Study of mural painting isolates, leading to the transfer of '*Bacillus maroccanus*' and '*Bacillus carotarum*' to *Bacillus simplex*, emended description of *Bacillus simplex*, re-examination of the strains previously attributed to '*Bacillus macroides*' and description of *Bacillus muralis* sp. nov.

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A group of 24 strains was isolated from deteriorated mural paintings situated in Spain (necropolis of Carmona) and Germany (church of Greene-Kreiensen). (GTG)₅-PCR genomic fingerprinting was performed on these strains to assess their genomic variability and the strains were delineated into four groups. Representatives were studied by 16S rRNA gene sequencing and were found to be closely related to *Bacillus simplex* and the species '*Bacillus macroides*' (strain NCIMB 8796) and '*Bacillus maroccanus*' (names not validly published) according to a FASTA search. The close similarity between *B. simplex*, '*B. macroides*' NCIMB 8796, '*B. maroccanus*' and the mural painting isolates was confirmed by additional (GTG)₅-PCR, ARDRA, FAME and SDS-PAGE analyses. Furthermore, these techniques revealed that strains of '*Bacillus carotarum*', another name that has not been validly published, also showed high similarity to this group of organisms. On the other hand, it was shown that the strains labelled '*B. macroides*' in different collections do not all belong to the same species. Strain NCIMB 8796 can be allocated to *B. simplex*, while strain DSM 54 (= ATCC 12905) shares the highest 16S rRNA gene sequence similarity with *Bacillus sphaericus* and *Bacillus fusiformis* (both around 98.6%). On the basis of further DNA–DNA hybridization data and the study of phenotypic characteristics, one group of five mural painting strains was attributed to a novel species in the genus *Bacillus*, for which the name *Bacillus muralis* sp. nov. is proposed. Finally, the remaining mural painting strains, one (LMG 18508 = NCIMB 8796) of two strains belonging to '*B. macroides*' and strains belonging to '*B. maroccanus*' and '*B. carotarum*' are allocated to the species *B. simplex* and an emended description of *B. simplex* is given.

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Bacillus muralis* sp. nov. LMG 20238^T is AJ628748.

An ARDRA-based dendrogram and details of DNA–DNA hybridization results are available as supplementary material in IJSEM Online.

INTRODUCTION

Gottheil (1901) described *Bacillus simplex* and believed that '*Bacillus carotarum*' (Koch, 1888) and '*Bacillus cohaerens*' were possibly synonyms. '*Bacillus capri*', '*Bacillus cobayae*' and '*Bacillus muscoli*' were mentioned as other possible synonyms of '*B. carotarum*' by Stapp (1920). Since the

original '*B. carotarum*' strains were lost, Gibson & Gordon (1974) proposed '*B. capri*' strain NRS 607 as the representative of the species. Hunger & Claus (1981) showed that the DNA–DNA relatedness between '*B. carotarum*' NRS 607 and *B. simplex* was around 70 %, the borderline for species delineation. Priest *et al.* (1988) studied 368 strains, including '*B. carotarum*' and *B. simplex*, by 118 phenotypic tests. In the resulting clustering, *B. simplex* and '*B. carotarum*' strains grouped together. Despite this close relatedness and regardless of the DNA–DNA hybridization data of Hunger & Claus (1981), Priest *et al.* (1988) concluded from their study that '*B. carotarum*' and *B. simplex* were distinct taxa and gave a species description of *B. simplex* (later validated; Priest *et al.*, 1989).

'*Bacillus maroccanus*' (Delaporte & Sasson, 1967) was included, together with *B. simplex*, in the phylogenetic studies of Ash *et al.* (1991) and Xu & Côté (2003). In both studies, '*B. maroccanus*' and *B. simplex* clustered closely together. However, to our knowledge no DNA–DNA hybridization studies between these two taxa have been published to date.

'*B. macroides*' was originally described as '*Lineola longa*' (Pringsheim & Robinow, 1947; Pringsheim, 1950), until Bennett & Canale-Parola (1965) demonstrated endospore formation and a positive Gram-stain in this ostensibly Gram-negative, non-sporulating taxon. Two different '*B. macroides*' strains are available: ATCC 12905 (= DSM 54), on which the original description is based, and NCIMB 8796 (= NCDO 1661) of which a 16S rRNA gene sequence is available (GenBank/EMBL accession number X70312). The sequence of the latter shows high similarity to sequences of *B. simplex* and '*B. maroccanus*', while Xu & Côté (2003) recently demonstrated that strain ATCC 12905 shares highest similarity with *Bacillus fusiformis* and *Bacillus sphaericus* based on comparison of 3'-end 16S rRNA gene and 5'-end 16S–23S ITS sequences. Since the phenotypic characters of '*B. macroides*' also conform to those of *B. sphaericus*, except for sporangial morphology (Claus & Berkeley, 1986), it can be concluded that ATCC 12905 is the original '*B. macroides*' strain.

The finding of isolates from deteriorated wall paintings with high 16S rRNA gene sequence similarity to '*B. macroides*' strain NCIMB 8796 (Heyrman & Swings, 2001) and consequently the need for their classification, led in this study to the revision of the taxonomic position of strains previously attributed to '*B. macroides*', '*B. maroccanus*' and '*B. carotarum*' and to the proposal of the novel species *Bacillus muralis* sp. nov. to accommodate five mural painting isolates.

METHODS

Strains and media. The mural painting isolates originate from two severely damaged mural painting sites, the Servilia tomb of the Roman necropolis of Carmona (Spain) and the Lutheran church of Greene-Kreinsen (Germany). The Carmona strains were isolated

from a sample of powdery growth on the painted wall at the left of the main entrance of the tomb, at a height of 1.8 m. The Greene strains originate from sampling of ochre-coloured, round, dry spots taken at the west wall of the church (depicting the Adoration of the Magi). Further isolate designations and methods applied are shown in Table 1. Isolates were maintained in Microbank tubes at -80°C . For phenotypic characterization studies, including microscopy, cultures were maintained on slopes of trypticase soy agar (TSA) containing $5\text{ mg MnSO}_4\cdot\text{H}_2\text{O l}^{-1}$ (to enhance sporulation).

DNA preparation. Total genomic DNA was purified for 16S rRNA gene sequencing, ARDRA and rep-PCR using a slight modification of the method of Pitcher *et al.* (1989), as described by Heyndrickx *et al.* (1996). For determination of the G+C content and DNA–DNA hybridization, approximately 1 g biomass was harvested from agar plates. DNA was purified by a combination of the protocols of Marmur (1961) and Pitcher *et al.* (1989), as described by Logan *et al.* (2000).

16S rRNA gene sequencing and phylogenetic analysis. Sequence analysis was performed as previously described by Heyrman & Swings (2001). For partial sequencing, two primers were used (reverse 358–339 and reverse 536–519; Heyrman & Swings, 2001) to obtain the first 400–500 base pairs of the 16S rRNA gene, which according to Goto *et al.* (2000) includes the hypervariable region for the genus *Bacillus*. A phylogenetic tree was constructed using the BioNumerics 3.0 software (Applied Maths) by applying the neighbour-joining method of Saitou & Nei (1987) on a multiple alignment similarity matrix. The stability of relationships was assessed by a bootstrap analysis of 1000 datasets.

ARDRA. Amplified 16S rRNA genes were obtained by PCR and analysed by restriction digestion with five restriction enzymes (*Hae*III, *Dpn*II, *Rsa*I, *Bfa*I and *Tru*9I) as described previously by Heyndrickx *et al.* (1996). The restriction patterns were analysed as described by Heyndrickx *et al.* (1998).

rep-PCR genomic fingerprinting. PCR was performed with the (GTG)₅ primer (Versalovic *et al.*, 1994) using the PCR conditions described previously by Rademaker & de Bruijn (1997). Electrophoretic conditions for the (GTG)₅-PCR of the mural painting isolates (see Fig. 1a) were those described by Heyrman *et al.* (2003); those for the additional (GTG)₅-PCR (see Fig. 1b) were described by Scheldeman *et al.* (2004). The resulting patterns were digitized and a Pearson correlation of the resulting band patterns was performed using the BioNumerics and the GelCompar software, respectively.

G+C content and percentage DNA–DNA binding. The G+C content of DNA was determined by HPLC (Mesbah *et al.*, 1989) using the further specifications given by Logan *et al.* (2000). DNA–DNA hybridization was performed using a modification of the microplate method of Ezaki *et al.* (1989), as described by Willems *et al.* (2001). A hybridization temperature of 37°C (calculated with correction for the presence of 50 % formamide) was used.

Chemotaxonomic characterization. Gas chromatographic analysis of fatty acid methyl esters was performed starting from strains grown on TSA for 24 h at 28°C . A quantitative analysis of cellular fatty acid compositions was further performed by the gas-liquid chromatographic procedure as previously described (Mergaert *et al.*, 1993). Computer analysis of the resulting profiles was performed as described by Heyrman *et al.* (1999). For SDS-PAGE of whole-cell proteins, cells were obtained as described by Heyndrickx *et al.* (1998). SDS protein extracts were prepared and electrophoresed according to Pot *et al.* (1994), and the data were collected and interpreted as described by Vauterin & Vauterin (1992).

Phenotypic characterization. The strains were phenotypically characterized by the methods of Logan & Berkeley (1984); other

Table 1. Strains studied and the methods used for characterization

Strain	Source/comments	Methods used for characterization						
		FAME	PAGE	API biovar	(GTG) ₅ -1	(GTG) ₅ -2	16S rRNA*	ARDRA
Novel isolates								
LMG 19489 (=R-5266)	Mural painting, Carmona	+	+	1	C	+	F	+
LMG 22046 (=R-5269)	Mural painting, Carmona	+	+	2	C	+	P	
R-5275	Mural painting, Carmona	+		2	C	+		
LMG 22045 (=R-5282)	Mural painting, Carmona	+		2	B	+	P	+
R-5307	Mural painting, Carmona	+	+	1	B	+		
R-8191	Mural painting, Greene	+		1	A			
LMG 20238 ^T (=R-8192 ^T)	Mural painting, Greene	+	+	3	D	+	F	+
R-8193	Mural painting, Greene	+		2	A			
R-8202	Mural painting, Greene	+		2	A			
R-8204	Mural painting, Greene	+		3 atypical	D			
R-8207	Mural painting, Greene	+	+	2	A			
R-8208	Mural painting, Greene	+	+	2	A	+		
R-8210	Mural painting, Greene	+	+	3	D	+		
R-8214	Mural painting, Greene	+		2	A			
LMG 21002 (=R-8216)	Mural painting, Greene	+	+	2	A	+	F	+
R-8218	Mural painting, Greene	+		2	A			
R-8220	Mural painting, Greene	+	+	2	A	+		
R-8225	Mural painting, Greene	+		-	A			
R-8231	Mural painting, Greene	+		2	A			
R-8234	Mural painting, Greene	+	+	2	A	+		
LMG 21004 (=R-8249)	Mural painting, Greene	+	+	3	D	+	P	
R-8251	Mural painting, Greene	+	+	3	D	+		
R-8253	Mural painting, Greene	+		2	A			
R-8254	Mural painting, Greene	+		1	A	+		
<i>B. simplex</i>								
LMG 11160 ^T (=B1879 ^T)	Obtained as DSM 1321 ^T	+	+	1		+	F	+
<i>‘B. maroccanus’</i>								
LMG 12364 (=B0342)	Warm arid soil, Morocco	+	+	2		+	EMBL	+
<i>‘B. macroides’</i>								
LMG 18474 (=B0341=DSM 54=ATCC 12905=1741-1b)		+	+	2		+	F	+
LMG 18508 (=NCIMB 8796 =1745-1b)		+		1			EMBL	+
<i>‘B. carotarum’</i>								
LMG 17633 (=B0079)	Dust, Gibson		+	2 atypical		+	F	+
LMG 17634 (=B0272)	Gibson	+	+	1		+		+
LMG 17636 (=B0976)	Unpasteurized milk, Gibson		+	1		+	F	+
LMG 17637 (=B0993)	=NRS 607, Gibson		+	2		+		
LMG 17638 (=B1044)	Gibson		+	1		+		+
LMG 17639 (=B1050)	Gibson		+	2		+		+
LMG 17640 (=B0081)	Soil, Gibson		+	1		+		+
LMG 17642 (=B0788)	Mucoid, Keates		+	2		+		
LMG 17643 (=B0984)	Gibson		+	1		+		+
LMG 17644 (=B0997)	Gibson			1		+		+
LMG 17646 (=B1373)	Flour, FMBRA		+	2		+		+
Unidentified strains								
<i>Bacillus</i> sp. R-15936 (=B2456)	Clinical isolate	+	+	1		+		+
<i>Bacillus</i> sp. R-15943 (=B2543)	Antarctic soil, Russel	+		1		+		+

*EMBL, Sequence obtained from EMBL database; F, sequenced fully; P, partial sequence.

Fig. 1. (a) Grouping of normalized (GTG)₅ patterns (using the electrophoretic conditions described by Heyrman *et al.*, 2003) of 24 mural painting isolates. (b) Grouping of normalized (GTG)₅ patterns (using the electrophoretic conditions described by Scheldeman *et al.*, 2004) of representative mural painting isolates together with the *B. simplex* type strain, '*B. macroides*' LMG 18474 (=DSM 54=ATCC 12905), '*B. maroccanus*', strains belonging to '*B. carotarum*' and additional isolates R-15936 and R-15943. Both dendrograms are based on a UPGMA clustering of Pearson's correlation similarity coefficients.

characters were determined, and the data numerically analysed, as described by Logan *et al.* (2000). For strains that give very few positive reactions in these phenotypic tests, the inclusion of negative results in similarity calculations tends to overemphasize the similarities of these organisms; data for unreactive strains in this study were therefore analysed by omitting negative matching characters from the calculations. Vegetative cells and sporangia were observed by phase-contrast microscopy. Strains were also examined for catalase and oxidase production and casein and starch hydrolysis using the methods of Gordon *et al.* (1973). Maximum and minimum growth temperatures were determined by incubating 10 ml TSA cultures in a water bath; pH ranges for growth were determined using 10 ml TSA cultures adjusted to pH 5.0, 7.0 and 9.0; both series were examined for turbidity at 24 h intervals. Anaerobic growth was tested for by incubating cultures on TSA plates in a GasPak jar, with aerobically incubated plates as controls. A small number of strains were also tested for assimilation of substrates as sole carbon sources, using the API Biotype 100 kit as described by Heyndrickx *et al.* (1997).

RESULTS AND DISCUSSION

Genomic profiling and 16S rRNA gene sequencing

Firstly, (GTG)₅-PCR fingerprinting was performed on the mural painting isolates to determine their genotypic diversity and to select representative strains for further analyses. The resulting electrophoretic patterns were used to construct a dendrogram based on a Pearson correlation (Fig. 1a). In this dendrogram, four clusters can be distinguished with an internal correlation above 65 %. These clusters of strains are called (GTG)₅-groups A to D. The mural painting strains had clustered according to geographical origin, with the Carmona (Spain) strains divided between (GTG)₅-groups B and C, and the Greene (Germany) strains between groups A and D.

Six representative strains were chosen for partial sequencing (463 bases) of the hypervariable region for *Bacillus* of the

16S rRNA gene (Goto *et al.*, 2000): one of (GTG)₅-groups A and B (LMG 21002 and LMG 22045, respectively) and two of groups C and D (LMG 19489 and LMG 22046; LMG 21004 and LMG 20238^T, respectively). The strains of groups A, B and C, had identical partial sequences, while the two strains of group D were almost identical to each other (one extra base in the sequence of LMG 20238^T). Comparison of LMG 21002 (group A) and LMG 20238^T (group D) revealed four base mismatches; these two strains and LMG 19489 (group C) were chosen for determination of the nearly complete 16S rRNA gene sequence. LMG 21002 showed respective sequence similarities of 99.7 and 99.9 % to LMG 19489 and LMG 20238^T, while the latter two strains had a sequence similarity of 99.5 %. A FASTA search (Pearson & Lipman, 1988) of the analysed sequences showed 99 % sequence similarity or higher for all three to sequences of *B. simplex* DSM 1321^T (AJ439078), '*B. macroides*' NCIMB 8796 (X70312), two sequences (AY030319 and AF157696) attributed to '*B. macroides*' on the basis of comparative analysis with X70312, and some additional unnamed strains (Fig. 2). The 16S rRNA gene sequences of *B. simplex* DSM 1321^T (X60638) and '*B. maroccanus*' NCIMB 10500 (X60626) analysed by Ash *et al.* (1991) could not be retrieved from the FASTA results, because these sequences contain large numbers of unknown bases (61 and 25, respectively). When their unknown bases were discarded, high sequence similarities (>99.5 %) to the sequences deposited as AJ439078 (*B. simplex*) and X70312 ('*B. macroides*') were also demonstrated. Since the sequence AJ439078 for *B. simplex* was only recently deposited in the international sequence databases, several strains were attributed previously to '*B. macroides*' because of their high sequence similarity to strain NCIMB 8796 (X70312). However, '*B. macroides*' NCIMB 8796 differs importantly from ATCC 12905, the strain on which the original description of '*B. macroides*' was based. Xu & Côté (2003) compared the 3'-end 16S rRNA gene and 5'-end 16S-23S

Fig. 2. Unrooted phylogenetic tree derived from the analysis of 16S rRNA gene sequences of mural painting isolates (in bold) together with strains that were attributed to *B. simplex* and the species '*B. macroides*', '*B. maroccanus*' and '*B. carotarum*'.

ITS nucleotide sequences of 40 species of the *Bacillaceae* and showed that '*B. macroides*' ATCC 12905 showed highest similarity to *B. sphaericus* and *B. fusiformis*. In the present study, the nearly complete 16S rRNA gene sequence of strain LMG 18474 (=DSM 54=ATCC 12905) was determined and *B. sphaericus* and *B. fusiformis* were indeed retrieved as the closest related species on the basis of the respective sequence similarities of 98.4 and 98.7%. In the original description, the phenotypic characters of '*B. macroides*' were similar to those of *B. sphaericus*, except for sporangial morphology (Claus & Berkeley, 1986). This led to the conclusion that strain NCIMB 8796 is taxonomically different from '*B. macroides*' as represented by LMG 18474 (=DSM 54=ATCC 12905). In a phylogenetic tree, strain NCIMB 8796 clustered closely with strains belonging to *B. simplex* and strains that were named '*B. maroccanus*'. In this tree, three sequences are present that are all presumed to belong to the *B. simplex* DSM 1321^T, although one of them (D78478) is clearly distinct from the other two. To assess which sequence was erroneously linked with the type strain of this species, we also determined the 16S rRNA gene sequence of *B. simplex* LMG 11160^T (AJ628743). The sequence with accession number D78478 remained separate and was therefore regarded as aberrant; the presence of such sequences in the databases that are erroneously attributed to type strains results in faulty interpretations. No 16S rRNA gene sequences of named '*B. carotarum*' strains were available in the international databases. Since a close relatedness could be expected from the literature between named '*B. carotarum*' strains and *B. simplex* (see Introduction), 16S rRNA gene sequences of LMG 17633 and LMG 17636 were determined. They were identical and showed 99.7% sequence similarity to *B. simplex* (AJ439078 and AJ628743), corresponding to 4 base mismatches, confirming the close relatedness between '*B. carotarum*' and *B. simplex*.

As a result of this sequence analysis that was supported by other data (see Introduction), a separate (GTG)₅-PCR fingerprinting experiment (using different electrophoretic conditions, see Methods) was performed on 14 representative mural painting isolates, the *B. simplex* type strain, strains previously attributed to '*B. macroides*' (strain DSM 54), '*B. maroccanus*' (LMG 12364) and '*B. carotarum*' and two additional isolates, R-15936 and R-15943 (Fig. 1b). Numerical analysis of this second set of (GTG)₅ data revealed that most strains named '*B. carotarum*', the '*B. maroccanus*' strain and the mural painting isolates attributed to (GTG)₅-group A formed a tight cluster with a Pearson correlation above 70%. Strains of (GTG)₅-group B clustered at more than 80% correlation and were joined by clinical isolate R-15943 at 60%. *B. simplex* LMG 11160^T, '*B. carotarum*' LMG 17638, '*B. macroides*' LMG 18474 (=DSM 54) and (GTG)₅-groups C and D clustered separately (<50% relatedness to other profiles).

In addition, the named '*B. carotarum*' strains, '*B. macroides*' strains LMG 18474 and LMG 18508 and '*B. maroccanus*'

were analysed together with *B. simplex* by ARDRA (see Supplementary Fig. A available in IJSEM Online). A cluster at 90% or more pattern similarity was obtained for all '*B. carotarum*' strains, '*B. maroccanus*' LMG 12364 and '*B. macroides*' LMG 18508 (=NCIMB 8796) that was joined by the *B. simplex* type at 88% similarity. The pattern of the named '*B. macroides*' strain LMG 18474 (=DSM 54) was only 70% similar to that of the other strains.

The results above showed that the mural painting strains belonging to (GTG)₅-group A grouped together with '*B. carotarum*' and '*B. maroccanus*'. Furthermore, these strains were also similar to the type strain of *B. simplex*, '*B. macroides*' NCIMB 8796 and the other mural painting strains. However, '*B. macroides*' strains LMG 18474 (=DSM 54=ATCC 12905) and LMG 18508 (=NCIMB 8796) clearly belong to separate taxa. DNA–DNA hybridization experiments were performed to determine the exact taxonomic position of the investigated strains.

DNA–DNA hybridization and DNA base composition

On the basis of rep-PCR fingerprinting, 16S rRNA gene sequencing and ARDRA, strains were selected for DNA–DNA relatedness studies. The results (Supplementary Table A) show that the mural painting strains belonging to (GTG)₅-groups A, B and C, '*B. maroccanus*' and '*B. carotarum*' belong to *B. simplex* (relatedness values >70%), according to the current species definition (Stackebrandt *et al.*, 2002). For '*B. carotarum*', these data are in accordance with those of Hunger & Claus (1981), who performed a DNA–DNA hybridization experiment between NRS 607, proposed as a representative strain of '*B. carotarum*' by Gibson & Gordon (1974), and *B. simplex* NRS 960^T and found a DNA–DNA relatedness value of 71%.

Strain LMG 20238^T, representative of (GTG)₅-group D, showed less than 50% DNA–DNA relatedness to *B. simplex*, '*B. maroccanus*' and the other mural painting representatives, and thus represents a novel genospecies. In the text, these strains, i.e. the (GTG)₅-group D strains, will be referred to as *Bacillus muralis*. As predicted from the previous analyses, '*B. macroides*' strains LMG 18474 and LMG 18508 do not belong to the same species (DNA–DNA relatedness below 10%). Strain LMG 18508 (=NCIMB 8796), of which a nearly complete 16S rRNA gene sequence is available (accession no. X70312), can be attributed to *B. simplex* (mean DNA–DNA relatedness value of 73.4%).

In this study, the G+C content of the *B. simplex* type strain (LMG 11160^T) was in the range of 39.5–41.6 mol%. All tested strains that were attributed to *B. simplex* by DNA–DNA hybridization study have a G+C content that falls within this range (Supplementary Table A). The G+C content of LMG 20238^T (*B. muralis*) is similar to that of *B. simplex* (40.5–41.8 mol%), while the G+C content of '*B. macroides*' LMG 18474 is slightly lower (37.6–38.9 mol%).

Fig. 3. Simplified dendrogram obtained by UPGMA clustering of Euclidean distances calculated between fatty acid profiles of mural painting isolates, additional isolates R-15936 and R-15943, *B. simplex* and named '*B. macroides*', '*B. maroccanus*' and '*B. carotarum*' strains. For *B. simplex* and '*B. macroides*', the MIDI reference profiles were also included.

Chemotaxonomic characteristics

Fatty acid analysis was performed on the mural painting isolates, *B. simplex* LMG 11160^T, '*B. macroides*' strains LMG 18508 (=NCIMB 8796) and LMG 18474 (=DSM 54=ATCC 12905), '*B. maroccanus*' LMG 12364, '*B. carotarum*' LMG 17634 and strains R-15936 (clinical isolate) and R-15943 (Antarctic soil isolate). In Fig. 3 the resulting profiles are grouped together with the MIDI database reference profiles of *B. simplex* and '*B. macroides*'. The outgroup (at a Euclidean distance of about 86 %) is formed by '*B. macroides*' LMG 18474 and the database reference profile of '*B. macroides*', which is in accordance with the earlier statement that strain LMG 18474 (=DSM 54=ATCC 12905) is the original '*B. macroides*' strain, while strain LMG 18508 (=NCIMB 8796) must be allocated to *B. simplex*. The remaining strains form a group that is divided into two subgroups at a Euclidean distance of 95 %. The first subgroup contains the mural painting isolates of (GTG)₅-groups B and D (*B. muralis*) together with profiles of the *B. simplex* type strain and of the atypical '*B. macroides*' strain, LMG 18508 (=NCIMB 8796). The second contains the mural painting isolates of (GTG)₅-groups A and C, two profiles of '*B. maroccanus*', '*B. carotarum*' LMG 17634, R-15936, R-15943 and the MIDI reference profile of *B. simplex*. Except for R-15943, which showed the highest similarity to the database profile of *B. simplex*, the strains of the second subgroup were identified by the TSBA 4.0 MIDI database as *Bacillus megaterium* with similarities ranging from 0.48 to 0.89. Kämpfer (1994) already noted the close similarity of the fatty acid profiles of *B. simplex* and *B. megaterium*. The mural painting isolates that belong to the novel species *B. muralis* form a tight distinct FAME cluster. However, this cluster is embedded in a heterogeneous larger FAME group composed of strains that may all be attributed to *B. simplex* on the basis of genotypic analyses. It is thus not

straightforward to distinguish *B. muralis* from *B. simplex* on the basis of fatty acid analysis, although small differences are clear: in comparison, *B. muralis* strains contain smaller amounts of the fatty acid anteiso-C_{15:0} and higher amounts of iso-C_{15:0} and C_{16:1}ω11c (Table 2).

Numerical analysis of SDS-PAGE patterns of whole-cell proteins (Fig. 4) revealed that, as expected from previous results, the *B. simplex* type strain, '*B. maroccanus*', '*B. carotarum*' and representative mural painting isolates showed very similar patterns (similarity > 90 %). '*B. macroides*' LMG 18474 (=DSM 54=ATCC 12905) showed a distinct pattern, which confirms our earlier findings. Again, the strains of the novel species *B. muralis* group together within the larger cluster of strains that must be attributed to *B. simplex* according to our polyphasic approach.

Phenotypic characterization

The strains listed in Table 1 were investigated by the API 20E and API 50CHB system (bioMérieux). The phenotypic patterns obtained were compared along with the profiles of other *Bacillus* species, using Gower's coefficient (*S_G*) and omitting negative matches. In this grouping (not shown), the strains were divided between three clusters, which will be called biovars 1 to 3 (biovar 1 comprised 14 strains, biovar 2 comprised 21 strains and biovar 3 comprised 5 strains; biovars 2 and 3 each possessed a single atypical strain; Table 1). Biovar 1 strains included the type strain of *B. simplex* (LMG 11160^T) and '*B. macroides*' LMG 18508 (=NCIMB 8796) and they differed from biovar 2 strains by generally weaker acid production from a slightly narrower range of carbohydrates (as listed in the emended description given below). Biovar 1 strains merged at only 70 % *S_G* and this rather heterogeneous cluster encompassed a strain of *Bacillus firmus* (B0823=NRS 854=R-15586). Strains attributed to biovar 2 formed a very loose cluster

Table 2. Comparison of the mean fatty acid profiles of the mural painting isolates and *B. simplex*

B. simplex profiles obtained in this study and the MIDI reference are shown. The data given are mean percentages of total fatty acids ($\pm$ SD if applicable). Only fatty acids accounting for at least 1.0 % of the total fatty acid content are listed. Strains belonging to (GTG)₅-groups A and C are listed together since they have similar profiles (see Fig. 3).

Fatty acid	<i>B. simplex</i>				<i>B. muralis</i> sp. nov.
	Group A + C (17 strains)	Group B (2 strains)	LMG 11160 ^T (3 profiles)	MIDI reference	Group D (5 strains)
iso-C _{14:0}	4.99 $\pm$ 0.65	8.93 $\pm$ 1.23	10.78 $\pm$ 4.34	3.92	8.67 $\pm$ 4.06
C _{14:0}	1.34 $\pm$ 0.12	1.36 $\pm$ 0.08	2.78 $\pm$ 0.24	2.95	4.35 $\pm$ 1.18
iso-C _{15:0}	15.02 $\pm$ 2.37	21.64 $\pm$ 1.22	13.53 $\pm$ 1.18	9.65	22.51 $\pm$ 4.47
anteiso-C _{15:0}	62.25 $\pm$ 2.67	51.76 $\pm$ 2.05	48.46 $\pm$ 4.12	61.01	42.69 $\pm$ 5.72
C _{15:0}	< 1.0	< 1.0	< 1.0	< 1.0	1.21 $\pm$ 0.79
C _{16:1} ω 7c alcohol	2.89 $\pm$ 0.36	2.87 $\pm$ 0.17	3.33 $\pm$ 0.60	1.84	4.11 $\pm$ 1.67
iso-C _{16:0}	1.75 $\pm$ 0.87	4.16 $\pm$ 0.35	4.86 $\pm$ 1.40	2.81	1.61 $\pm$ 1.00
C _{16:1} ω 11c	4.55 $\pm$ 1.34	3.08 $\pm$ 0.32	6.57 $\pm$ 0.74	6.22	10.33 $\pm$ 2.73
C _{16:0}	1.67 $\pm$ 0.49	2.32 $\pm$ 0.21	5.41 $\pm$ 0.62	6.49	2.27 $\pm$ 0.72
Summed feature	1.61 $\pm$ 0.42	< 1.0	< 1.0	< 1.0	< 1.0
iso-C _{17:0}	< 1.0	1.74 $\pm$ 0.16	1.02 $\pm$ 0.16	1.04	< 1.0
anteiso-C _{17:0}	1.88 $\pm$ 0.50	2.14 $\pm$ 0.04	1.94 $\pm$ 0.48	2.70	< 1.0

that merged at only 65 % S_G, with one atypical strain (LMG 17633) outlying at 60 % S_G. This cluster contained ‘*B. maroccanus*’ LMG 12364. The mural painting strains belonging to (GTG)₅-groups A, B and C and the ‘*B. carotarum*’ strains were distributed between both biovars 1 and 2. ‘*B. macroides*’ strain LMG 18474 (=DSM 54=ATCC 12905), which is believed to represent the authentic ‘*B. macroides*’, was unreactive in the API 50CHB and 20E tests. These results again confirm that the two strains received as ‘*B. macroides*’ (the unreactive LMG 18474, and

LMG 18508, which was reactive in the phenotypic tests) do not belong to the same species. When the groupings based on API 50CHB and 20E test patterns were compared with those obtained by other analyses [(GTG)₅, ARDRA, SDS-PAGE, FAME], biovars 1 and 2 were not distinguishable, but appeared scattered among the groups that had been recognized by those other methods.

Strains belonging to biovar 3 showed generally stronger acid production reactions over a wider range of substrates in

Fig. 4. Normalized computer profiles of protein patterns (PAGE) of mural painting isolates, additional isolates R-15936 and R-15943, *B. simplex* and named ‘*B. macroides*’ (strain LMG 18474 =DSM 54 =ATCC 12905), ‘*B. maroccanus*’ and ‘*B. carotarum*’ strains. The group containing strains belonging to the novel species *B. muralis* is presented as a shaded triangle.

comparison with strains of biovars 1 and 2. Biovar 3 contained only strains belonging to (GTG)₅-group D (*B. muralis*), but *B. muralis* strain R-8204 was an outlier of this cluster. When positive and negative reactions were given equal weights in the similarity calculations, *B. muralis* strains LMG 20238^T, LMG 21004, R-8210 and R-8251 merged at 90 % S_G, and this group was joined by R-8204 at 85 %, but strains of *Bacillus shackletonii* and *B. simplex* (LMG 17636 and LMG 17643) also joined at this level. However, when individual API tests are considered, *B. muralis* R-8204 can be differentiated from *B. simplex* strains (including LMG 17636 and LMG 17643) by acid production from D-galactose, lactose, D-mannose and melibiose. In addition, all of the *B. muralis* strains can be separated from *B. simplex* on the basis of the ONPG test and the colour of the diffusible pigment produced on skimmed milk agar (brown for *B. simplex* strains and salmon-orange for *B. muralis* strains), and from

B. shackletonii on the basis of several API tests and some non-API characters (Table 3). The differentiation of *B. simplex* and *B. muralis* from other related *Bacillus* species can also be achieved using the characters shown in Table 3.

To investigate further the differentiation of *B. muralis* from *B. simplex*, two biovar 3 strains (LMG 20238^T and R-8204) and six biovar 1 and 2 strains (LMG 11160^T, LMG 17633, LMG 17636, LMG 17644, LMG 17646 and R-8208) were characterized using the Biotype 100 carbon source assimilation kit (bioMérieux). The patterns of substrate utilization of the tested *B. simplex* biovar 1 and 2 strains were similar, but they were quite distinct from those of the biovar 3 (*B. muralis*) strains. *B. muralis* could be differentiated from *B. simplex* by assimilation of D-galactose, α -lactose, D-mannose, α -D-melibiose and D-turanose, and failure to assimilate L-aspartate, caprylate, fumarate, gentisate, *m*-hydroxybenzoate, L-malate, mucate,

Table 3. Characters that distinguish between *B. simplex* (divided into biovars 1 and 2), *B. muralis* sp. nov. and some phenotypically similar and phylogenetically related *Bacillus* species

Reference species: 1, *B. megaterium*; 2, *B. flexus* (morphological characters, starch and casein hydrolysis data taken from Priest *et al.*, 1988); 3, *B. shackletonii*; 4, *B. firmus* (starch hydrolysis data from Priest *et al.*, 1988); 5, *B. sporothermodurans*; 6, *B. oleronius*. Symbols: +, >85 % positive; +/w, positive or weakly positive; w, weakly positive; v, variable (26–74 % positive); v/w, variable and when positive weak; –, 0–15 % positive. With the exception of microscopic observations, anaerobic growth, starch and casein hydrolysis, all characters were determined using tests in API 20E and 50CHB.

Characteristic	<i>B. simplex</i>		<i>B. muralis</i> (5 strains)	1	2	3	4	5	6
	Bv. 1	Bv. 2							
Chain formation	+	+	+	+	+	–	–	Filaments	–
Sporangia swollen	–	–	w	–	–	w	w	w	w
Anaerobic growth	v	v	w	–	–	+	+	+	–
Pink pigmentation	–	–	+	–	–	–	–	–	–
Starch hydrolysis	+	+	+	+	+	–	+	–	–
Casein hydrolysis	v	v	v	+	+	w	+	–	–
ONPG	–	–	+	+	+	w	–	–	–
Voges–Proskauer	–	–	–	+	–	–	–	w	–
Nitrate reduction	+	+	+	v	–	–	+ /w	–	+
Acid from:									
Amygdalin	–	–	–	+	–	w	v	v	–
L-Arabinose	v/w	+ /w	w	+	–	–	–	–	–
Cellobiose	–	v/w	+	–	–	+	–	v	+ /w
Galactose	–	–	w	+	+	–	–	–	–
Gentiobiose	–	–	–	+	–	–	–	v	–
Glycogen	–	–	–	+	+	–	v	–	–
meso-Inositol	–	v/w	–	+	–	–	–	–	–
Inulin	w	+	–	v	–	–	–	v	–
Lactose	–	–	+ /w	+	+	–	–	–	–
Mannose	–	–	+	v	–	w	v	v	v/w
Melibiose	–	–	w	+	+	–	–	–	–
Raffinose	v/w	w	w	+	+	–	–	–	–
Starch	–	–	–	+	+	–	v	v	v
D-Tagatose	–	–	–	–	–	w	–	v	+
D-Turanose	–	–	–	+	+	–	–	v	–
D-Xylose	–	w	–	+	–	–	v	–	–

D-saccharate and succinate. The authentic '*B. macroides*' strain LMG 18474 (= DSM 54 = ATCC 12905) was unable to grow on most of the carbon sources. This again confirms its distinctness from *B. simplex* and *B. muralis*.

Taxonomy

The possible synonymy of the species '*B. carotarum*' (Koch, 1888) and *B. simplex* (Gottheil, 1901) was already recognized in the original description of the latter species. Several other possible synonyms were also mentioned in the early literature for '*B. carotarum*': '*B. cohaerens*' (Gottheil, 1901), '*B. capri*', '*B. cobayae*' and '*B. musculi*' (Stapp, 1920). Since the original '*B. carotarum*' strains were lost, Gibson & Gordon (1974) proposed '*B. capri*' strain NRS 607 as the representative strain of this species. For some time, '*B. carotarum*' and its synonyms were thought to belong to *B. megaterium* (Smith *et al.*, 1946). *B. megaterium* was considered to consist of two main groups of strains with similar properties as well as some intermediate strains (Gordon *et al.*, 1973). One main group of strains corresponded closely to the original description of *B. megaterium* (de Bary, 1884), while the other included strains of '*B. capri*' (including NRS 607), '*B. cobayae*', *B. simplex* and '*Bacillus teres*'. Later, Hunger & Claus (1981) showed that the two main *B. megaterium* groups differed in G+C content and that the DNA-DNA relatedness between the groups was well below 70%. Within the second group, these authors found DNA-DNA relatedness values of 70% or higher between '*B. capri*' (strain NRS 607), '*B. cobayae*' and '*B. musculi*'. Between *B. simplex* strains and '*B. capri*' values of 67–71% were obtained. '*B. teres*' gave 57 and 64% DNA-DNA relatedness values with '*B. capri*' and '*B. musculi*', respectively. Thus, Hunger & Claus (1981) showed that '*B. carotarum*' (represented by '*B. capri*') could be attributed to a single genus/species together with *B. simplex*, '*B. cobayae*' and '*B. musculi*'. Further, it was shown that this genus/species differed from *B. megaterium*. However, the authors did not propose taxonomic changes in their paper, but rather stated that more DNA pairing studies had to be performed. Priest *et al.* (1988) studied 368 strains, at that time attributed to *Bacillus*, by 118 phenotypic tests. This study included strains named as '*B. carotarum*', *B. simplex* and '*B. teres*'. In the resulting clustering, *B. simplex* and '*B. teres*' grouped together and were closely joined by '*B. carotarum*' strains. Despite this close relatedness and regardless of the DNA-DNA hybridization data of Hunger & Claus (1981), Priest *et al.* (1988) concluded from their study that '*B. carotarum*' and *B. simplex* were distinct taxa and gave a species description of *B. simplex* (including the '*B. teres*' strain). The name *B. simplex* was later validated (Priest *et al.*, 1989).

In the present study '*B. carotarum*' strain NRS 607 (= LMG 17637) was included together with other named '*B. carotarum*' strains and the *B. simplex* type strain. The data led to the conclusion that strains belonging to '*B. carotarum*' and its suggested synonyms '*B. capri*', '*B. cobayae*' and '*B. musculi*' must be allocated to *B. simplex*. In addition, strains

belonging to '*B. maroccanus*' and '*B. macroides*' NCIMB 8796 (= NCDO 1661 = LMG 18508) should be reclassified as *B. simplex*, as could already be suspected from their close phylogenetic relatedness (Ash *et al.*, 1991; Xu & Côté, 2003). Another '*B. macroides*' strain, ATCC 12905 (= DSM 54 = LMG 18474), that can most probably be regarded as the original representative of this species, shares highest 3'-end 16S rRNA gene and 5'-end 16S–23S ITS sequence similarity with *B. fusiformis* and *B. sphaericus* (Xu & Côté, 2003). Whether this '*B. macroides*' representative deserves separate species rank is still open to investigation. Most mural painting isolates investigated in this study, as well as isolates R-15936 and R-15943, were also attributed to *B. simplex*. In addition, a group of five mural painting isolates, recognized on the basis of genotypic and phenotypic analyses, is proposed as the novel species *Bacillus muralis* and a description follows.

Description of *Bacillus muralis* sp. nov.

Bacillus muralis (mu.ra'lis. L. masc. adj. *muralis* pertaining or belonging to walls).

Cells are rod-shaped, 0.7–0.8 µm in diameter, and occur singly, in pairs or chains. They are straight with round or occasionally slightly tapered ends. They are motile when occurring singly but non-motile when in chains. Endospores are ellipsoidal, lie centrally or paracentrally and do not swell the sporangia or swell them only slightly. Gram reaction is variable. Colonies grown for 2 days on nutrient agar at 30 °C are 3–6 mm in diameter, butyrous, pink-pigmented, glossy, with irregular margins, slightly raised and umbonate. Growth under anaerobic conditions on nutrient agar is weak. Optimal growth at 30 °C, weak growth at 20 °C and no growth at 45 °C. Strains grow at pH 9 less profusely than at pH 7 and growth at pH 5 is negative. Casein hydrolysis is variable and a diffusible salmon-pink tint appears in the medium. Starch is hydrolysed. Strains tolerate 7% NaCl, but grow weakly. Oxidase and catalase reactions are positive.

In the API 20E strip, the ONPG reaction is positive. Arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase reactions are negative, citrate is not utilized, hydrogen sulphide and indole are not produced, urease production and the Voges-Proskauer reaction are negative and gelatin is not hydrolysed. Nitrate is reduced to nitrite. In the API 50 CHB gallery, hydrolysis of aesculin is positive and acid is produced (sometimes weakly) without gas from the following carbohydrates: L-arabinose, arbutin, cellobiose, fructose, galactose, N-acetylglucosamine, glucose, glycerol, lactose, maltose, mannitol, mannose, melibiose, raffinose, ribose, trehalose and salicin. Results are variable for acid production from sucrose. Acid is not produced from the following carbohydrates: adonitol, amygdalin, D-arabinose, D- and L-arabitol, dulcitol, erythritol, D- and L-fucose, gentiobiose, gluconate, glycogen, inositol, inulin, 2- and 5-ketogluconate, D-lyxose, melezitose, methyl α-D-glucoside, methyl α-D-mannoside, methyl β-D-xyloside, rhamnose,

starch, sorbitol, sorbose, D-tagatose, D-turanose, D- and L-xylose and xylitol.

The major cellular fatty acids are anteiso- $C_{15:0}$ and iso- $C_{15:0}$, present at about 43 and 23 % of the total fatty acids, respectively. The following fatty acids are present at ≥ 1 %: iso- $C_{14:0}$, $C_{14:0}$, $C_{15:0}$, $C_{16:1\omega7c}$ alcohol, iso- $C_{16:0}$, $C_{16:1\omega11c}$ and $C_{16:0}$. The G + C content is 41.2 mol% for the type strain.

The type strain, strain LMG 20238^T (= DSM 16288^T), was isolated from mural paintings in the Lutheran church of Greene-Kreienzen, Germany. In the characters for which a variable result was obtained, the type strain does not hydrolyse casein and does not produce acid from sucrose. In the Biotype 100 kit, the type strain hydrolyses aesculin and is able to grow on the following substrates as sole carbon sources: *cis*- and *trans*-aconitate, D- and L-alanine, 4-aminobutyrate, 5-aminovalerate, L-arabinose, L-arabitol, D-cellobiose, citrate, β -D-fructose, D-galactose, β -gentiobiose, D-gluconate, D-glucosamine, α -D-glucose, glycerol, L-histidine, 3-hydroxybutyrate, 2-oxoglutarate, DL-lactate, α -lactose, maltose, maltotriose, D-mannitol, D-mannose, α -D-melibiose, methyl α -D-glucopyranoside, *N*-acetyl-D-glucosamine, palatinose, phenylacetate, L-proline, putrescine, D-raffinose, D-ribose, L-serine, sucrose, D-trehalose, L-tryptophan, D-turanose, L-tyrosine and D-xylose.

Emended description of *Bacillus simplex* (ex Meyer and Gottheil 1901) Priest *et al.* 1989

Bacillus simplex (sim'plex. L. masc. adj. *simplex* simple).

Rods are straight, 0.7–0.9 μ m in diameter, round-ended or occasionally slightly tapered and occur in chains and sometimes singly or in pairs. Motile when occurring singly but non-motile when growing in chains. Endospores are ellipsoidal, occasionally spherical, lie centrally, paracentrally or subterminally, and do not obviously swell the sporangia. Gram reaction is variable amongst cells. Colonies, grown for 2 days on nutrient agar at 30 °C, are 3–6 mm in diameter, cream-coloured, glossy, with irregular margins, slightly raised and umbonate. Most strains are strictly aerobic, although some strains may grow weakly on nutrient agar under anaerobic conditions. They grow at 20 and 30 °C but are not able to grow at 45 °C. Strains grow at pH 9 as profusely as at pH 7 and growth at pH 5 is variable. Casein hydrolysis is variable and a brown tint, of varying strength, diffuses into the medium. Starch is hydrolysed. Tolerance to 5 % NaCl (w/v) is variable and no growth occurs in media supplemented with 7 % NaCl (w/v). Oxidase reaction is negative and catalase is positive. In the API 20E strip, the ONPG, arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase reactions are negative, citrate is not utilized, hydrogen sulphide and indole are not produced, urease production and the Voges–Proskauer reaction are negative and gelatin hydrolysis is variable. Nitrate is reduced to nitrite. In the API 50 CHB gallery, hydrolysis of aesculin is variable and weak. For all strains tested, acid is

produced, mostly weakly, without gas from the following carbohydrates: fructose, *N*-acetylglucosamine, glucose, inulin, trehalose and sucrose. Strains produce acid weakly and variably from salicin. Acid production is always negative from adonitol, amygdalin, D-arabinose, D- and L-arabitol, arbutin, dulcitol, erythritol, D- and L-fucose, galactose, gentiobiose, gluconate, 2- and 5-keto-D-gluconate, glycogen, lactose, D-lyxose, mannose, melezitose, melibiose, methyl α -D-glucoside, methyl α -D-mannoside, methyl β -D-xyloside, rhamnose, starch, sorbose, D-tagatose, D-turanose, L-xylose and xylitol. On the basis of reaction patterns for other substrates, two biovars may be recognized: strains belonging to biovar 1 produce acid weakly and variably from L-arabinose, mannitol, raffinose, ribose and sorbitol, while acid production is always negative from cellobiose, glycerol, inositol, maltose and D-xylose; strains of biovar 2 produce acid, weakly, without gas from the following carbohydrates: L-arabinose, mannitol, raffinose, ribose, sorbitol and D-xylose; biovar 2 strains are variable for weak acid production from cellobiose, glycerol, inositol and maltose.

The major cellular fatty acids are anteiso- $C_{15:0}$ and iso- $C_{15:0}$, present (means $\pm$ SD) at 59.03 ± 5.88 and 15.55 ± 2.95 % of total fatty acids, respectively. The following fatty acids are present (mean values) at ≥ 1 %: iso- $C_{14:0}$ (5.94 ± 2.82 %), $C_{14:0}$ (1.56 ± 0.59 %), $C_{16:1\omega7c}$ alcohol (2.97 ± 0.74 %), iso- $C_{16:0}$ (2.26 ± 1.42 %), $C_{16:1\omega11c}$ (4.80 ± 1.30 %), $C_{16:0}$ (2.36 ± 1.58 %), iso- $C_{17:1}$ I and/or anteiso- $C_{17:1}$ B (1.12 ± 0.82 %), and anteiso- $C_{17:0}$ (1.82 ± 0.80 %). The G + C content is in the range of 39.5–41.8 mol%.

The type strain is strain NRS 960^T (= ATCC 49097^T = DSM 1321^T = LMG 11160^T). For the variable characters in the API kits, the results for the type strain are as follows: weak or moderate acid production for L-arabinose, mannitol, ribose and sorbitol. No acid is produced from raffinose. In the Biotype 100 kit, the type strain hydrolyses aesculin very weakly and is able to grow on the following substrates as sole carbon sources: *cis*- and *trans*-aconitate, D- and L-alanine, 4-aminobutyrate, 5-aminovalerate, L-aspartate, benzoate, caprylate, citrate, *m*-coumarate, ethanolamine, β -D-fructose, fumarate, gentisate, D-gluconate, D-glucosamine, α -D-glucose, D-glucuronate, L-glutamate, glutarate, DL-glycerate, glycerol, L-histidine, *m*-hydroxybenzoate, 3-hydroxybutyrate, *myo*-inositol, 2-keto-D-gluconate, DL-lactate, L-malate, maltose, maltotriose, D-mannitol, methyl α -galactopyranoside, mucate, *N*-acetyl-D-glucosamine, palatinose, phenylacetate, 3-phenylpropionate, L-proline, propionate, protocatechuete, putrescine, quinate, α -L-rhamnose, D-ribose, D-saccharate, L-serine, D-sorbitol, succinate, L-tartrate, D-trehalose, tricarballylate, L-tryptophan, L-tyrosine and D-xylose.

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