of a group of moderately thermophilic actinomycetes isolated from vegetable matter was determined using a suite of genotypic and phenotypic properties. The organisms were found to share a range of chemical and morphological markers typical of members of the genus *Amycolatopsis*. A representative of the group, strain K24^T, formed a distinct phyletic line within the range of variation occupied by the genus *Amycolatopsis* in the 16S rDNA tree. The strains have many phenotypic properties in common and some of these distinguish the group from representatives of the validly described species of *Amycolatopsis*. It is clear from the combined datasets that the strains merit recognition as a new species of *Amycolatopsis*. The name proposed for the new species is *Amycolatopsis sacchari*; the type strain is K24^T (= DSM 44468^T = KCTC 9863^T).

Keywords: *Amycolatopsis sacchari* sp. nov., polyphasic taxonomy, 16S rDNA sequencing

INTRODUCTION

The family *Pseudonocardiaceae* was proposed by Embley *et al.* (1988a) to accommodate actinomycetes which contained *meso*-diaminopimelic acid (*meso*-A_{2pm}), arabinose and galactose in their wall peptidoglycan (wall chemotype IV *sensu* Lechevalier & Lechevalier, 1970) but lacked mycolic acids. Members of the family are currently classified in the genera *Actinobispora*, *Amycolatopsis*, *Kibdelosporangium*, *Prauserella*, *Pseudonocardia* (including *Amycolata*), *Saccharomonospora*, *Saccharopolyspora* and *Thermocrisum* (Kim & Goodfellow, 1999; Xu *et al.*, 1999; Labeda & Kroppenstedt, 2000).

The genus *Amycolatopsis* was established by Lechevalier *et al.* (1986) to accommodate four species which had been misclassified in the genus *Nocardia*. At present, the genus contains 10 validly described species which form a phyletic line within the evolutionary radiation occupied by the family *Pseudonocardiaceae* (Embley *et al.*, 1988a; Chun *et al.*, 1999). Members of

three of these species, namely, *Amycolatopsis fastidiosa* (ex Celmer *et al.* 1977) Henssen *et al.* 1987, *Amycolatopsis methanolica* De Boer *et al.* 1990 and *Amycolatopsis thermoflava* Chun *et al.* 1999 grow well between 50 and 60 °C and hence can be considered as thermophilic actinomycetes (Cross, 1968; Brock, 1986). In contrast, strains assigned to the remaining species, i.e. *Amycolatopsis alba* Mertz and Yao 1993, *Amycolatopsis azurea* (Omura *et al.* 1983) Henssen *et al.* 1987, *Amycolatopsis coloradensis* Labeda 1995, *Amycolatopsis japonica* Goodfellow *et al.* 1997, *Amycolatopsis mediterranei* (Margalith and Beretta 1960) Lechevalier *et al.* 1986, *Amycolatopsis orientalis* (Pittinger and Brigham 1956) Lechevalier *et al.* 1986 and *Amycolatopsis sulphurea* (ex Oliver and Sinclair 1964) Lechevalier *et al.* 1986, are mesophilic and do not grow above 45 °C.

The genus *Amycolatopsis* can be distinguished from members of other genera classified in the family *Pseudonocardiaceae* using a combination of chemical and morphological markers (Kim & Goodfellow, 1999). Similarly, members of validly described species of *Amycolatopsis* can be differentiated from one another using a battery of phenotypic properties (Chun *et al.*, 1999). Some *Amycolatopsis* strains are the

The GenBank accession number for the 16S rDNA sequence of strain K24^T (= DSM 44468^T = KCTC 9863^T) is AF223354.

source of commercially significant antibiotics (Embley, 1992).

The present investigation was designed to establish the taxonomic position of *Amycolatopsis*-like isolates which formed a distinct cluster in numerical phenetic surveys of the family *Pseudonocardiaceae* and related taxa (Mattinson-Rose, 1986; Whitehead, 1989). Genotypic and phenotypic data show that these strains should be recognized as a new species of *Amycolatopsis* for which the name *Amycolatopsis sacchari* sp. nov. is proposed.

METHODS

Test strains and culture conditions. *Amycolatopsis*-like strains K23–K25 were isolated from floor dust of a hemp factory in Lucknow, India, by Dr J. Lacey (Rothamsted Experimental Station, Harpenden, Herts, UK) who also isolated *Amycolatopsis*-like strains K87–K89 and *Amycolatopsis*-like isolates N751, N759 and N761 from sugar-cane bagasse in Reunion and Trinidad, respectively. The strains were isolated using a wind-tunnel technique (Lacey, 1971; Lacey & Dutkiewicz, 1976); the Andersen sampler was loaded with Petri dishes containing half-strength nutrient agar supplemented with 50 µg actidione ml⁻¹ (Gregory & Lacey, 1963). *Amycolatopsis*-like strain N749 was provided by Dr E. Eklund of the Department of Microbiology, University of Helsinki, Finland. The strains were maintained on glucose-yeast extract agar (GYEA) slopes (Gordon & Mihm, 1962) at room temperature and as suspensions of mycelial fragments and spores in glycerol (20%, v/v) at -20 °C. Biomass for the chemosystematic studies was prepared by growing strains K23 (= DSM 44467 = KCTC 9862), K24^T (= DSM 44468^T = KCTC 9863^T), K88 (= DSM 44469 = KCTC 9864) and N759 (= DSM 44470 = KCTC 9865) in shake flasks of nutrient broth (Oxoid) at 100 r.p.m. for 2 d at 30 °C. Cultures were checked for purity, killed by shaking with formalin (1%, v/v), harvested by centrifugation, washed three times in distilled water and freeze-dried. Biomass for the 16S rRNA sequencing study was obtained after growing *Amycolatopsis*-like strain K24^T on a Czapek–Dox-yeast extract-casein agar plate (CYC; Cross & Attwell, 1974) for 7 d at 30 °C. The phenotypic tests were incubated at 30 °C for 21 d unless otherwise stated.

Morphology. The morphological properties of the strains were examined by eye and light microscopy on modified Bennett's (Jones, 1949) and CYC agars (Cross & Attwell, 1974) after 21 d at 30 °C.

Phenotypic characterization. Most of the phenotypic tests were carried out using media and methods described previously (De Boer *et al.*, 1990; Goodfellow *et al.*, 1997). Acid production from sugars was determined according to Gordon *et al.* (1974); the sugars were sterilized by Seitz filtration prior to addition to the cooled molten sterile basal medium. The staining properties were determined using standard procedures after growth on GYEA plates for 7 d at 30 °C.

Chemotaxonomy. The isomeric form of diaminopimelic acid (A_{2pm}) was determined by TLC of whole-organism hydrolysates following the procedure described by Stanek & Roberts (1974). Whole-organism sugars were analysed by GC as their alditol acetates using the method of Englyst & Cummings (1984). Freeze-dried biomass (about 50 mg) was degraded by acid methanolysis (Minnikin *et al.*, 1980) and

the non-hydroxylated fatty acid methyl esters purified by preparative TLC (O'Donnell *et al.*, 1982), then separated and quantified by GC as described by Embley *et al.* (1987). The TLC method of O'Donnell *et al.* (1982) was used to check for the absence of mycolic acids. Isoprenoid quinones and polar lipids were extracted from dried biomass (about 50 mg) using the small-scale procedure of Minnikin *et al.* (1984). Polar lipid patterns were determined using established TLC procedures (Minnikin *et al.*, 1984). Purified menaquinones (1 µl) were analysed using a model Bio-Rad 1330 HPLC instrument fitted with a variable UV detector (model 1305A; Bio-Rad). Separation was achieved using a 10 cm (4 mm i.d.) Techsil reverse phase octadecyl silane (5 µm particle size) analytical column (HPLC Technology) with acetonitrile/propan-2-ol (3:1, v/v) as solvent. The chromatograph was coupled to a Shimadzu CE1B electronic integrator (Dyson Instruments) which was used to calculate the retention time and peak area of each component. Data were presented as the percentage of the total peak area occupied by each individual peak and the corresponding retention time relative to tetrahydrogenated menaquinone with nine isoprene units [MK-9(H₄)].

16S rDNA sequencing. Chromosomal DNA was extracted from strain K24^T using a procedure (Chun & Goodfellow, 1995) slightly modified from that of Pitcher *et al.* (1989). PCR, cloning and sequencing of the resultant 16S rDNA preparations were performed using a *Taq* Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystems) and the Applied Biosystems 373 DNA sequencer, as described previously (Chun & Goodfellow, 1995). The resultant 16S rDNA sequence was aligned manually, using the AL16S program (Chun, 1995), against corresponding sequences of representative members of the family *Pseudonocardiaceae* and related taxa retrieved from the GenBank, EMBL and DDBJ databases.

Unrooted evolutionary trees were inferred by using three treeing algorithms, namely the Fitch–Margoliash (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1981) and neighbour-joining (Saitou & Nei, 1987) methods. The distance model of Jukes & Cantor (1969) was used to generate an evolutionary distance matrix. The topologies of the resultant trees were evaluated by bootstrap analyses (Felsenstein, 1985) of the neighbour-joining method based on 1000 resamplings. All of the phylogenetic analyses were carried out using the PHYLIP package (Felsenstein, 1993). The root position of the unrooted tree based on the neighbour-joining method was estimated by using three outgroup organisms, namely *Pseudonocardia thermophila* ATCC 19285^T (accession no. X53195), *Saccharomonospora viridis* NCIB 9602^T (Z38007) and *Saccharopolyspora hirsuta* ATCC 27875^T (X53196), as described by Swoffold & Olsen (1990).

RESULTS AND DISCUSSION

The 10 strains shared many phenotypic properties in common as they consistently fell into a distinct, homogeneous cluster that was most closely related to a corresponding phenon that was equated with *Amycolatopsis orientalis*, the type species of the genus, in extensive numerical phenetic surveys of representatives of the family *Pseudonocardiaceae* and related taxa (Mattinson-Rose, 1986; Whitehead, 1989). The strains are aerobic, non-motile, Gram-positive, non-acid-alcohol-fast, catalase-positive actinomycetes which

Fig. 1. Neighbour-joining tree (Saitou & Nei, 1987) based on 16S rDNA sequences showing relationships between *Amycolatopsis* strain K24^T and representatives of the validly described species of *Amycolatopsis*. The numbers at the nodes indicate the percentage bootstrap values based on 1000 resampled datasets. The arrow indicates the estimated root position based on three outgroup organisms, namely *Pseudonocardia thermophila* ATCC 19285^T (X53195), *Saccharomonospora viridis* NCIB 9602^T (Z38007) and *Saccharopolyspora hirsuta* ATCC 27875^T (X53196). The bar represents 0.1 substitutions per nucleotide position.

form branched substrate hyphae that fragmented into rod-like elements when grown on modified Bennett's (Jones, 1949) and CYC (Cross & Attwell, 1974) agars. The substrate mycelium carried moderate to abundant white aerial hyphae which differentiated into flexuous chains of spore-like structures on CYC agar. All of these properties are consistent with the classification of the strains in the genus *Amycolatopsis* (Lechevalier *et al.*, 1986; Holt *et al.*, 1994).

The assignment of the strains to the genus *Amycolatopsis* is also supported by the chemotaxonomic data. All four representative strains contained *meso*-A_{2pm} as the wall diamino acid, arabinose (53–61 % of total whole-organism sugars) and galactose (12–37 %)

as major sugars, predominant amounts of tetrahydrogenated menaquinones with nine isoprene units (75–96 % of total menaquinone composition) and a phospholipid pattern consisting of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol and a number of uncharacterized glyco- and polar lipids and a minor ninhydrin-positive phospholipid which possibly corresponds to hydroxyphosphatidylethanolamine (i.e. phospholipid pattern type 2 *sensu* Lechevalier *et al.*, 1977). The organisms also contained 14-methylpentadecanoic acid (30–58 % of total cellular fatty acids), monounsaturated hexadecanoic acid (1–3 %), hexadecanoic acid (4–14 %), 15-methylhexadecanoic acid (7–9 %), 14-methylhexadecanoic acid (7–11 %) and heptadecanoic acid (2–5 %) but lacked mycolic acids. These chemical markers serve to distinguish the strains from all other chemotype IV actinomycetes, apart from those classified in the genus *Amycolatopsis* (Lechevalier *et al.*, 1986; Embley *et al.*, 1988b; Yassin *et al.*, 1991; Kim & Goodfellow, 1999).

An almost complete 16S rDNA sequence was obtained for strain K24^T (1484 nt). Phylogenetic comparison of this sequence with corresponding nucleotide sequences of the representatives of the family *Pseudonocardaceae* clearly showed that strain K24^T belongs to this family since the organism has all of the 16S rDNA signature nucleotides characteristic of this taxon (Stackebrandt *et al.*, 1997). The high 16S rDNA gene sequence similarity values to previously described members of the genus *Amycolatopsis* (94.2–96.7 %) support the addition of strain K24^T to this genus.

It is apparent from Fig. 1 that strain K24^T forms a distinct phyletic line towards the periphery of the evolutionary branch that encompasses the type strains of the mesophilic *Amycolatopsis* species; this relationship was supported by the results obtained with the Fitch–Margoliash and maximum-likelihood treeing algorithms. Strain K24^T is most closely related to *Amycolatopsis sulphurea* DSM 46092^T with which it shares a 16S rDNA nucleotide similarity of 97.7 %, a value that corresponds to 29 nucleotide differences. This clearly indicates that strain K24^T forms a new centre of taxonomic variation within the genus *Amycolatopsis* as the type strains of *Amycolatopsis methanolica* and *Amycolatopsis thermoflava* show a much higher 16S rDNA nucleotide similarity of 98.8 %, which corresponds to 16 nucleotide differences; these strains belong to distinct genomic species (Chun *et al.*, 1999).

It is evident from the numerical phenetic study of Whitehead (1989) that the tested strains form a homogeneous group which corresponds to multi-membered clusters equated to *Amycolatopsis mediterranei* and *Amycolatopsis orientalis* and to single-membered clusters formed by the type strains of *Amycolatopsis azurea*, *Amycolatopsis fastidiosa*, *Amycolatopsis methanolica* and *Amycolatopsis sulphurea*. The tested strains can be separated from the type

Table 1. Characters separating species of the genus *Amycolatopsis*

+, Positive or present; w, weak positive; —, negative or absent. Strains: 1, *Amycolatopsis sacchari* K23, K24^T, K25, K87–K89, N749, N751, N759, N761; 2, *Amycolatopsis alba* DSM 44262^T; 3, *Amycolatopsis azurea* NRRL 11412^T; 4, *Amycolatopsis coloradensis* NRRL 3218^T; 5, *Amycolatopsis fastidiosa* NRRL B-16697^T; 6, *Amycolatopsis japonica* DSM 44213^T; 7, *Amycolatopsis mediterranei* ATCC 13685^T; 8, *Amycolatopsis methanolica* IFO 15065^T; 9, *Amycolatopsis orientalis* NRRL 2450^T; 10, *Amycolatopsis sulphurea* DSM 46092^T; 11, *Amycolatopsis thermoflava* IFO 14333^T.

Character	1*	2	3	4	5	6	7	8	9	10	11
Colour of aerial mycelium:											
Blue	0	—	+	—	—	—	—	—	—	—	—
Pink	0	—	—	—	+	—	—	—	—	—	—
White	10	+	—	—	+	+	+	+	+	+	+
White to olive buff	0	—	—	+	—	—	—	—	—	—	—
Yellowish green	0	—	—	—	—	—	—	—	—	+	—
Production of soluble pigment	0	—	+	+	+	—	—	—	—	—	+
Acid production from:											
Adonitol	9	+	+	—	—	+	—	+	+	—	+
L(+)-Arabinose	10	+	+	—	w	+	+	—	+	—	+
D(+)-Cellobiose	10	+	+	+	+	+	+	+	+	—	+
Dextrin	10	+	+	+	w	+	+	—	+	+	—
meso-Erythritol	10	+	+	—	—	+	—	+	+	—	+
D(+)-Fructose	10	+	+	+	w	+	+	+	+	+	+
D(+)-Galactose	10	+	+	+	w	+	+	+	+	+	+
meso-Inositol	0	+	+	+	—	+	+	—	+	—	—
D(+)-Lactose	10	+	+	—	—	+	+	—	+	—	+
D(+)-Maltose	10	+	+	+	w	+	+	—	+	+	—
D(–)-Mannitol	10	+	+	+	—	+	+	+	+	+	+
D(+)-Melibiose	0	+	+	—	—	+	+	—	—	—	+
α-Methyl-D-glucoside	10	+	+	+	w	+	—	—	+	—	+
α-L(+)-Rhamnose	9	—	—	—	—	—	+	+	+	—	—
Salicin	10	+	+	+	—	+	+	w	+	—	+
D(–)-Sorbitol	0	—	—	—	—	—	w	+	—	—	+
D(–)-Sucrose	10	+	+	+	w	+	+	+	+	+	—
D(+)-Trehalose	10	w	+	+	w	+	+	+	+	+	+
D(+)-Xylose	10	+	+	+	—	+	+	+	+	—	+
Decomposition of:											
Aesculin	10	+	+	+	+	+	+	w	+	—	+
Allantoin	8	+	—	—	—	+	—	+	—	—	+
Casein	10	+	+	+	+	+	+	—	+	+	+
Gelatin	10	+	+	+	—	+	+	+	+	+	—
Hypoxanthine	2	+	+	+	—	+	+	+	+	—	+
Xanthine	10	+	—	—	—	+	—	—	+	—	+
Growth at:											
10 °C	5	—	+	+	+	+	+	—	+	—	—
45 °C	10	—	—	—	+	—	—	+	—	—	+
Growth in the presence of:											
NaCl (5%, w/v)	10	+	+	+	—	w	w	+	w	—	+
Production of:											
Amylase	0	+	—	+	—	+	—	—	+	—	—
Nitrate reductase	10	—	+	+	+	—	—	+	+	+	—
Urease	10	+	+	—	+	—	+	—	+	—	+

* No. of strains positive.

strains of all validly described species of *Amycolatopsis* using a combination of phenotypic properties (Table 1). These findings, together with the 16S rDNA nucleotide sequence data, clearly show that the strains

merit species status within the genus *Amycolatopsis*. It is proposed that the strains be classified in the genus *Amycolatopsis* as *Amycolatopsis sacchari* sp. nov., with strain K24^T as the type strain.

Description of *Amycolatopsis sacchari* Goodfellow et al.

Amycolatopsis sacchari (sac'char.ri. M.L. n. *Saccharum* generic name of sugar cane; M.L. gen. n. *sacchari* of sugar cane).

Aerobic, non-motile, Gram-positive, non-acid-alcohol-fast, catalase-positive actinomycete which forms a branched substrate mycelium that fragments into rod-like elements. The substrate mycelium carries moderate to abundant white aerial hyphae which differentiate into straight chains of spore-like structures on CYC agar. Brown, orange or yellow irregular colonies with highly filamentous margins are formed on modified Bennett's agar. Diffusible pigments are not produced. Aesculin and urea are hydrolysed, hydrogen sulfide is formed from peptone and nitrate is reduced. Most strains also hydrolyse allantoin; casein, elastin, gelatin, testosterone, Tweens 20, 40, 60 and 80, L-tyrosine and xanthine are degraded but starch is not. Acid is formed from adonitol, L-arabinose, D-cellobiose, dextrin, *meso*-erythritol, D-fructose, D-galactose, D-lactose, D-maltose, D-mannitol, α -methyl-D-glucoside, α -L-rhamnose, salicin, D-sucrose, D-trehalose and D-xylose, but not from *meso*-inositol, D-melibiose or D-sorbitol. The organism cleaves 4-methylumbelliferone (4MU)-2-acetamido-2-deoxy- β -D-glucopyranoside, 4MU-*N*-acetyl- β -D-glucosaminide, 4MU- β -D-glucopyranoside, 4MU-heptanoate, 4MU-nonanoate, 4MU-palmitate, 4MU-phosphate (acid) and 4MU- β -D-xyloside, but not 4MU- α -L-arabinofuranoside, 4MU- β -D-fucopyranoside, 4MU- β -L-fucopyranoside, 4MU- α -D-galactopyranoside, 4MU- β -D-galactopyranoside or 4MU- β -D-glucuronide. Similarly, all of the strains cleaved L-alanine-7 amino-methylcoumarin (7AMC), L-arginine-7AMC, benzyloxycarbonyl-L-arginine-L-arginine-7AMC, L-cysteine-(benzyl)-7AMC, L-lysine-7AMC, L-methionine-7AMC, benzyloxycarbonyl-L-phenylalanine-L-arginine-7AMC, L-tyrosine-7AMC and *tert*-butyloxycarbonyl-L-valine-L-proline-L-arginine-7AMC, but none attacked β -alanine-7AMC, L-aspartate-7AMC, L-glutamate-7AMC, *tert*-butyloxycarbonyl-glutamyl-glycine-glycine-L-phenylalanine-7AMC, L-ornithine-7AMC or L-pyroglutamate-7AMC. Adonitol, D- and L-arabinose, D-cellobiose, dextrin, *meso*-erythritol, D-fructose, D- and L-fucose, D-galactose, gentiobiose, D-glycerol, glycogen, D-lactose, D-maltose, D-mannitol, D-mannose, α - and β -D-methylglucoside, α -L-rhamnose, D-ribose, salicin, L-sorbose, D-sucrose, D-trehalose, xylitol and D-xylose (all at 1%, w/v) are used as sole carbon sources for energy and growth, but not amygdalin, arabinol, dulcitol, *meso*-inositol, D-melibiose, D-sorbitol or D-turanose. Similarly, at 0.1% (w/v), growth was observed on L-alanine, androsterone, arbutin, butan-1-ol, ergosterol, ethanol, propan-1-ol, propan-2-ol, propylene glycol, protocatechuic acid, quinic acid, L-serine, shikimic acid, sodium butyrate, sodium hippurate, sodium propionate, sodium pyruvate, sodium succinate, testosterone and L-tyrosine as sole carbon

sources, but not on acetamide, benzamide, catechol, L-citrulline, *p*-cresol, *m*- or *p*-hydroxybenzoic acid, β -hydroxybutyric acid, D-mandelic acid, progesterol, sodium fumarate, sodium tartrate, squalene, syringaldehyde, trimethylamine or vanillin. Growth occurs between pH 5 and 8 and between 20 and 45 °C, but not at 55 °C. Growth also occurs in the presence of cephaloridine hydrochloride (250 μ g ml⁻¹), chloramphenicol (50 μ g ml⁻¹), demeclocycline hydrochloride (8 μ g ml⁻¹), gentamicin sulfate (5 μ g ml⁻¹), kanamycin sulfate (5 μ g ml⁻¹), lincomycin hydrochloride (10 μ g ml⁻¹), neomycin sulfate (10 μ g ml⁻¹), oleandomycin phosphate (2 μ g ml⁻¹), penicillin G (20 μ g ml⁻¹), polymyxin β sulfate (50 μ g ml⁻¹), rifampicin (2 μ g ml⁻¹), streptomycin sulfate (16 μ g ml⁻¹), tobramycin sulfate (8 μ g ml⁻¹) and vancomycin hydrochloride (0.25 μ g ml⁻¹), but not in the presence of kanamycin sulfate (50 μ g ml⁻¹) or tetracycline hydrochloride (5 μ g ml⁻¹). The organism is resistant to bismuth citrate (10 μ g ml⁻¹), crystal violet (1 μ g ml⁻¹), phenol (100 μ g ml⁻¹), phenyl ethanol (4000 μ g ml⁻¹), potassium tellurite (10 μ g ml⁻¹), sodium azide (10 μ g ml⁻¹), sodium chloride (7%, w/v), teepol (100 μ g ml⁻¹), tetrazolium (100 μ g ml⁻¹) and thallos acetate (10 μ g ml⁻¹) but is sensitive to bismuth citrate (100 μ g ml⁻¹), crystal violet (10 μ g ml⁻¹), sodium chloride (10%, w/v), tetrazolium (1000 μ g ml⁻¹) and thallos acetate (100 μ g ml⁻¹). The type strain, K24^T (DSM 44468^T = KCTC 9863^T), exhibits all of the properties cited above. Isolated from sugar cane bagasse and from floor dust of a hemp factory.

ACKNOWLEDGEMENTS

M. G. and Z.-H. Z. are grateful for support received through the Royal Society-Chinese Academy of Sciences Exchange Scheme. This work was also underpinned by BBSRC grant R185/05688/01. All of the authors are indebted to Drs E. Eklund and J. Lacey for the gift of strains.

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