Erysipelothrix inopinata sp. nov., isolated in the course of sterile filtration of vegetable peptone broth, and description of *Erysipelotrichaceae* fam. nov.

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Based on 16S rRNA gene sequence comparison, an isolate that was detected in sterile-filtered vegetable broth was classified as a novel member of the *Erysipelothrix* line of descent of the *Firmicutes*. Strain MF-EP02^T resembles members of the two species of *Erysipelothrix* with validly published names, *Erysipelothrix rhusiopathiae* and *Erysipelothrix tonsillarum*, in morphology, fatty acid composition, lack of menaquinones in aerobically and anaerobically grown cultures, DNA G+C content and peptidoglycan amino acid composition. Distinct differences in physiological characteristics, however, support the allocation of this isolate to a novel species of the genus *Erysipelothrix*, for which the name *Erysipelothrix inopinata* sp. nov. (type strain, MF-EP02^T = DSM 15511^T = CIP 107935^T) is proposed. Members of the *Erysipelothrix* line of descent are included in the family *Erysipelotrichaceae* fam. nov.

The genus *Erysipelothrix* contains two species with validly published names, *Erysipelothrix rhusiopathiae* (Migula, 1900; Skerman *et al.*, 1980) and *Erysipelothrix tonsillarum* (Takahashi *et al.*, 1987). Whilst the former species is the causative agent of swine erysipelas and a pathogen for other animals and humans (Wood & Shuman, 1975), strains of the latter species are avirulent and have been isolated from the tonsils of apparently healthy pigs. *E. rhusiopathiae* was classified among the regular, non-spore-forming, Gram-positive rods (Jones, 1986) before its phylogenetic position within the radiation of the clostridia as a deeply branching lineage adjacent to members of cluster XVI (*Eubacterium biforme*, *Streptococcus pleomorphus* and *Clostridium innocuum*) was determined (Collins *et al.*, 1994).

Isolation

In the course of validation of production processes in aseptic manufacturing of pharmaceuticals, a vegetable-based growth medium was tested for dilution performance. In the course of preparation of the vegetable CSB medium (peptone vegetable, 20·0 g; (+)-D-glucose, 2·5 g; K_2HPO_4, 2·5 g; water, 1000 ml), the water used for dilution was heated to 80 °C for 1 h and allowed to cool to room temperature. Dehydrated medium was then added to the water and the solution was filtered through a membrane filter (pore width, 0·2 μm). Following incubation of a medium sample at room temperature for 3 days, the medium became turbid. Microscopic analysis and plating in TSA (tryptic soy agar: casein peptone, 15 g; soy peptone, 5 g; NaCl, 5·0 g; agar, 15·0 g; water, 1000 ml; pH 7·3) and TSS (TSA + 5 % sheep blood) media indicated the presence of a single contaminant, strain MF-EP02^T.

Phylogenetic analyses

Sequencing methods and analyses of phylogenetic relatedness followed described procedures (Rainey *et al.*, 1996). The almost-complete sequence (1424 nt) of strain MF-EP02^T was aligned to the ARB database of 16S rRNA gene sequences (Ludwig *et al.*, 2003) and subsequently to the DSMZ database of Gram-positive bacteria. The new isolate showed moderate sequence similarity to members of the genus *Erysipelothrix* (96·4 %) and lower similarity (91 %) to *Holdemania filiformis* (Willems *et al.*, 1997), a species of *Clostridium* cluster XVI as defined by Collins *et al.* (1994). The 16S rRNA gene sequences of *E. rhusiopathiae* strains ATCC 19414^T (= DSM 5055^T) and DSM 5056 were identical and were highly similar to the sequence of *E. tonsillarum* ATCC 43339^T (99·8 % similarity). The gene sequence of strain MF-EP02^T is 99·9 % similar to that of strain Pecs 56

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Erysipelothrix inopinata* MF-EP02^T is AJ550617.

A table showing fatty acid compositions is available as supplementary material in IJSEM Online.

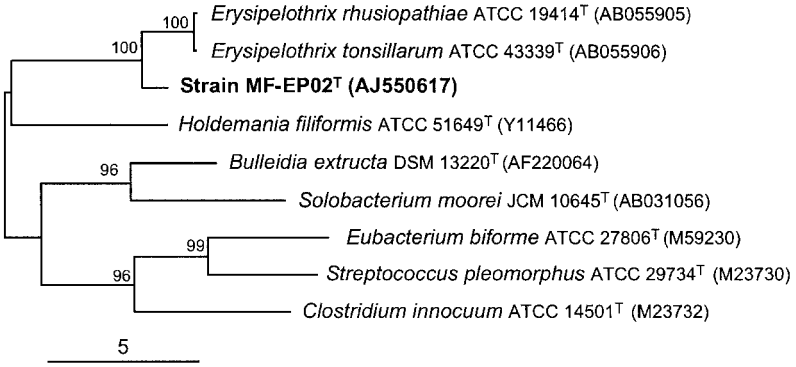

Fig. 1. 16S rRNA gene sequence dendrogram (DeSoete, 1983), displaying the phylogenetic position of strain MF-EP02^T among some phylogenetic neighbours. Numbers indicate percentage of 1000 bootstrap resamplings. GenBank accession numbers of 16S rRNA gene sequences are shown in parentheses. Bar, 5 % sequence divergence.

(AB055907), which is listed as 'unpublished' in GenBank/EMBL. The 16S rRNA gene sequences of *E. rhusiopathiae* strains of serotypes 13 (AB019249) and 18 (AB019250) (Takeshi *et al.*, 1999), which cover only the 3′ half of the molecule (about 790 nt), share 97·5 and 97·8 % similarity, respectively, with the corresponding fragment of strain MF-EP02^T. Similarity values obtained for almost-complete sequences were transformed into phylogenetic distance values that compensated for multiple substitutions at any given site in the sequence (Jukes & Cantor, 1969). A distance matrix dendrogram (DeSoete, 1983), which contains the GenBank accession numbers of reference strains used in the phylogenetic analysis, indicates that the phylogenetic position of strain MF-EP02^T lies adjacent to *Erysipelothrix* species (Fig. 1). Most of the less deeply branching points are supported by bootstrap values of >95 % (Felsenstein, 1993).

Based on moderate sequence similarity of $<97{\cdot}5$ %, the novel isolate can be considered as a taxonomic entity that is separate from the two species of *Erysipelothrix* with validly published names (Stackebrandt & Goebel, 1994).

In silico analysis of the 16S rRNA gene sequence of strain MF-EP02^T for target sites for a primer pair that was designed to identify members of the genus *Erysipelothrix* (Makino *et al.*, 1994) indicates that these primers would also identify strain MF-EP02^T.

RiboPrint analysis

Automated ribotyping was carried out with the RiboPrinter microbial characterization system (Qualicon; DuPont). Sample preparation and analysis were performed according to the manufacturer's instructions; *Eco*RI was used to generate restriction fragments. The RiboPrint pattern of strain MF-EP02^T confirmed the differentiation of this strain from the two *Erysipelothrix* species (Fig. 2).

Chemotaxonomic analyses

In order to circumscribe the novel Gram-positive isolate, chemical properties of taxonomic relevance for members of the *Clostridium* subphylum were analysed. Cells for menaquinone analysis were obtained from biomass that was grown under aerobic and anaerobic conditions on TSB agar + 5 % sheep blood. Isoprenoid quinones were extracted by chloroform/methanol (2 : 1, v/v) from lyophilized cells, purified by preparative TLC on silica gel and analysed by HPLC (Collins *et al.*, 1977; Groth *et al.*, 1996). None of the *Erysipelothrix* strains, including isolate MF-EP02^T, contained significant amounts of menaquinones, no matter what conditions cells were grown under for the preparation of isoprenoid quinones. Traces of MK-7 were detected in cells of *E. tonsillarum* DSM 14972^T following aerobic cultivation. The lack of significant amounts of menaquinones in strains of *E. rhusiopathiae* confirms the results of Collins & Jones (1981).

Fatty acid methyl esters were prepared from 40–80 mg wet cells (Miller, 1982) that were grown on TSBA/blood agar and columbia agar. Extracts of the methanolysates were analysed by the MIDI microbial identification system as described by Sasser (1990). The fatty acid composition of strain MF-EP02^T reveals similarity to those of strains of *Erysipelothrix* species (see Supplementary Table in IJSEM Online). A dendrogram of fatty acid methyl ester relationships is depicted in Fig. 3. The pattern is dominated by $C_{18:1}9cis$ (>30 %), $C_{16:0}$ (>24 %) and $C_{18:0}$ (>10 %) fatty acid methyl esters; quantitative values for strain MF-EP02^T are given in the species description. This pattern differs from that of *H. filiformis* ATCC 51649^T, which contains higher amounts of $C_{18:1}9cis$ (50 %), additional minor components and significant amounts of dimethyl acetal [$C_{18:1}9cis$ (12 %) and $C_{16:0}$ (4 %)] (Willems *et al.*, 1997).

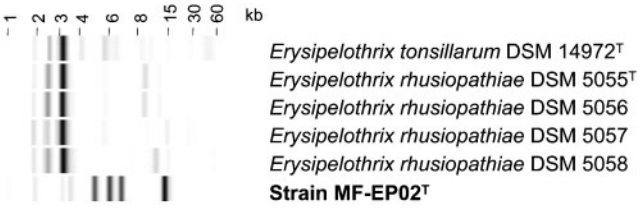

Fig. 2. Diversity of normalized *Eco*RI ribotype patterns found for members of the genus *Erysipelothrix* and strain MF-EP02^T.

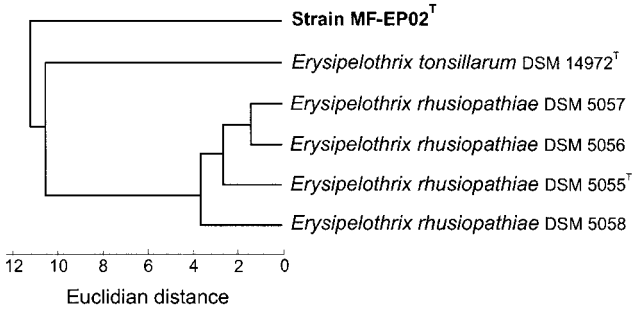

Fig. 3. Dendrogram of relationships based on Euclidian distances between fatty acid methyl ester patterns.

Analysis of the peptidoglycan structure followed the method described by Schleifer & Kandler (1972), modified as described by Willems *et al.* (1997). Two-dimensional TLC of the partial acid hydrolysate of strain MF-EP02^T revealed, besides the presence of lysine, glutamic acid, glycine, serine, alanine, muramic acid and glucosamine, the presence of, besides others, the fragments D-Glu→Gly, L-Ser→D-Glu and L-Lys→L-Lys, whereas aspartic acid or fragments that contained aspartic acid were missing. The quantitative amino acid composition (MacKenzie, 1987) is Ala : Gly : Ser : Glu : Lys = 1·7 : 0·7 : 0·9 : 1·0 : 1·5. It can thus be deduced that the peptidoglycan type is B1δ, with the interpeptide bridge being Gly→L-Lys→L-Lys, which is identical to that reported for *E. rhusiopathiae* (Schubert & Fiedler, 2001) but different from that reported for *H. filiformis* ATCC 51649^T, in which the interpeptide bridge consists of L-Asp→L-Lys.

The DNA G+C content of strain MF-EP02^T was determined by HPLC (Mesbah *et al.*, 1989) to be 37·4 mol%, which corresponds to the range of G+C contents found in members of the genus *Erysipelothrix* (Takahashi *et al.*, 1992).

Physiological properties

Utilization of substrates and enzyme activities of strain MF-EP02^T and strains of *Erysipelothrix* species were done with the Biolog GP and API STREPT microtitre plate panels, respectively. Catalase and oxidase tests followed the description of Smibert & Krieg (1994). Although many test reactions were identical for these strains, isolate MF-EP02^T differed in a significant number of tests from both *Erysipelothrix* species with validly published names, which confirms its phenotypic uniqueness (Table 1).

Taxonomic conclusions

16S rRNA gene sequence analysis indicates a close phylogenetic relationship of strain MF-EP02^T to members of the genus *Erysipelothrix*. Phylogenetic position *per se*, however, is not a reliable indication of genus affiliation, as similarity values change with each novel species that is added to the genus. The hallmark of *Erysipelothrix* is the presence of a type B cell wall, in which the peptide bridge is formed between amino acids at positions 2 and 4 of adjacent peptide side-chains and not, as in the vast majority of bacteria, between amino acids at positions 3 and 4. In order to link the two carboxylic groups of amino acids at positions 2 and 4, the interpeptide bridge of B types must contain at least one diamino acid residue. The B type occurs within the family *Microbacteriaceae*, order *Actinomycetales*, and in some members of the *Clostridium* subphylum, e.g. *Erysipelothrix*, *Holdemania*, *Acetobacterium*, *Clostridium barkeri* and *Eubacterium limosum* (Schleifer & Kandler, 1972; Willems *et al.*, 1997). Strain MF-EP02^T has exactly the same peptidoglycan amino acid composition as that reported previously for *E. rhusiopathiae* and that found for *E. tonsillarum* in this study, which is different from that of the nearest (although remote) neighbour, *Holdemania filiformis* (Willems *et al.*, 1997). Although the type strains of *E. rhusiopathiae* and *E. tonsillarum* (Takahashi *et al.*, 1987) share almost-identical 16S rRNA gene sequences (99·8 % similarity) and RiboPrint patterns (Fig. 2), their DNA–DNA reassociation values are only 18–36 %, which confirms their difference at the physiological level and, hence, their separate species status. Considering the distinct RiboPrint pattern of strain MF-EP02^T and its moderate 16S rRNA gene sequence similarities to type strains of the genus *Erysipelothrix*, we refrained from performing DNA–DNA reassociation studies.

The decision to propose strain MF-EP02^T as a member of a novel *Erysipelothrix* species has been made on the basis of its lack of menaquinone. Chemotaxonomic properties play a decisive role in the delineation of Gram-positive taxa and the absence of isoprenoid quinones under aerobic and anaerobic growth appears to be sufficiently significant to justify the affiliation of strain MF-EP02^T to the genus *Erysipelothrix*. Members of this genus form an individual line of descent within the phylogenetic confines of clostridia and bacilli, for which a novel family, *Erysipelotrichaceae* fam. nov., is proposed. This family, aside from its phylogenetic distinctiveness, is defined predominantly by its unique peptidoglycan type. As the novel family is isolated phylogenetically, a set of signature nucleotides that define the novel family is not indicated, but may be defined once neighbouring members have been described.

Description of *Erysipelotrichaceae* fam. nov.

Erysipelotrichaceae (E.ry.si.pe.lo.tri.cha′ce.ae. N.L. fem. n. *Erysipelothrix* type genus of the family; *-aceae* ending to denote a family; N.L. fem. pl. n. *Erysipelotrichaceae* the *Erysipelothrix* family).

The description is based on the generic description of *Erysipelothrix* (Jones, 1986; data obtained in this study). Straight or slightly curved, slender rods; some strains have a tendency to form long filaments. Non-motile. Endospores are not produced. Menaquinones are absent. Murein belongs to the B-cross-linking type, having L-alanine in position 3 of the peptide subunit and an interpeptide

Table 1. Phenotypic properties that differentiate the novel isolate from *Erysipelothrix* strains, as determined by the API 32 STREPT and Biolog GP microplate panels

Taxa: 1, *E. inopinata* MF-EP02^T; 2, *E. rhusiopathiae* DSM 5055^T; 3, *E. rhusiopathiae* DSM 5056; 4, *E. rhusiopathiae* DSM 5057; 5, *E. rhusiopathiae* DSM 5058; 6, *E. tonsillarum* DSM 14972^T. According to API 32 STREPT, all strains were positive for glycyl tryptophan arylamidase, pyroglutamic acid arylamidase and acid production from glucose. All strains were negative for oxidase, aminopeptidase, hydrolysis of starch, gelatin, DNA and casein, urease, acid from mannitol, sorbitol, raffinose, sucrose, L-arabinose, D-arabitol, cyclodextrin, glycogen, pullulan, maltose, melibiose, melezitose and tagatose, β-glucuronidase, production of acetoin and hydrolysis of hippurate. As determined with Biolog GP, all strains use the following substrates: adenosine, uridine, methyl pyruvate, *N*-acetylglucosamine and α-D-glucose. All strains are negative for methyl β-D-glucoside, D-tagatose, lactamide, alaninamide, D-arabitol, lactulose, methyl α-D-mannoside, D-lactic acid methyl ester, D-alanine, β-cyclodextrin, maltose, palatinose, turanose, L-lactic acid, L-alanine, dextrin, maltotriose, xylitol, D-malic acid, L-asparagine, glycogen, D-mannitol, D-raffinose, L-malic acid, inulin, L-fucose, L-rhamnose, acetic acid, L-glutamic acid, adenosine 5′-monophosphate, mannan, D-melezitose, α-hydroxybutyric acid, monomethyl succinate, glycyl-L-glutamic acid, thymidine 5′-monophosphate, Tween 40, D-galacturonic acid, D-melibiose, β-hydroxybutyric acid, propionic acid, L-pyroglutamic acid, uridine 5′-monophosphate, Tween 60, methyl α-D-galactoside, sedoheptulosan, γ-hydroxybutyric acid, pyruvic acid, L-serine, fructose 6-phosphate, D-gluconic acid, methyl β-D-galactoside, D-sorbitol, *p*-hydroxyphenyl acetic acid, succinamic acid, putrescine, glucose 1-phosphate, stachyose, α-ketoglutaric acid, succinic acid, 2,3-butanediol, glucose 6-phosphate, amygdalin, *m*-inositol, methyl α-D-glucoside, sucrose, α-ketovaleric acid, *N*-acetyl L-glutamic acid and DL-α-glycerol phosphate. +, Positive; −, negative; W, weak.

Characteristic	1	2	3	4	5	6
API STREPT:						
β-Glucosidase	+	−	−	−	−	+
Alkaline phosphatase	−	−	−	−	−	+
Ribose (acid)	W	−	−	−	−	+
Lactose (acid)	−	+	+	+	+	−
Trehalose (acid)	+	−	−	−	−	−
N-Acetyl-β-glucosaminidase	+	+	−	W	+	+
β-Mannosidase	W	−	−	−	−	−
Utilization of (Biolog GP Microplate panel):						
L-Arabinose	−	+	+	+	+	W
N-Acetyl-D-mannosamine	−	+	+	+	+	+
Arbutin	+	−	−	−	−	−
Cellobiose	+	−	−	−	−	−
D-Fructose	−	+	+	+	+	+
D-Galactose	−	+	+	+	+	+
Gentiobiose	+	−	−	−	−	−
α-D-Lactose	−	+	+	+	+	−
D-Mannose	−	+	+	W	+	−
3-Methyl glucose	−	−	−	−	+	−
D-Psicose	−	+	+	+	+	+
D-Ribose	W	−	+	+	+	+
Salicin	+	−	−	−	−	−
D-Trehalose	+	−	−	−	−	−
Xylose	−	−	+	+	+	W
Glycerol	+	−	−	−	−	−

bridge that consists of Gly→L-Lys→L-Lys. $C_{16:0}$, $C_{18:1}$9*cis* and $C_{18:0}$ are predominant fatty acids. Aerobic to facultatively anaerobic. Chemoorganotrophic; metabolism is respiratory and weakly fermentative. Acid, but no gas, is produced from glucose and other carbohydrates. DNA G+C content is 36–40 % (HPLC, T_m, Bd). Some strains are pathogenic for mammals and birds. The 16S rRNA gene-directed primer pair (forward, 5′-TGATGCCATAGAAACTGGTA-3′; reverse, 5′-CTGTATCCGCCATAACTA-3′) specifically amplifies the DNA of members of the genus *Erysipelothrix*. Belongs phylogenetically to the *Firmicutes*. Type genus is *Erysipelothrix* (Migula 1900) Buchanan 1918, 55.

Description of *Erysipelothrix inopinata* sp. nov.

Erysipelothrix inopinata (in.o.pi.na′ta. L. fem. adj. *inopinata* unexpected).

Cells are Gram-positive, catalase- and oxidase-negative, non-motile, non-spore-forming rods, approximately 0·5 μm in width and 1·5–3·0 μm in length. Surface colonies on

BHI (Difco) after 2 days incubation are punctiform to approximately 1·5 mm in diameter, creamy white, undulate, convex, translucent and soft. Growth occurs under aerobic and anaerobic conditions in BHI and columbia blood media, preferably at pH 8. Growth occurs at 20 and 40 °C, but not at 45 °C. The optimal temperature for growth is 25–30 °C. Physiological properties are indicated in Table 1. DNA G+C content is 37·5 mol% (HPLC).

Type strain is MF-EP02^T (=DSM 15511^T=CIP 107935^T). Isolated from vegetative broth used for preparation of growth media.

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