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NOTE

Corynebacterium efficiens sp. nov., a glutamic-acid-producing species from soil and vegetables

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Three glutamic-acid-producing coryneform strains were isolated from soil and vegetable samples. Chemotaxonomic investigations indicated that these strains belonged to the genus *Corynebacterium*. Phylogenetic studies, based on 16S rDNA analysis, demonstrated that the three strains formed a distinct cluster within the genus *Corynebacterium* and that their nearest relatives were *Corynebacterium glutamicum* and *Corynebacterium callunae*, also known as glutamic-acid-producing species. The data from 16S rDNA sequence and DNA–DNA relatedness studies clearly indicated that the three isolates represented a new species within the genus *Corynebacterium*. All of the isolates could grow at 45 °C and produced acid from dextrin; these were the most significant characteristics differentiating the three isolates from their neighbours. On the basis of the data presented here, it is proposed that the three glutamic-acid-producing isolates together be classified as *Corynebacterium efficiens* sp. nov., the type strain of which is YS-314^T (= AJ 12310^T = JCM 11189^T = DSM 44549^T).

Keywords: *Corynebacterium efficiens* sp. nov., glutamic-acid-producing corynebacteria

The genus *Corynebacterium* represents a large group of Gram-positive asporogenous, rod-shaped bacteria with a high DNA G+C content (Collins *et al.*, 1986; Liebl, 1992). Based on 16S rDNA analyses, species of the genus *Corynebacterium* have been reported to constitute a monophyletic group among the actinomycete taxa, although there is considerable intrageneric heterogeneity reflecting the diversity of the phenotypic characteristics and habitats of the different species (Pascual *et al.*, 1995). Some non-clinical species of the genus *Corynebacterium*, namely *Corynebacterium glutamicum* (= '*Brevibacterium lactofermentum*') and *Corynebacterium callunae*, are capable of producing large amounts of L-glutamic acid and have been exploited for the commercial production of this acid (Liebl, 1992). Worldwide production of monosodium glutamate (MSG), which is used as an enhancer of taste, exceeded 1 million tonnes in 1996. To prevent the loss of bacterial activity in the industrial production of L-glutamic acid, a cooling system has to be employed to remove the heat generated during bacterial fermentation and growth. Thus, if fermentation at a higher temperature without the loss of microbial activity could be achieved, it would reduce cooling costs and provide an economic advantage in the industrial production of L-glutamic acid. For this reason, we have searched for new glutamic-acid-producing bacteria that have the ability to grow at an elevated temperature. We have succeeded in obtaining three strains which could grow substantially even at 45 °C, a temperature at which previously isolated strains of glutamic-acid-producing corynebacteria could not grow. Based on the phylogenetic and biochemical studies presented here, we conclude that the three new isolates constitute a new species within the genus *Corynebacterium*, for which the name *Corynebacterium efficiens* is proposed.

Three strains, YS-52 (= AJ 12308 = JCM 11187 =

The DDBJ accession numbers for the 16S rRNA gene sequences of isolates YS-314^T, YS-52 and YS-155 are AB055963, AB055964 and AB055965, respectively.

DSM 44547), YS-155 (= AJ 12309 = JCM 11188 = DSM 44548) and YS-314^T (= AJ 12310^T = JCM 11189^T = DSM 44549^T), were originally isolated on CM2 medium [which contained (per litre of distilled water) 10 g yeast extract, 10 g polypeptone, 5 g NaCl and 20 g agar (Difco)] incubated aerobically at 45 °C. Strain YS-52 was isolated from onion bulbs and strains YS-155 and YS-314^T were isolated from soils collected at Kanagawa, Japan. The three strains had been tentatively named '*Corynebacterium thermoaminogenes*' in a Japanese patent application (Yamada & Seto, JP 63-240779, 6 October, 1987). For phenotypic characterization and the collection of biomass, the strains were cultivated aerobically in CM2 broth at 37 °C unless otherwise specified. The abilities of the strains to produce acid from carbohydrates, to assimilate organic acids and to hydrolyse casein and tyrosine were determined according to the methods of Yamada & Komagata (1972); hippurate hydrolysis and phosphatase activity were determined as described by Barksdale *et al.* (1979). For the determination of some biochemical characteristics the API CORYNE system (API bioMérieux) was also employed, according to the manufacturer's instruction. The production of L-glutamic acid by the three strains was investigated under biotin-limiting conditions (Shiio *et al.*, 1962); the amounts of L-glutamic acid produced by the strains were determined using a Bioteck Analyser AS-210 (Asahi Chemical Industry). Amino-acid and sugar analyses of the cell walls of the three strains followed the method described by Harper & Davis (1979); mycolic acids were detected by the method of Minnikin *et al.* (1980). The cellular fatty-acid composition of the strains was determined by GLC (model GC-353B, GL Science, Japan) using a capillary column (TC-70, 0·25 mm I.D. × 30 m; GL Science, Japan). Isoprenoid quinones were extracted from the three strains with acetone and were analysed by TLC and HPLC, according to the method of Hiraishi *et al.* (1984). Phospholipids were extracted and examined by two-dimensional TLC, as described previously (Yamanaka *et al.*, 1988). The 16S-rRNA-encoding gene of each isolate was amplified by PCR from crude cell lysates, as described by Iizuka *et al.* (1998), and the amplified products were cycle-sequenced using a SequiTherm Long-Range Cycle Sequencing Kit (Epicentre Technologies) and detected with a Pharmacia DNA sequencer according to the manufacturers' instruction. The determined sequences were aligned with the sequences of related corynebacterial strains obtained from the DDBJ. CLUSTAL W was used to generate a multiple-sequence alignment (Thompson *et al.*, 1994), and a phylogenetic tree was constructed by the neighbour-joining method. The topology of the tree was confirmed by bootstrap analysis (Felsenstein, 1985) with 1000 replications. Maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Swofford, 1991) methods were also used to evaluate the robustness of the tree constructed using the neighbour-joining method. Genomic DNA was extracted and purified according to the modified method of Saito & Miura (1963). The DNA obtained was further purified using a QIAGEN Genomic-tip Kit. The DNA base composition was determined spectrophotometrically by the method of Yamada & Komagata (1970). DNA–DNA relatedness was measured by the dot-blot hybridization method using a Biodyne A membrane (Gibco-BRL), principally according to the method of Hiraishi *et al.* (1991). Labelling of the DNA and the detection of hybridized DNA were performed by using the AlkPhos Direct system for chemifluorescence (Amersham Pharmacia Biotech), according to the manufacturer's instructions. Quantification of the dots was performed using a FLA 3000 fluoroimage analyser (Fuji Film).

All of the isolates (i.e. YS-52, YS-155 and YS-314^T) were Gram-positive, asporogenous rods that measured 0·8–1·1 μm in width and 1·0–4·5 μm in length (not shown). Some of the cells were arranged in a V formation, due to their snapping division. Neither a rod–coccus cell cycle nor aerial mycelium were found. Cell motility was not observed at any stage of growth. When cultured on nutrient agar plates at 37 °C for 2 days, the isolates grew as yellow, smooth, entire, circular and slightly glistening colonies of up to 2–3 mm in diameter. The isolates were aerobic and facultatively anaerobic chemo-organotrophic thermotolerant bacteria. They showed optimum growth between 30 and 40 °C and showed substantial growth at 15 or 45 °C. No growth was observed above 47 °C. The isolates also produced L-glutamic acid (more than 1·0 g l^{-1}) when they were cultured aerobically at 45 °C and provided with 6 % glucose as a carbon source. The isolates grew well between pH 7 and 10, but not at pH 6·5 or below. They showed good growth in the presence of 10 % NaCl. YS-155 grew slightly in the presence of 15 % glucose, but YS-314^T and YS-52 did not; growth was not observed for any of the isolates in the presence of 30 % glucose. The organic acids acetate, pyruvate and L-lactate were assimilated by all of the strains, whereas D-lactate, succinate, 2-oxoglutarate, citrate, formate, propionate, butyrate, oxalate, glutarate, adipicate, pimelate, glycolate and glyoxylate were not. Malate, fumarate and gluconate were assimilated by strains YS-52 and YS-314^T, but not by strain YS-155. All three isolates produced acid from glucose, fructose, mannose, ribose, maltose and dextrin. None of the isolates produced acid from xylose, mannitol, lactose, salicin, galactose, starch or glycogen. All three isolates showed positive results for catalase activity, pyrazinamidase activity, hippurate hydrolysis and nitrate reduction. They were negative for the activities of pyrrolidonyl arylamidase, alkaline phosphatase, β-galactosidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, tyrosinase and oxidase. Gelatin was not hydrolysed. The production of urease was variable. Only strain YS-155 gave positive results for aesculin hydrolysis and for β-glucuronidase activity.

The cell wall of the isolates contained arabinose and galactose. *meso*-Diaminopimelic acid was the major diamino acid of the cell wall. The major cellular fatty

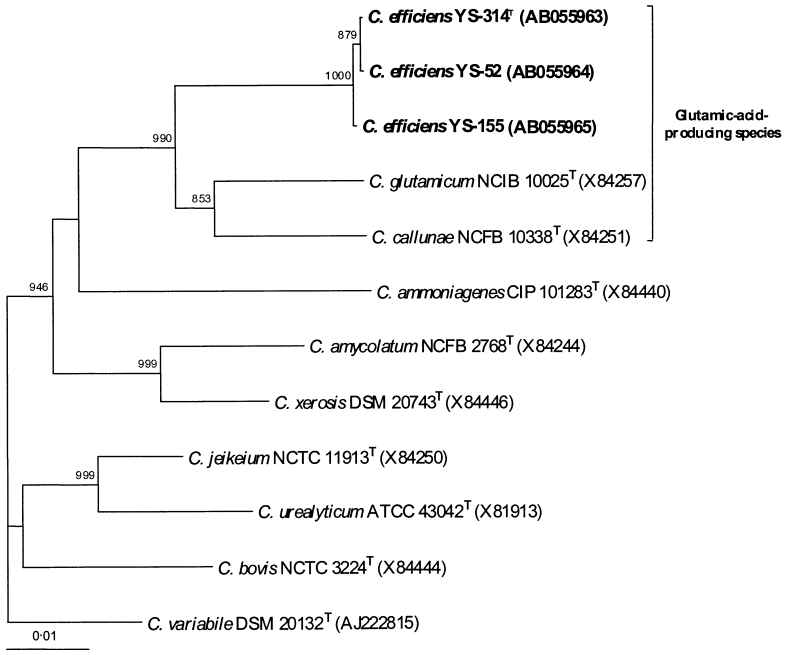

Fig. 1. Unrooted tree, based on 16S rRNA gene sequences, showing the phylogenetic relationship of *Corynebacterium efficiens* sp. nov. with its closest relatives. Bootstrap values (based on 1000 replications) are shown at the branching points. Bar, one nucleotide substitution per 100 nucleotide sites.

acids were hexadecanoic acid ($C_{16:0}$, 39–45 %) and octadecenoic acid ($C_{18:1\omega9}$, 45–48 %); smaller amounts of octadecanoic acid ($C_{18:0}$, 0·8–1·5 %) were also found. Neither tuberculostearic acid nor hydroxy acids were detected. The mycolic acids of the short-chain type were detected. MK-9(H2) was the major menaquinone. Phosphatidylinositol, and its mannoside, was present as the main component of the polar lipids. The DNA base ratios of the strains were 59·0, 60·2 and 59·5 mol % G+C for strains YS-314^T, YS-52 and YS-155, respectively. Within the group of asporogenous, Gram-positive irregular rods with a high G+C content, the genus *Corynebacterium sensu stricto* is chemotaxonomically confined to those species that contain arabinogalactan and *meso*-diaminopimelic acid in the cell wall (chemotype IV), short-chain mycolic acids (22–36 carbon atoms), dihydrogenated menaquinones with eight and/or nine isoprene units, straight-chain saturated and monounsaturated fatty acids and genomic DNA with a G+C content of 51–63 mol % (Collins & Cummins, 1986). The chemotaxonomic characteristics of the new glutamic-acid-producing strains described here coincide with the definition of the genus *Corynebacterium*, indicating that the three isolates are members of this genus.

The almost complete sequences of the 16S rRNA genes from the three isolates were determined (> 1400 nt). Pairwise analyses revealed that strains YS-314^T and YS-52 had exactly the same sequence; only three nucleotides differed between YS-155 and YS-314^T and YS-52. Fig. 1 shows a dendrogram, constructed using the neighbour-joining method, for the 16S rDNA sequences of the three isolates and their closest relatives. Phylogenetically, the three isolates were positioned within the genus *Corynebacterium*, and they were most closely related to the group of 'glutamic-acid-producing species' mentioned by Pascual *et al.* (1995). A similar tree topology was obtained by reconstruction of the phylogeny using the maximum-likelihood and maximum-parsimony methods (data not shown). However, the sequence similarity observed between the new isolates and their closest relative, *C. glutamicum*, was 95·3 % – this is a much lower value than the borderline used for defining bacterial species (i.e. 97 %) as proposed by Stackebrandt & Goebel (1994). Our isolates also showed low DNA–DNA relatedness with their closest relatives (< 20 % hybridization), whereas high hybridization values (> 80 %) were obtained among the three new isolates (Table 1). These results strongly suggest that the three isolates described here represent a genetically distinct species that is most closely related to the 'glutamic-acid-producing species' *C. glutamicum* and *C. callunae*.

The phenotypic characteristics that are useful for distinguishing the three new glutamic-acid-producing isolates from *C. glutamicum* and *C. callunae* are summarized in Table 2. Among these characteristics, the ability to grow at 45 °C is not only useful for the differentiation of these isolates from other *Corynebacterium* spp., but it is also beneficial from an economic point of view, since fermentation using these new isolates would reduce the need for a cooling system in industrial fermenters and could possibly lead to the development of a low-cost alternative for the production of L-glutamic acid.

Based on the phylogenetic, physiological and biochemical findings presented here, we conclude that the three new isolates should be classified as a new species of the genus *Corynebacterium*, for which we propose the name *Corynebacterium efficiens*.

Table 1. DNA–DNA relatedness and 16S rDNA sequence similarity between strains of *Corynebacterium efficiens* and other glutamic-acid-producing bacteria

Species: 1, *C. efficiens* YS-314^T; 2, *C. efficiens* YS-52; 3, *C. efficiens* YS-155; 4, *C. glutamicum* ATCC 13032^T; 5, *C. callunae* ATCC 15991^T.

	1	2	3	4	5
Percentage reassociation with:					
Strain YS-314^T	100	97	82	5	11
C. glutamicum ATCC 13032^T	11	12	20	100	12
16S rDNA similarity with strain YS-314^T (%)	100	100	99·5	95·3	95·2

Table 2. Characteristics that differentiate *Corynebacterium efficiens* from other glutamic-acid-producing *Corynebacterium* spp.

Species: 1, *C. efficiens*; 2, *C. glutamicum*; 3, *C. callunae*.

Characteristics	1	2	3
Acid produced from:			
Salicin	−	−	+
Dextrin	+	−	−
Assimilation of:			
D-Lactic acid	−	+	+
Succinic acid	−	+	+
Nitrate reduced to nitrite	+	+	−
Growth:			
At pH 6	−	+	+
At 45 °C	+	−	−
With 30 % glucose	−	+	+
DNA G+C content (mol %)	59·0–60·2	55·0–57·7	51·0

Description of *Corynebacterium efficiens* sp. nov.

Corynebacterium efficiens (effi.ci′ens. L. part. adj. *efficien*s from efficient or effective).

Cells are Gram-positive, non-motile, non-spore-forming, club-shaped rods that are 0·8–1·1 × 1·0–4·5 μm in size. Colonies on nutrient agar are smooth, entire, circular, dull to slightly glistening and generally yellow. Aerobic and facultatively anaerobic. All strains require biotin for growth. Good growth at 30–40 °C; growth occurs up to 45 °C. No growth occurs when the pH is below 6 or in the presence of 30 % glucose. All strains assimilate acetate, pyruvate and L-lactate, but they do not assimilate D-lactate, succinate, 2-oxoglutarate, citrate, formate, propionate, butyrate, oxalate, glutarate, adipicate, pimelate, glycolate or glyoxylate. Acid is formed from glucose, fructose, mannose, ribose, maltose and dextrin, but not from xylose, mannitol, lactose, salicin, galactose, starch or glycogen. Nitrate is reduced to nitrite. Hydrolysis of urea and aesculin is variable. Catalase and pyrazinamidase are detected, but pyrrolidonyl arylamidase, alkaline phosphatase, β-galactosidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, tyrosinase and oxidase are not detected. Produces large amounts of L-glutamic acid under aerobic conditions. The cell wall contains *meso*-diaminopimelic acid. Mycolic acids are present. MK-9(H2) is the major menaquinone. Major fatty acids are hexadecanoic acid ($C_{16:0}$) and octadecenoic acid ($C_{18:1\omega9}$). G+C content of DNA is 59·0–60·2 mol %. Strains were isolated from soil and vegetables. The type strain of *Corynebacterium efficiens* is YS-314^T (= AJ 12310^T = JCM 11189^T = DSM 44549^T).

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