Demetria terragena gen. nov., sp. nov., a New Genus of Actinomycetes Isolated from Compost Soil

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A novel actinomycete was isolated from compost soil and was studied taxonomically and phylogenetically. Cells of this organism were gram positive, not acid fast, nonmotile, nonsporulating, irregular coccoid to short rod shaped, and microaerophilic. The cell wall peptidoglycan contained lysine and was cross-linked via an L-Lys←L-Ser←D-Asp interpeptide bridge. The major menaquinone was MK-8(H₄). The polar lipids were phosphatidylinositol, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, and two unknown phospholipids. Mycolic acids were absent. The cellular fatty acid profile was complex, with large amounts of saturated and monounsaturated straight-chain acids and smaller amounts of iso and anteiso branched-chain acids. The G+C content of the DNA was 66 mol%. Comparative 16S ribosomal DNA studies revealed that strain HKI 0089^T represents a novel lineage within *Actinobacteria* (32) distinct from all previously described genera and most closely related to members of the genera *Kytococcus*, *Dermacoccus*, and *Dermatophilus* of the family *Dermatophilaceae*. On the basis of our results, we suggest that strain HKI 0089 should be classified in a new genus and species, for which we propose the name *Demetria terragena*. The type strain and the only strain of the genus and species is HKI 0089 (DSM 11295).

In a program aimed at isolating rare or novel actinomycetes as a potential resource of bioactive secondary metabolites, isolates were studied by morphological, physiological, and chemotaxonomic methods. It is generally accepted that the results of chemotaxonomic analyses of cellular compounds are extremely useful for delimiting genera within the order *Actinomycetales* (6, 30). In 115 of 900 isolates of actinomycetes originating from 33 soil samples from different geographic regions, lysine was found to be the diagnostic diamino acid in the peptidoglycan. Most of these strains could be identified as members of the genera *Arthrobacter*, *Kocuria*, *Micrococcus*, *Nesterenkonia*, *Oerskovia*, *Promicromonospora*, and *Microbacterium*. We focused our interest on two of these isolates commonly containing lysine and having MK-8(H₄) as the main menaquinone but differing remarkably from each other by morphological, physiological, and further biochemical properties. One of these isolates from a sample of soda soil has been recently described as a member of the new genus and species *Bogoriella caseilytica* (13). In this paper, we describe the taxonomic and phylogenetic characterization of the second isolate, HKI 0089^T, with the combination of the cell wall diamino acid lysine and the menaquinone MK-8 (H₄). On the basis of our results, we suggest that this strain should be assigned to a new genus and species. The name *Demetria terragena* is proposed for this organism, which has been deposited in the German Collection of Microorganisms and Cell Cultures as strain DSM 11295^T. Strain HKI 0089 is the type strain and the only strain of *D. terragena*.

MATERIALS AND METHODS

Bacterial strain and cultural conditions. Strain HKI 0089^T was isolated from a sample of frozen compost soil obtained near Jena, Germany, at a depth of about 10 cm. The organism was isolated by dilution plating on nutrient agar

containing (per liter) 20.0 g of peptone, a pancreatic digest (meat or fish), 5.0 g of NaCl, and 12.0 g of agar. For morphological and chemotaxonomic studies, strain HKI 0089^T was cultivated in liquid or on solidified rich (R) medium (36), peptone-yeast extract-brain heart infusion medium (37), and Bacto tryptic soy broth (Difco Laboratories, Detroit, Mich.).

Morphological and physiological characteristics. Cell morphology was examined by phase-contrast microscopy (Olympus BH-2 microscope; Tokyo, Japan). Motility was studied with a hanging drop. Cell dimensions were measured with an ocular (×10) and objective micrometer (×100/1.25) device. Gram staining was performed according to Hucker's modification (4) with reagents produced by bioMérieux (Marcy l'Etoile, France). Ninety-six percent (wt/vol) ethanol was substituted for the decolorizer of the Gram staining. Acid fastness was determined by Ziehl-Neelsen staining (18). Colony morphology was studied with a stereo microscope (Olympus SZ 11). For scanning electron microscopy, a 48-h-old culture of strain HKI 0089^T from R medium was suspended in a phosphate-buffered salt solution. The cells were fixed with 0.5% glutaraldehyde (pH 7.4), washed several times, and dehydrated in a series of increasing ethanol concentrations. After being sputter coated with gold-palladium, the cells were observed with a scanning electron microscope (JSM 6300 F; JEOL, Tokyo, Japan) at 3 kV at a working distance of 7 mm. Tolerance to NaCl was tested at concentrations of between 2 and 14% (wt/vol) in combination with R medium. The effect of temperature on growth was tested at 28 and 37°C on R medium. Oxygen requirements were studied with Generbag microaer and Generbag anaer incubation systems (bioMérieux) on R agar plates. Acid production from carbohydrates was examined with the oxidation-fermentation (O-F) medium of Hugh and Leifson (15) as recommended by Lanyi (18). Enzyme activities from a 72-h-old culture on solid R medium were examined with API ZYM galleries (bioMérieux). Utilization of organic acids was studied with the medium described by Cowan and Steel (4) by adding the sodium salts of the organic acids to a final concentration of 0.2% (wt/vol). In the case of tartrate, the potassium salt was added. Nitrate reduction; urease activity; indole production; hydrogen sulfide production; hydrolysis of Tween 80, esculin, and gelatin; and the methyl red and Voges-Proskauer reactions were examined as described by Lanyi (18). In the case of nitrate reduction, the reagents NIT 1 and NIT 2 (bioMérieux) were used. Indole production and methyl red and Voges-Proskauer reactions were examined by using Kovacs reagent and reagents VP 1 and VP 2 (bioMérieux), respectively. Catalase production and hydrolysis of casein and potato starch were studied by the methods of Gledhill and Casida (9). Decomposition of adenine, hypoxanthine, xanthine, and tyrosine was studied by the method recommended by Gordon et al. (11). Cytochrome *c* oxidase activity was studied by monitoring the oxidation of *N,N,N',N'*-tetramethyl-phenylene-diamine dihydrochloride on DrySlide oxidase (Difco). Hydrolysis of hippurate was tested on hippurate agar (4). Susceptibility to antibiotics was studied by placement of antibiotic discs (Oxoid) on Bacto nutrient agar plates seeded with suspensions of strain HKI 0089^T.

Cell wall analysis. Purified cell wall preparations were obtained by the method of Schleifer and Kandler (28). The amino acids and peptides in cell wall hydro-

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lysates were analyzed by two-dimensional ascending thin-layer chromatography on cellulose plates with the solvent systems described in reference 28. The amino-terminal amino acid of the interpeptide bridge was determined by dinitrophenylation as described by Schleifer (27). Whole-cell sugars were determined as alditol acetates by gas chromatography and gas chromatography-mass spectrometry as described previously (14). The molar ratios of amino acids were determined by gas chromatography and gas chromatography-mass spectrometry of *N*-heptafluorobutyl amino acid isobutyl esters (20). Analysis of enantiomers of cell wall amino acids was performed by gas chromatography of *N*-pentafluoropropionyl amino acid isopropyl esters (8) on an L-Chirasil-Val column (25 m by 0.25 mm [inside diameter]; Macherey-Nagel, Düren, Germany) with He as the carrier gas at a linear velocity of 200 mm/s and at a temperature programmed from 80°C to 190°C at a rate of 2°C/min. The instruments used for gas chromatography and gas chromatography-mass spectrometry were specified previously (14). The glycolate content of bacterial cell walls was determined by the colorimetric method of Uchida and Aida (35).

Lipid analysis. Cellular fatty acid methyl esters obtained from cells grown in Bacto tryptic soy broth at 28°C by the method described by Stead et al. (33) were separated by gas chromatography (14) and identified as described previously (29). Menaquinones were extracted as described by Collins et al. (3) and were analyzed by high-performance liquid chromatography (14). Polar lipids extracted by the method of Minnikin et al. (24) were identified by two-dimensional thin-layer chromatography and by being sprayed with specific reagents (2). The absence of mycolic acids was demonstrated by thin-layer chromatography (23).

DNA base composition. DNA was isolated, and its G+C content was determined by high-performance liquid chromatography of deoxyribonucleosides as described previously (14).

16S rDNA sequence determination and phylogenetic analysis. Genomic DNA extraction, PCR-mediated amplification of the 16S ribosomal DNA (rDNA), and sequencing of the PCR products were performed as described previously (25). The sequence reactions were electrophoresed with a model 373A automatic DNA sequencer (Applied Biosystems, Foster City, Calif.). The 16S rDNA sequences were manually aligned with the sequences of members of the *Actinobacteria* by using the ae2 editor (21). Evolutionary distances were calculated by the method of Jukes and Cantor (16). Phylogenetic dendrograms were reconstructed with treeing algorithms contained in the PHYLIP package (7).

Nucleotide sequence accession number. The 16S rDNA sequence determined in this study has been deposited in the EBI database under accession no. Y14152. The accession numbers of the reference strains used in the phylogenetic analyses are as follows: *Arthrobacter globiformis* DSM 20124^T, M23411; *B. caseilytica* HKI 0088^T, Y09911; *Brachybacterium faecium* DSM 4810^T, X83810; *Brevibacterium linens* DSM 20425^T, X777451; *Cellulomonas flavigena* DSM 20109^T, X83799; *Clavibacter michiganense* subsp. *michiganense* DSM 46364^T, X77435; *Dermabacter hominis* DSM 7083^T, X91034; *Dermaococcus nishinomiyaensis* DSM 20448^T, X87757; *Dermatophilus congolensis* ATCC 14637^T, M59057; *Jonesia denitrificans* DSM 20603^T, X83811; *Kocuria rosea* DSM 20447^T, X87756; *Kyococcus sedentarius* DSM 20547^T, X87755; *Microbacterium lacticum* DSM 20427^T, X77441; *Micrococcus luteus* ATCC 381, M38242; *Nesterenkonia halobia* DSM 20541^T, X80747; *Renibacterium salmoninarum* ATCC 33209^T, X51601; *Rothia dentocariosa* ATCC 17931^T, M59055; *Sanguibacter keddietii* ST-74^T, X79450; and *Terrabacter tumescens* DSM 20308^T, X83812.

RESULTS

Morphological and cultural characteristics. Strain HKI 0089^T formed circular entire convex smooth colonies that were white to yellow on R medium. Cells were gram-positive, non-acid fast, irregular coccoid to short rod shaped, and varied in size (0.8 by 1.2 to 0.8 by 2.2 µm). They occurred as single cells, pairs, short chains, or irregular clusters (Fig. 1). Scanning electron micrographs revealed the existence of filopodia at the bottom sides of the cells (Fig. 2). We assume that these structures are probably responsible for the adhesion of the cells to the substrate and/or for the communication between neighbouring cells, provided the distance between the cells is less than 1 µm. Further studies are necessary to prove this assumed function and to clarify these structures. Up to now, comparable external cell structures in actinomycetes were only reported for the alkaliphilic species *B. caseilytica* (13).

Strain HKI 0089^T grew well on complex organic media at 28°C under aerobic and microaerophilic conditions. At 37°C and under anaerobic conditions, no growth occurred on R medium. The addition of NaCl to R medium stimulated growth in concentrations of 4 to 6% (wt/vol). Beginning with a concentration of 8% (wt/vol) NaCl, a decrease in growth was

FIG. 1. Micrograph of strain HKI 0089^T grown on solid R medium at 28°C for 48 h. Bar, 10 µm.

observed, and at a concentration of 14% (wt/vol), no growth occurred.

Physiological characteristics. The physiological properties of strain HKI 0089^T are listed in the formal description of the strain at the end of the Discussion section.

Chemotaxonomic characteristics. The peptidoglycan of strain HKI 0089^T contained Lys, Ala, Asp, Glu, and Ser in a molar ratio of 0.8:1.8:1.2:1.0:0.8, respectively. Asp was found to be the amino-terminal amino acid of the interpeptide bridge. The enantiomeric analysis of cell wall amino acids resulted in the detection of L-Lys, D-Ala and L-Ala (molar ratio, 1:1.3), D-Asp, D-Glu, and L-Ser. From these results and from two-dimensional thin-layer chromatographic peptide patterns of partial hydrolysates of cell walls, it was concluded that strain HKI 0089^T represents variation A11.36 (5) of the peptidoglycan type A4α (27) with an L-Lys←L-Ser←D-Asp interpeptide bridge. The acyl type is acetyl. Galactose was found only as whole-cell sugar. The polar lipid pattern consisted of phosphatidylinositol, phosphatidylglycerol, diphosphatidylglycerol,

FIG. 2. Scanning electron micrograph of cells from a 48-h-old culture of strain HKI 0089^T grown at 28°C on solid R medium. Bar, 1 µm.

FIG. 3. 16S rDNA sequence-based phylogenetic dendrogram reconstructed from evolutionary distances by the neighbor-joining method (26). Scale bar, 5 inferred nucleotide substitutions per 100 nucleotides.

phosphatidylethanolamine, and two unknown phospholipids. The complex fatty acid profile was characterized by the occurrence of large amounts of saturated and monounsaturated straight-chain acids as well as smaller amounts of iso and anteiso branched-chain acids. The percentages fatty acid composition of strain HKI 0089^T are as follows: C_{14:0}, 0.5; i-C_{15:0}, 1.9; C_{15:0}, 1.5; i-C_{16:0}, 7.4; C_{16:0}, 9.8; C_{16:1}, 3.4; i-C_{17:0}, 6.2; ai-C_{17:0}, 9.9; C_{17:0}, 13.9; C_{18:0}, 14.2; C_{18:1}, 25.2; t₁₉, 1.8; and C_{19:0}, 2.2. (Note that C_{16:0} is hexadecanoic acid, C_{18:1} is octadecanoic acid, i-C_{15:0} is 13-methyl tetradecanoic acid, ai-C_{17:0} is 14-methyl hexadecanoic acid, and t₁₉ is 10-methoxyl octadecanoic acid.) Mycolic acids were not present. The only menaquinone was MK-8(H₄). The G+C content of the DNA of strain HKI 0089^T was 66 mol%.

16S rDNA sequence analysis. An almost complete 16S rDNA sequence comprising 1,476 nucleotides (>95% of the *Escherichia coli* sequence (1) was determined for strain HKI 0089^T. The phylogenetic dendrogram shown in Fig. 3 was reconstructed from evolutionary distances (16) by the neighbor-joining method (26). A total of 1,315 nucleotides present in all strains between positions 41 and 1452 (*E. coli* positions [1]) was used for this analysis. The phylogenetic dendrogram (Fig. 3) shows that strain HKI 0089^T falls within the radius of the order *Actinomycetales*. Its closest phylogenetic neighbors are the genera of the family *Dermatophilaceae*, i.e., *Kytococcus*, *Dermacoccus*, and *Dermatophilus*. Strain HKI 0089^T shows 16S rDNA sequence similarities of 93.8, 92.8, and 90.8% to the sequences of *D. nishinomiyaensis*, *K. sedentarius*, and *D. congolensis*, respectively, when compared directly with these taxa. The grouping of strain HKI 0089^T within the cluster comprising the family *Dermatophilaceae* is supported by the presence of all

signature nucleotides previously defined for the family *Dermatophilaceae* (32).

DISCUSSION

Within the peptidoglycan types of the actinomycetes, those with lysine as the diagnostic diamino acid are the most numerous (5). It is clear that lysine is mainly associated with coryneform and nocardioform morphologies. In sporoactinomycetes, lysine is found only in the genera *Catenuloplanes* (38) and *Couchioplanes* (34). To date, all known lysine-containing genera of actinomycetes were found to belong to group A peptidoglycan, with the exception of the genus *Microbacterium*, which possesses the group B peptidoglycan (28).

Diamino acids in combination with menaquinone types are extremely useful in differentiating genera of the coryneform bacteria from each other. Menaquinone patterns occurring together with lysine comprise partially saturated and unsaturated menaquinones. The number of isoprene units of unsaturated menaquinones may vary in a range from 6 to 12. The menaquinone type MK-8(H₄) and the occurrence of lysine up to now have only been reported for the actinomycete *B. caseilytica* (13). The menaquinone MK-8(H₄) has been described, however, for G+C-rich bacteria in combination with LL-diaminopimelic acid (A₂pm) (*Aeromicrobium* [29], *Nocardioides* [29], *Terrabacter* [29]) and with meso-A₂pm in the peptidoglycan (*Dermatophilus* [31], *Nocardia* [19], *Pseudonocardia* [19], *Saccharomonospora* [12], and *Saccharopolyspora* [19], *Pseudonocardia* [19], *Saccharomonospora* [12], and *Saccharopolyspora* [19] and the coryneform genera *Janibacter* [22] and *Microsphaera* [22]).

On the basis of the chemotaxonomic characteristics (Table 1), especially because of the rare structure of the peptidoglycan with the L-Lys←L-Ser←D-Asp interpeptide bridge in combination with the menaquinone MK-8(H₄), strain HKI 0089^T can be clearly differentiated from its phylogenetic neighbors (Fig. 3) and from all of the genera mentioned above with lysine or menaquinone type MK-8(H₄). The results of the 16S rDNA sequence comparisons clearly demonstrate the distinct position of strain HKI 0089^T within the radius of the actinomycetes. This is seen in the phylogenetic dendrogram, the range of 16S rDNA sequence similarity values to the closest relatives within the family *Dermatophilaceae*, and the presence of all signature nucleotides as defined for this family. Strain HKI 0089^T differs significantly from the distantly related genus *Dermatophilus* in the G+C content of the DNA, the diagnostic diamino acid of the peptidoglycan, the composition of fatty acids and phospholipids, the lack of diagnostic cell sugars, and the morphological and physiological properties. The closest phylogenetic neighbor of HKI 0089^T is *D. nishinomiyaensis*, which differs from our isolate in the amino acids of the interpeptide bridge, in having menaquinone type MK-8(H₄), and in lacking the characteristic phospholipid phosphatidylethanolamine, as well as in fatty acid composition. Furthermore, differences in morphology and in physiological properties such as the presence of oxidase and urease, the toxic effect of more than 7% (wt/vol) NaCl in the culture medium, the good growth at 37°C, and obvious differences in the utilization of carbon sources justify its separation from HKI 0089^T on the genus level. Pronounced differences in chemotaxonomic and physiological characteristics were found equally between HKI 0089^T and *K. sedentarius*. Therefore, we concluded that on the basis of our results and remarkable differences in taxonomic characteristics between HKI 0089^T and all genera of actinomycetes, this organism should be assigned to a new genus and species, for which the name *Demetria terragena* is proposed.

TABLE 1. Characteristics of the phylogenetic neighbors of HKI 0089^{Ta}

Taxon	Morphology	Peptidoglycan (group)	Major menaquinone(s)	Fatty acids	Polar lipids	Mycolic acids	G+C content (mol%)
HKI 0089 ^T	Coccoid, short rods, nonsporulating	Lys, MCA, DCA (A4 α)	MK-8(H ₄)	S, U, A, I	PI, PG, DPG, PE, PL	—	66
<i>Dermacoccus</i> ^{b,c}	Coccoid, nonsporulating	Lys-MCA, DCA (A4 α)	MK-8(H ₂)	S, A, I	DPG, PG, PI	—	66–71
<i>Kytococcus</i> ^{b,c}	Coccoid, nonsporulating	Lys-DCA (A4 α)	MK-8, MK-9, MK-10	(S), A, I	DPG, PG, PI	—	68–69
<i>Dermatophilus</i> ^{d,e}	Mycelium, motile spores	meso-A ₂ pm (A1 γ)	MK-8(H ₄)	S, U	PG, DPG, PI	ND ^f	57–59

^a MCA, monocarboxylic amino acid; DCA, dicarboxylic amino acid; S, straight-chain saturated; U, monounsaturated; A, anteiso methyl branched; I, iso methyl branched; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, unknown phospholipid(s).

^b Data from reference 10.

^c Data from reference 17.

^d Data from reference 30.

^e Data from reference 31.

^f ND, not determined.

Description of *Demetria* gen. nov. *Demetria* (De.mē.tries Gr. M. n. *Demeter*, Greek female god of agriculture and wives; -ia, M.L. fem., *Demetria*, a bacterium being responsible for fertility). Cells are gram positive; not acid fast; irregular coccoid to short rod shaped (0.8 to 1.2 by 0.8 to 3.0 μ m); and occur singly, in pairs, in short chains, or in small irregular clusters. Nonmotile and nonsporulating. Colonies are circular, convex, entire, smooth, 1 to 3 mm in diameter, and white to pale yellow. Aerobic to microaerophilic. Catalase positive, oxidase negative. The cell wall peptidoglycan contains lysine as the characteristic diamino acid and a dicarboxylic amino acid in the interpeptide bridge. The acyl type is acetyl. Phospholipids are phosphatidylinositol, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, and two unknown phospholipids. Mycolic acids are absent. The cellular fatty acid profile is complex, with saturated and monounsaturated straight-chain acids, and iso and anteiso branched-chain acids. The menaquinone type is MK-8 (H₄). The G+C content of the DNA is 66 mol%. Phylogenetically, this genus is a member of the family *Dermatophilaceae* (32). The type species is *D. terrigena*.

Description of *D. terrigena* sp. nov. *D. terrigena* (ter.ra.ġen.a L. n. terra, soil; L. suffix *gena*, origin; M.L. n. *terrigena*, coming from soil). Cells are gram positive; not acid fast; irregular coccoid to short rod shaped (0.8 to 1.2 by 0.8 to 3.0 μ m); and occur singly, in pairs, in short chains, or in small irregular clusters. Nonmotile and nonsporulating. Colonies are circular, convex, entire, smooth, 1 to 3 mm in diameter, and white to pale yellow. Aerobic to microaerophilic. Catalase positive, oxidase negative. Acids are produced from D-fructose, D-galactose, D-glucose, glycerol, D-mannitol, D-mannose, and D-ribose. No acid production from L-arabinose, D-cellobiose, dextrin, D-glucitol, inulin, lactose, maltose, D-raffinose, L-rhamnose, salicin, sucrose, potato starch, trehalose, and D-xylose. Acetate, aconitate, citrate, malate, and succinate are utilized as carbon sources; benzoate, formate, and DL-tartrate are not utilized. Nitrate is not reduced to nitrite, catalase and hydrogen sulfide are produced, and oxidase and indole are not. Methyl red and Voges-Proskauer reactions are negative. Casein, potato starch, tyrosine, xanthine, and Tween 80 are decomposed; adenine, esculin, gelatin, hippurate, hypoxanthine, and urea are not. NaCl in combination with R medium is well tolerated at concentrations of 2, 4, 6, and 8% (wt/vol). Growth is weak at 10 and 12% NaCl, and there is no growth at 14% NaCl. The optimal growth temperature is 28°C. Cells are susceptible to ampicillin (10 μ g), chloramphenicol (30 μ g), ciprofloxacin (5 μ g), erythromycin (15 μ g), gentamicin (10 μ g), kanamycin (30

μ g), lincomycin (weakly susceptible [2 μ g]), neomycin (30 μ g), nitrofurantoin (300 μ g), oxacillin (weakly susceptible [5 μ g]), oxytetracycline (30 μ g), penicillin G (2 IU), polymyxin B (300 IU), rifampin (2 μ g), and streptomycin (10 μ g). Cells are resistant to sulfonamide (300 μ g). The peptidoglycan is of the A4 α type, with L-Lys←L-Ser←D-Asp in the interpeptide bridge. The fatty acid pattern is complex, with saturated and monounsaturated straight-chain acids and smaller amounts of iso and anteiso branched-chain acids. According to the API ZYM enzyme assay, HKI 0089^T is positive for alkaline phosphatase, chymotrypsin, esterase lipase (C₈), leucine arylamidase, phosphatase acid, naphthol-AS-BI-phosphohydrolase, and α -glucosidase; weakly positive for esterase (C₄), lipase (C₁₄), valine arylamidase, trypsin, and α -mannosidase; and negative for cystine arylamidase, α -galactosidase, β -galactosidase, β -glucuronidase, β -glucosidase, N-acetyl- β -glucosaminidase, and α -fucosidase. The G+C content of the DNA is 66 mol%. The habitat is soil. The type strain is HKI 0089, which has been deposited in the German Collection of Microorganisms and Cell Cultures as strain DSM 11295.

ACKNOWLEDGMENTS

This work was supported by grant 0310591A from the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie, Germany.

We acknowledge Regine Vettermann for critical review of the manuscript. We thank Christiane Weigel and Carmen Schult for excellent technical assistance.

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