Nonomuraea bangladeshensis sp. nov. and *Nonomuraea coxensis* sp. nov.

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Two novel bacterial strains were isolated from sandy soil from Cox's Bazar, Bangladesh. Strains 5-10-10^T and 5-38-42^T were Gram-positive, aerobic, non-motile actinomycetes that form branched substrate and aerial mycelium. On the basis of 16S rRNA gene sequence similarity studies, the novel strains were shown to belong to the genus *Nonomuraea*, being most closely related to *Nonomuraea fastidiosa*. Chemotaxonomic data supported the assignment of the novel strains as members of the genus *Nonomuraea*. Strain 5-10-10^T contained MK-9(H₄) and strain 5-38-42^T contained MK-9(H₆) and MK-9(H₄) as the major menaquinones. Major polar lipids were phosphatidylethanolamine and hydroxyphosphatidylethanolamine. The major cellular fatty acid for strain 5-10-10^T was iso-C_{16:0} (26.4 %); C_{16:0} (17.4 %) was the major cellular fatty acid for strain 5-38-42^T. The results of DNA–DNA hybridization and physiological tests enabled strains 5-10-10^T and 5-38-42^T to be differentiated genotypically and phenotypically from each other and from the closely related species, *N. fastidiosa*. On the basis of these results, strains 5-10-10^T and 5-38-42^T represent two novel species of the genus *Nonomuraea*. Following an evaluation of morphological, physiological and chemotaxonomic characteristics, 16S rRNA gene sequence comparisons and DNA–DNA hybridization experiments, the new isolates are proposed as two novel species, *Nonomuraea bangladeshensis* sp. nov. [type strain, 5-10-10^T (=MTCC 8089^T = JCM 13930^T)] and *Nonomuraea coxensis* sp. nov. [type strain, 5-38-42^T (=MTCC 8090^T = JCM 13931^T)].

The genus *Nonomuraea* was originally proposed by Zhang *et al.* (1998) as a member of the family *Streptosporangiaceae* which forms extensively branched substrate and aerial mycelia. On the basis of detailed phylogenetic analyses, the genus presently comprises 17 species and two subspecies (Gyobu & Miyadoh, 2001; Quintana *et al.*, 2003; Stackebrandt *et al.*, 2001).

In the course of an investigation into novel actinomycetes present in soil from the southern area of Bangladesh, we isolated strains 5-10-10^T and 5-38-42^T that showed morphological characteristics that are typical of the genus *Nonomuraea*. In this paper, the characterization and classification of these strains is described and two novel species of the genus *Nonomuraea* are proposed.

Strains 5-10-10^T and 5-38-42^T were isolated from sandy soil collected at a sea beach in Cox's Bazar, Bangladesh. The strains were isolated using the standard dilution plate method and grown on humic acid-vitamin agar (Hayakawa & Nonomura, 1987) supplemented with cycloheximide (50 mg l⁻¹), nystatin (50 mg l⁻¹) and nalidixic acid (20 mg l⁻¹). After 21 days of aerobic incubation at 30 °C, the strains were transferred and purified on yeast extract-malt extract agar (medium 2 of the International *Streptomyces* Project; ISP-2). The strains were maintained as working cultures on yeast-starch agar (pH 7.2) containing (l⁻¹), 15.0 g soluble starch, 4.0 g yeast extract, 0.5 g

Abbreviations: DAP, diaminopimelic acid; ISP, International *Streptomyces* Project; OH-PE, hydroxyphosphatidylethanolamine; PE, phosphatidylethanolamine.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 5-10-10^T and 5-38-42^T are AB274966 and AB274967 respectively.

Supplementary tables detailing cultural, physiological and chemotaxonomic characteristics, cellular fatty acid contents, 16S rRNA gene sequence similarities and DNA–DNA relatedness of strains 5-10-10^T and 5-38-42^T and related taxa are available with the online version of this paper.

K₂HPO₄, 0.5 g MgSO₄·7H₂O and 15.0 g agar. *Nonomuraea fastidiosa* JCM 3321^T was used for comparative purposes in this study.

In order to determine cell morphology, strains 5-10-10^T and 5-38-42^T were grown on oatmeal (ISP-3) agar and yeast extract-malt extract (ISP-2) agar media at 30 °C for 21 days and then observed by using light (S-2400; Hitachi) and scanning electron (JSM-5600; JEOL) microscopes. Samples for scanning electron microscopy were prepared as described by Ito *et al.* (1989) and Ara & Kudo (2006).

The phenotypic properties of the two novel strains were examined using the following standard methods. For cultural characterization, the isolates were grown for 21 days at 30 °C on various agar media as described by Waksman (1950, 1961), Shirling & Gottlieb (1966) and Asano & Kawamoto (1986). The *Colour Harmony Manual* (Jacobson *et al.*, 1958) was used to determine the names and designations of colony colours. The temperature range and NaCl tolerance for growth were determined on oatmeal agar. Utilization of carbohydrates as sole carbon sources was tested by using ISP-9 medium as a basal medium according to the modified method of Stevenson (1967). Production of melanoid pigments was examined using tyrosine agar (ISP-7).

For the analysis of chemotaxonomic characteristics, freeze-dried cells were obtained from cultures grown in yeast-glucose or trypticase soy broth on a rotary shaker at 28 °C. The isomers of diaminopimelic acid (DAP) in the cell-wall peptidoglycan were determined by TLC as described by Stanek & Roberts (1974). Reducing sugars from whole-cell hydrolysates were analysed by the paper chromatography method of Becker *et al.* (1965). The *N*-acyl group of the muramic acid in the peptidoglycan was determined according to the method of Uchida & Aida (1984). Phospholipids in cells were extracted and identified according to the method of Minnikin *et al.* (1984). Methyl esters of cellular fatty acids were prepared and analysed by TechnoSuruga Co., Ltd. according to the instructions of the Microbial Identification System (MIDI) using GC (HP6890; Hewlett Packard) (Sasser, 1990). Isoprenoid quinones were extracted according to the method of Collins *et al.* (1977, 1984) and were analysed by HPLC (802-SC; Jasco) using a CAPCELL PAK C18 column (Shiseido) (Tamaoka *et al.*, 1983). Preparation and detection of methyl esters of mycolic acids was carried out as described by Tomiyasu (1982).

The G+C content of the DNA was determined using the HPLC method of Tamaoka & Komagata (1984). An equimolar mixture of nucleotides for analysis of DNA base content (Yamasa Shoyu) was digested by bacterial alkaline phosphatase and used as the quantitative standard.

For DNA–DNA hybridization experiments, chromosomal DNA of strains 5-10-10^T and 5-38-42^T was isolated from biomass. Cells of the novel strains were freeze-dried and ground mechanically for preparation of chromosomal DNA as described by Raeder & Broda (1985). DNA–DNA

relatedness was measured fluorometrically using the microplate hybridization method devised by Ezaki *et al.* (1989). Hybridization was carried out overnight at 52 °C.

Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and sequencing of the PCR products were carried out as described by Nakajima *et al.* (1999) and Ara & Kudo (2006). The sequences were multiple-aligned with selected sequences obtained from the GenBank/EMBL/DBJ databases using the CLUSTAL_X program (Thompson *et al.*, 1997). The alignment was manually verified and adjusted prior to construction of a phylogenetic tree. A phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) in CLUSTAL_X based on a comparison of 1262 nucleotides present in all of the strains as a result of elimination of gaps and ambiguous nucleotides from the sequences between positions 34 and 1491 (position numbering according to *Escherichia coli*). *Thermopolyspora flexuosa* DSM 43186^T was used as an outgroup. The confidence values of the branches of the phylogenetic tree were determined using bootstrap analyses based on 1000 resamplings (Felsenstein, 1985). The values for 16S rRNA gene sequence similarity (%) between *Nonomuraea* strains were calculated after pairwise alignment using CLUSTAL_X.

Analysis of cell morphology revealed that strains 5-10-10^T and 5-38-42^T produced well-developed branched substrate and aerial mycelia and did not fragment into bacillary or coccoid elements. Spiral chains of strain 5-10-10^T (covered by a smooth sheath) were composed of about 8–12 non-motile spores that were borne directly, singly or in clusters, on aerial mycelia (Fig. 1a). For strain 5-38-42^T, spiral to hooked chains composed of 12–17 non-motile spores with a smooth surface were observed (Fig. 1b). A spore sheath was not observed for strain 5-38-42^T.

Strains 5-10-10^T and 5-38-42^T grew well on various inorganic and organic media and produced spore chains; the vegetative and aerial mycelia grew well on the agar media tested (see Supplementary Table S1 in IJSEM Online). The physiological and biochemical properties of strains 5-10-10^T and 5-38-42^T are listed in Table 1 and in Supplementary Table S2 (available in IJSEM Online).

Chemotaxonomic analyses revealed that strains 5-10-10^T and 5-38-42^T exhibited characteristics that are typical for members of the genus *Nonomuraea*, such as the presence of *meso*-DAP and *N*-acetyl muramic acid in the cell-wall peptidoglycan and the presence of madurose, galactose, glucose, mannose and ribose as whole-cell sugars (see Supplementary Tables S3 and S4 in IJSEM Online). The diagnostic phospholipids are phosphatidylethanolamine (PE) and hydroxyphosphatidylethanolamine (OH-PE). The major fatty acids for strain 5-10-10^T are iso-C_{16:0} (26.4 %), iso-C_{15:0} (14.7 %) and 10-methyl C_{17:0} (10.5 %). Small amounts of iso-C_{17:0} (9.9 %), iso-C_{16:1} (7.3 %), 10-methyl C_{16:0} (5.7 %), C_{17:1ω6c} (4.5 %), C_{17:1ω8c} (3.8 %), C_{15:0} (1.8 %), summed feature 3 (C_{16:1ω7c}/iso-C_{15:0} 2-OH) (1.8 %), 10-methyl C_{18:0} (1.6 %), anteiso-C_{17:0} (1.6 %),

Fig. 1. Scanning electron micrographs of spiral spore chains covered with a sheath on aerial mycelia of *Nonomuraea bangladeshensis* sp. nov. 5-10-10^T (a) and spiral spore chains of *Nonomuraea coxensis* sp. nov. 5-38-42^T (b) grown on oatmeal agar at 30 °C for 3 weeks. Bars, 2 µm.

iso-C_{14:0} (1.5 %), C_{15:0} (1.2 %) and C_{18:1ω9c} (1.1 %) are present in strain 5-10-10^T. For strain 5-38-42^T, the major fatty acids are C_{16:0} (17.4 %), C_{17:1ω8c} (11.5 %) and 10-methyl C_{17:0} (8.4 %), with smaller amounts of C_{15:0} (7.5 %), C_{17:0} (6.7 %), summed feature 3 (C_{16:1ω7c}/iso-C_{15:0} 2-OH) (5.8 %), C_{14:0} (4.6 %), C_{18:1ω9c} (4.5 %), 10-methyl C_{16:0} (3.3 %), 10-methyl C_{18:0} (2.5 %), anteiso-C_{17:0} (2.4 %), C_{17:1ω6c} (2.4 %), iso-C_{15:0} (2.2 %), anteiso-C_{15:0} (2.0 %), C_{18:0} (1.9 %), 2-OH C_{16:0} (1.9 %) and C_{16:1ω9c} (1.3 %) (Table 1 and Supplementary Table S4 in IJSEM Online). The major menaquinones observed were MK-9(H₄) for strain 5-10-10^T and MK-9(H₆) and MK-9(H₄) for strain 5-38-42^T.

For the phylogenetic analysis, almost complete 16S rRNA gene sequences were determined for strains 5-10-10^T and 5-38-42^T consisting of 1513 and 1461 nucleotides, respectively. Phylogenetic analysis based on the 16S rRNA gene sequence indicated that strains 5-10-10^T and 5-38-42^T formed a coherent and monophyletic clade with *N. fastidiosa* that was supported by a bootstrap value of 57 % (Fig. 2). Levels of gene sequence similarities between these strains ranged from 96.7 % to 98.0 % (see Supplementary Table S5 in IJSEM Online).

The DNA G + C contents of strains 5-10-10^T and 5-38-42^T were 72.7 mol% and 72.3 mol% respectively. DNA–DNA relatedness value between strains 5-10-10^T and 5-38-42^T was 57.7–58.2 %. DNA–DNA relatedness between the novel strains and the closely related type strain of *N. fastidiosa* ranged from 30.1 to 31.9 % (mean value of four hybridizations) (see Supplementary Table S6 in IJSEM Online).

Strains 5-10-10^T and 5-38-42^T displayed morphological characteristics that are characteristic of other species of the genus *Nonomuraea*. In addition, based on their menaquinones and the predominant fatty acids in whole-cell methyl esters (Gyobu & Miyadoh, 2001; Quintana *et al.*, 2003; Kämpfer *et al.*, 2005), strains 5-10-10^T and 5-38-42^T should be placed in the genus *Nonomuraea*. The quinone system and fatty acid profiles also supported the affiliation of strains 5-10-10^T and 5-38-42^T as members of the genus *Nonomuraea*. Phylogenetic analysis based on 16S rRNA gene sequences showed that strains 5-10-10^T and 5-38-42^T clustered with *N. fastidiosa* in a group under the genus *Nonomuraea*.

Strains 5-10-10^T and 5-38-42^T could be distinguished from each other by morphological and cultural characteristics (see Fig. 1, Table 1 and Supplementary Table S1). Based on their menaquinones and the predominant fatty acids in whole-cell methyl esters (see Table 1 and Supplementary Tables S3 and S4), strains 5-10-10^T and 5-38-42^T represent two separate species in the genus *Nonomuraea*. The two novel isolates appeared to be relatively distantly related to other recognized representatives of the genus *Nonomuraea*. DNA–DNA hybridization is the standard criterion for the delineation of species with members of the same species having values of ≥70 % DNA–DNA relatedness (Wayne *et al.*, 1987). The DNA–DNA relatedness of strains 5-10-10^T and 5-38-42^T to the closely related type strain of *N. fastidiosa* was <70 % (30–32 %) and the relatedness value between strains 5-10-10^T and 5-38-42^T was 58 %.

On the basis of cultural, morphological and physiological characteristics, biochemical reactions, chemotaxonomic characteristics and 16S rRNA gene sequences similarity, it is apparent that strains 5-10-10^T and 5-38-42^T represent separate species. It is proposed that the two isolates represent separate novel species of the genus *Nonomuraea*, *Nonomuraea bangladeshensis* sp. nov. (type strain, 5-10-10^T) and *Nonomuraea coxensis* sp. nov. (type strain, 5-38-42^T). Characteristics that allow the differentiation of the two novel species from *N. fastidiosa* are listed in Table 1.

Description of *Nonomuraea bangladeshensis* sp. nov.

Nonomuraea bangladeshensis (ban.gla.desh.en'sis. N.L. fem. adj. *bangladeshensis* pertaining to Bangladesh, from where the strain was isolated).

Forms a well-developed, branched substrate mycelium. Abundant aerial mycelium is present. Spore chains are spiral with one or two turns (8–12 spores). Sporangia are not

Table 1. Differential physiological, morphological and chemotaxonomic characteristics of strains 5-10-10^T, 5-38-42^T and the phylogenetically related species, *N. fastidiosa*

Strains: 1, strain 5-10-10^T; 2, strain 5-38-42^T; 3, *N. fastidiosa* JCM 3321^T (data are from Kämpfer *et al.*, 2005, Kroppenstedt *et al.*, 1990, Poschner *et al.*, 1985 and Zhang *et al.*, 1998). ND, Not determined; +, positive; –, negative; ±, weakly positive; tr, trace; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; OH-PE, hydroxyphosphatidylethanolamine; GlcNU, phospholipids of unknown structure containing glucosamine. None of the strains produce melanin pigments.

Characteristic	1	2	3
Spore-chain morphology:			
Spore chain	Spiral with one or two turns	Straight to hooked	Spiral with one or five turns
Spore ornamentation	Smooth	Smooth	Irregular
Spore sheath	Present	Absent	ND
Number of spores	8–12	12–17	4–10
Growth on ISP-3 medium:			
Aerial mycelium	Pale brown	Pink to white	White/pink
Substrate mycelium	Pale brown	Orange	Colourless
Polar lipids	PE, OH-PE, GlcNU	PE, OH-PE, GlcNU	DPG, PE, PI (tr), OH-PE, PG
Major menaquinone(s)	MK-9(H ₄)	MK-9(H ₆), MK-9(H ₄)	MK-9(H ₄)
Major fatty acids (%)	iso-C _{16:0} (26.4 %), iso-C _{15:0} (14.7 %), 10-methyl C _{17:0} (10.5 %)	C _{16:0} (17.4 %), C _{17:1ω8c} (11.5 %), 10-methyl C _{17:0} (8.4 %)	iso-C _{16:0} (35.8 %), 10-methyl C _{17:0} (17.6 %), C _{18:1ω9c} (9.5 %)
DNA G + C content (mol%)	72.7	72.3	67.0
Utilization of:			
L-Arabinose	±	±	–
Cellobiose	+	+	–
D-Fructose	±	±	–
Glycerol	±	±	ND
myo-Inositol	±	±	+
Mannitol	+	+	ND
D-Mannose	+	+	–
L-Rhamnose	+	+	–
D-Raffinose	+	±	+
Sucrose	±	+	–
D-Xylose	±	+	+

detected. Spores are spherical to cylindrical and the spore sheath surface is smooth. Good growth occurs on tyrosine agar (ISP-7), yeast extract-malt extract agar (ISP-2), oatmeal agar (ISP-3), Bennett's agar, glucose-yeast extract agar, Hickey–Tresner agar, Waksman agar, oatmeal-nitrate agar and Seino (yeast-starch) agar. Moderate growth is found on glycerol-asparagine agar, inorganic salts-starch agar (ISP-4), nutrient agar and water agar. Poor growth occurs with glucose-asparagine agar and sucrose-nitrate agar. The colour of the substrate mycelium is shell (3ca) to cinnamon brown (3lg) on various agar media. Pale brown aerial mycelium and sporulation occur on ISP-7 agar, ISP-2 agar, ISP-3 agar, water agar and oatmeal-nitrate agar media after 14 days incubation at 28 °C. A soluble pigment is not produced. Aerobic, Gram-positive and able to grow at pH 5.0–9.0. The temperature range for growth is between 15 and 45 °C. No production of melanoid pigments. No growth is observed at 5 % NaCl. The diagnostic diamino acid isomer of the

cell-wall peptidoglycan is *meso*-DAP. Madurose, galactose, glucose, mannose and ribose are present in whole-cell hydrolysates. The *N*-acyl type of the muramic acid is acetyl. Mycolic acids are absent. The polar lipids include PE and OH-PE. Phosphatidylcholine is absent. The major menaquinone is MK-9(H₄), with minor amounts of MK-9(H₆), MK-9(H₂) and MK-9(H₀). Major fatty acids are iso-C_{16:0} (26.4 %), iso-C_{15:0} (14.7 %) and 10-methyl C_{17:0} (10.5 %). The G + C content of the DNA is 72.7 mol%.

The type strain, strain 5-10-10^T (= MTCC 8089^T = JCM 13930^T), was isolated from sandy soil from Cox's Bazar, Bangladesh.

Description of *Nonomuraea coxensis* sp. nov.

Nonomuraea coxensis (cox.en'sis. N.L. fem. adj. *coxensis* pertaining to Cox's Bazar, Bangladesh, the origin of the soil from where the type strain was isolated).

Fig. 2. Neighbour-joining tree (Saitou & Nei, 1987) based on nearly complete 16S rRNA gene sequences showing relationships between *Nonomuraea bangladeshensis* sp. nov. 5-10-10^T, *Nonomuraea coxensis* sp. nov. 5-38-42^T and all recognized species of the genus *Nonomuraea*. *Thermopolyspora flexuosa* was used as an outgroup. Numbers at nodes indicate the levels of bootstrap support (%) based on a neighbour-joining analysis of 1000 resampled datasets; only values above 40% are shown. Bar, 0.01 substitutions per site.

Forms a well-developed, branched substrate mycelium. Abundant aerial mycelium is present. Spore chains are spiral to hooked with 12–17 spores. Sporangia are not detected. Spores are spherical to oval and the spore surface is smooth. Good growth on yeast extract-malt extract agar (ISP-2), oatmeal agar (ISP-3), Bennett's agar, glucose-yeast extract agar, Hickey–Tresner agar, Waksman agar, 1/5 yeast-starch agar, oatmeal-nitrate agar and Seino (yeast-starch) agar. Moderate growth on tyrosine agar (ISP-7), inorganic salts-starch agar (ISP-4) and nutrient agar; poor growth on water agar, glycerol-asparagine agar and glucose-asparagine agar. Substrate mycelium is light melon yellow (4ca) to apricot (orange-brown) (4ga) on various agar media. Pale pink to white aerial mycelium and sporulation occur on ISP-2 agar, ISP-3 agar, Bennett agar's, Hickey–Tresner agar and 1/5 yeast-starch agar media after 14 days incubation at 28 °C. A soluble pigment is not produced. Aerobic. Gram-positive. Growth occurs at pH 5.0–9.0. The temperature range for growth is between 15 and 45 °C. No production of melanoid pigments. No growth on 4% NaCl. The diagnostic diamino acid isomer of the cell-wall peptidoglycan is *meso*-DAP. Madurose, galactose, glucose, mannose and ribose are present in whole-cell hydrolysates. The *N*-acyl type of muramic acid is acetyl. Mycolic acids are absent. The polar lipids include PE and OH-PE. Phosphatidylcholine is absent. The

major menaquinones are MK-9(H₆) and MK-9(H₄). The fatty acid profile is characterized by the presence of significant amounts of C_{16:0} (17.4%), C_{17:1}ω8c (11.5%) and 10-methyl C_{17:0} (8.4%). The G + C content of the DNA is 72.3 mol%.

The type strain, strain 5-38-42^T (= MTCC 8090^T = JCM 13931^T), was isolated from sandy soil from Cox's Bazar, Bangladesh.

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