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Characterization of a Wild, Novel Nisin A-Producing *Lactococcus* Strain with an *L. lactis* subsp. *cremoris* Genotype and an *L. lactis* subsp. *lactis* Phenotype, Isolated from Greek Raw Milk

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Several molecular taxonomic studies have revealed that many natural (wild) *Lactococcus lactis* strains of dairy origin which are phenotypically representative of the *L. lactis* subspecies *lactis* cluster genotypically within subspecies *cremoris* and *vice versa*. Recently, we isolated two wild nisin-producing (Nis$^+$) *L. lactis* strains, M78 and M104, of the *lactis* phenotype from Greek raw milk (J. Samelis, A. Lianou, A. Kakouri, C. Delbès, I. Rogelj, B. B. Matijašic, and M. C. Montel, J. Food Prot. 72:783–790, 2009); strain M78 possess a novel nisin A sequence (GenBank accession number HM219853). In this study, the actual subspecies identity of M78 and M104 isolates was elucidated, using 16S rRNA and *acmA* (encoding lactococcal *N*-acetylmuramidase) gene and histidine biosynthesis operon polymorphisms and 16S rRNA and *ldh* (encoding lactate dehydrogenase) gene phylogenies. Except the *acmA* gene analysis, molecular tools revealed that isolates M78 and M104 clustered with strains of the *cremoris* genotype, including the LMG 6897^T strain, while they were distant from strains of the *lactis* genotype, including the LMG 6890^T strain. The two wild isolates had identical repetitive sequence-based PCR (rep-PCR), randomly amplified polymorphic DNA (RAPD), plasmid, and whole-cell protein profiles and shared high 16S rRNA (99.9%) and *ldh* (100%) gene sequence homologies. In contrast, they exhibited identical sugar fermentation and enzymatic patterns which were similar to those of the subspecies *lactis* LMG 6890^T strain. To our knowledge, this is the first complete identification report on a wild *L. lactis* subsp. *cremoris* genotype of the *lactis* phenotype which is capable of nisin A production and, thus, has strong potential for use as a novel dairy starter and/or protective culture.

Lactococcus lactis is the most important and most extensively studied lactococcal species of high technological and safety significance for the dairy industry (1, 2). It currently includes three subspecies (*L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, and *L. lactis* subsp. *hordniae*) plus a diacetyl-forming biovariety (*L. lactis* subsp. *lactis* biovar diacetylactis) (2, 3). Natural (wild) *L. lactis* strains are naturally present in raw cow, ewe, or goat milk (4, 5, 6) and commonly found in artisanal cheeses and other traditional dairy foods, where they often dominate over the microbiota of other lactic acid bacteria (LAB) (7, 8, 9, 10, 11). Therefore, *L. lactis* subsp. *lactis* and, to a lesser extent, *L. lactis* subsp. *cremoris* have long been extensively used in starter cultures for milk fermentation (cheeses, sour cream, and butter) composed of single or multiple lactose-fermenting strains with or without other LAB (1, 12, 13). Wild antipathogenic *L. lactis* strains are also naturally present or have been applied as bioprotective/costarter cultures in cheese and other foods to produce bacteriocins *in situ*, with nisin being the commonest and best characterized (14, 15, 16, 17). Nisin has been permitted to be used and applied as a natural generally recognized as safe (GRAS) preservative in many countries (17, 18, 19). In general, dairy lactococci, and *L. lactis* in particular, are considered safe for human health and consumption, and thus, their multiple applications in foods as well as probiotics are of continuously increasing scientific and industrial interest (2).

Based on classical phenotypic and biochemical criteria used in early identification keys, the important dairy subspecies of *L. lactis*, *lactis* and *cremoris*, are distinct and well differentiated from each other, including their type strains (20, 21). An early DNA homology study (22), plus several molecular studies in later years, have indicated, however, that these two subspecies are rather intermixed phylogenetically (5, 12, 23, 24). In particular, many wild *L. lactis* isolates from raw milk and cheese which are well identifiable as *L. lactis* subsp. *lactis* phenotypically actually cluster within subspecies *cremoris* genotypically (3, 4, 22) and *vice versa* (12, 25). Moreover, while the subspecies *cremoris* phenotype may have evolved in association with dairy-related practices and thus occurs in milk habitats only (12, 25, 26), wild *cremoris* genotypes having biochemical traits and capabilities similar to those of the subspecies *lactis* phenotype are frequent in dairy but also in nondairy niches (25, 27). This makes differentiation between the two subspecies problematic and often unreliable if based on phenotypic or genotypic criteria at the species level only (23, 28). Consequently, several, mainly molecular, approaches have been proposed and applied since early 1990s as specific taxonomic tools for analysis of the high heterogeneity of *L. lactis* intraspecies (23, 24, 28, 29, 30, 31, 32, 33, 34). A very recent polyphasic taxonomic study (3) confirmed the aforementioned diversity of wild dairy lactococci and further suggested that the *lactis* and *cremoris* genotypes of phenotypic *L. lactis* subsp. *lactis* may actually represent new true subspecies.

In accordance with those findings, in 2009, in the course of a research project on traditional European foods (www.truefood.eu),

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we reported the isolation from Greek raw milk of two wild *L. lactis* isolates, M78 and M104, typical of the subspecies *lactis* phenotype, which were of great interest due to their strong ability to produce nisin in synthetic media but also in milk and cheese; both isolates were genotypically confirmed to belong to *L. lactis* and to be genotypically distant from *L. garvieae* (35), whereas these first molecular results were not able to discriminate them on the subspecies level. Since then, the expression of nisin genes in *L. lactis* M78 in a cheese-like medium has been quantified by quantitative real-time PCR (qRT-PCR) (36), and its novel nisin A sequence has been published (GenBank accession number HM219853) and compared to other nisin sequences (37). *L. lactis* M104, which showed nearly the same phenotype as *L. lactis* M78 (35), has also been evaluated as a costarter and/or protective adjunct in pilot traditional Greek cheese trials (38, 39).

Reports of numerous food (including dairy) ecology studies on wild *L. lactis* strains which possess nisin-encoding genes, either singly or in copresence with lacticin 481, or on nisin characterization by chemical methods that have been published so far have identified the producer strains as *L. lactis* subsp. *lactis* (15, 16, 17, 40, 41, 42, 43). To the best of our knowledge, molecular identification studies specifically on wild nisin-producing (Nis^+) *L. lactis* subsp. *cremoris* strains do not exist. All Nis^+ strains of the *cremoris* genotype that have been reported so far are either genetically modified for this trait (14) or are natural (wild) isolates which, however, produce nisin Z (44), with the possible exception of one Italian strain claimed to produce nisin A in a very recent technological study by Dal Bello et al. (45). However, to claim detection of such a novel wild $NisA^+$ *L. lactis* subsp. *cremoris* strain, a complete molecular identification study of the producer strain(s) would be necessary.

The present study was therefore undertaken to elucidate the subspecies identity of our wild *L. lactis* isolates M78 and M104, both possessing strong nisin-mediated antipathogenic activities. Identification was based on size polymorphisms of 16S rRNA (28), *acmA* (encoding lactococcal *N*-acetylmuramidase) (31, 34), and *ldh* (encoding lactate dehydrogenase) (33) genes and of a histidine biosynthesis operon (24, 32) previously reported in the literature to be effective for this purpose and on 16S rRNA and *ldh* gene phylogenies and was also accompanied by repetitive sequence-based PCR (rep-PCR) (46) plus randomly amplified polymorphic DNA (RAPD) (47), plasmid DNA, whole-cell protein (29), and phenotypic profiling of the strains. Based on the results, this report is the first complete identification study on natural (wild) strains of *L. lactis* subsp. *cremoris* which are capable of nisin A production.

TABLE 1 *Lactococcus lactis* strains used in this study

Strain	Comment(s) (reference)
L. lactis M78	Strain under investigation; isolated from Greek raw milk (35)
L. lactis M104	Strain under investigation; isolated from Greek raw milk (35)
L. lactis subsp. *lactis* LMG 6890^T	Type strain
L. lactis subsp. *cremoris* LMG 6897^T	Type strain
NCFB = NCDO 1402, *L. lactis* subsp. *lactis*	Nisin-producing (Nis^+); nisin resistant; lactose and sucrose positive; proteinase negative; was among the Nis^+ strains evaluated as starters in Cheddar cheese manufacture during the early 1990s (16); kindly provided by the Leatherhead Food Research Association (LFRA; Surrey, United Kingdom) microbiology laboratory for use as a bacteriocin-positive (Nis^+) control strain indicator in our previous sakacin B studies (48)
F1 *L. lactis* subsp. *lactis*	Nis^+, proteinase negative; sucrose-positive but lactose-negative strain, like NCFB 912, NIZO R5L0, and a few other natural Nis^+ *L. lactis* subsp. *lactis* strains (15); recovered as a "contaminant" from a stock culture of NCFB 1402; thus, F1 is either a lactose-negative variant of NCFB 1402 or a strain like NCFB 912 since exptl Cheddar cheese trials with mixed Nis^+ *L. lactis/cremoris* starter strains, such as those performed in the United States (14), were conducted also at the LFRA
CNRZ 125 *L. lactis* subsp. *lactis*	Strain of dairy origin provided by INRA, Jouy-en Josas, France
CNRZ 258 *L. lactis* subsp. *lactis*	Strain of dairy origin provided by INRA, Jouy-en Josas, France
CNRZ 301 *L. lactis* subsp. *lactis*	Strain of dairy origin provided by INRA, Jouy-en Josas, France
CNRZ 1075 *L. lactis* subsp. *lactis*	Strain of dairy origin provided by INRA, Jouy-en Josas, France
1L8 *L. lactis* subsp. *cremoris*	Raw cow milk isolate provided by INRA, Poligny, France

MATERIALS AND METHODS

Lactococcal strains and culture conditions. Strains of *L. lactis* used in this study are presented in Table 1. All lactococcal strains were maintained at −30°C in MRS broth (LAB M, Lancashire, United Kingdom) or M-17 broth (Merck, Darmstadt, Germany) with 20% glycerol added (Merck). Stock cultures were subcultured at least twice by transferring 0.1 ml of inoculum to 10 ml of fresh broth, and their purity was checked by streaking on the respective agar media, before use in the experiments. Unless otherwise stated, all lactococcal cultures for use in the biochemical and molecular tests of this study were grown in M-17 broth and agar media (Merck) at 30°C without shaking.

Biochemical characterization of the wild Nis^+ lactococcal strains. The phenotypic taxonomy within subspecies *lactis* of the Nis^+ *L. lactis* isolates M78 and M104 (35) was reconfirmed by testing them for cell morphology, gas production from glucose, arginine hydrolysis, growth at 15°C and 45°C, growth at 4% and 6.5% NaCl, growth on kanamycin esculin azide agar, and survival at 60°C for 30 min under the same culture conditions as those used with the reference strains of *L. lactis* included in Table 1. The media and methods used were as described by Samelis et al. (35). Moreover, the entire sugar fermentation patterns and main enzymatic activities profiles of the wild strains compared to those of the *L. lactis* reference strains were determined by the use of API 50 CHL and API ZYM kits (bioMérieux, Marcy l'Etoile, Lyon, France), respectively. In addition, strains M78 and M104 along with strains LMG 6890^T and LMG 6897^T were subjected to SDS-PAGE of whole-cell crude protein extracts, as follows. Lactococcal cultures at an optical density of 0.5 to 0.6 grown in M-17 broth at 30°C were centrifuged at 12,000 × *g* for 10 min at room temperature, washed, and resuspended in 1 ml of distilled water (29). Cells were further disrupted in a mini Bead-Beater (Biospec Products, Bartlesville, OK) (using a total of 10 1-min vibrations performed using 0.1-mm-di-

ameter zirconium beads). The cell debris homogenates were centrifuged at 12,000 × *g* for 15 min at 4°C, and the supernatants were used as protein extracts. Phenylmethylsulfonyl fluoride (PMSF) was added to the protein extracts at a final concentration of 1 mM immediately after centrifugation. The protein concentration was further determined by the Bradford method (49), and 20 μg of whole-cell proteins was loaded on the gels. Electrophoresis was carried out in a 12% (wt/vol) polyacrylamide gel at a constant current of 120 V until the dye front reached the bottom of the gel. Each gel was stained with a silver staining protocol as previously reported by Parapouli et al. (50). The profiles of the two wild and the two type strains were then compared macroscopically to detect the main differences in their protein bands.

Molecular characterization of the wild Nis$^+$ lactococcal strains. Identification of M78 and M104 isolates was verified by analysis of 16S rRNA and *acmA* (encoding lactococcal *N*-acetylmuramidase) gene and histidine biosynthesis operon polymorphisms and 16S rRNA and *ldh* (encoding lactate dehydrogenase) gene phylogenies as well as by rep-PCR, RAPD, and plasmid DNA analysis profile analyses. Strains LMG 6890^T and LMG 6897^T and a few additional strains (Table 1) served as controls. The genomic DNA used as the template for the amplification of 16S rRNA gene regions was extracted according to Pu et al. (28), whereas for *acm* and *ldh* gene amplifications as well as for RAPD analysis, DNA extraction was performed using a NucleoSpin tissue kit (Macherey-Nagel, Germany) according to the manufacturer's recommendations for Gram-positive bacteria. Plasmid DNA was purified using a NucleoSpin plasmid kit (Macherey-Nagel) according to the manufacturer's recommendations for Gram-positive bacteria. The heterogeneity of 16S rRNA gene sequences of the lactococcal species was studied using primers LacF, CreF, and LacreR according to the method of Pu et al. (28), whereas primers PALA 4 and PALA 14 (31) were employed for the detection of *acm* gene polymorphisms in comparisons of the different *L. lactis* subspecies as described by Garde et al. (34). For the amplification of the 16S rRNA gene, primers 8F and 1492R were used, while the PCR mixture was cycled using the following conditions: initial denaturation at 95°C for 5 min, 35 cycles consisting of 95°C for 1 min, 58°C for 2 min, and 72°C for 2 min, and a final extension step at 72°C for 10 min. The *ldh* gene sequences were amplified using primers LDHF1 and LDHR1 according to Urbach et al. (33). RAPD typing was performed with primer M13, as reported by Rossetti and Giraffa (47). All amplification reactions were carried out in a PTC-100 (version 7.0) thermocycler (MJ Research Inc.) using a Phusion High Fidelity DNA polymerase system (Finnzyme, Finland). The amplicons produced were purified using NucleoSpin Extract 2 in 1 (Macherey-Nagel). Molecular cloning was performed using a Zero Blunt kit (Invitrogen) according to the manufacturer's recommendations. *Escherichia coli* strain DH5α, grown aerobically in LB media at 37°C, served as the recombinant plasmid host (51). Amplified or extracted DNAs were separated by electrophoresis in 1% agarose gels, stained with ethidium bromide, and visualized using standard protocols (52). Plasmid DNA digestion by restriction enzymes and separation of fragments by agarose gel electrophoresis were also carried out using a standard methodology (52). Cloned 16S or *ldh* gene fragments were sequenced by VBC-Biotech (Austria). Taxonomic analysis was conducted by using the GenBank BLAST program (53). For phylogenetic and molecular evolutionary analysis, MEGA version 4.1 (54) was used. The 16S rRNA and *ldh* gene sequences of strains M78 and M104 were aligned with sequences from related taxa by using the CLUSTAL W program (55). The resultant tree topology was evaluated by bootstrap analysis by using the neighbor-joining method based on 1,000 resamplings. The size polymorphism of the histidine biosynthesis operon was checked using primers Lhis5F and Lhis6R, according to Beimfohr et al. (32). Rep-PCR analysis was performed using primers Rep-1R-Dt and REP2-D (46), as reported by Callon et al. (9). The dendrogram was derived from the unweighted-pair group method using average linkages (UPGMA) and based on Dice coefficients. Band position tolerance and optimization were set at 1.0 and 0.5%, respectively.

TABLE 2 Differentiating phenotypic/biochemical identification reactions of the wild Nis$^+$ *Lactococcus lactis* strains M78 and M104 in comparison with reference strains[a]

Phenotypic test	*Lactococcus lactis* strain					
	M78	M104	LMG 6890^T	LMG 6897^T	NCFB 1402	F1
NH_3 from arginine	+	+	+	−	+	+
Growth in 4% salt	+	+	+	−	+	+
Fermentation of:						
D-Ribose	+	+	+	−	+	+
D-Xylose	+	+	+	−	−	−
D-Galactose	+	+	+	−	+	+
D-Mannitol	+	+	−	−	+	+
Amygdalin	+	+	−	−	−	−
Arbutin	+	+	+	−	(+)	(+)
Esculin	+	+	+	−	+	+
Salicin	+	+	+	−	+	+
D-Cellobiose	+	+	+	−	+	+
D-Maltose	+	+	+	−	+	−
D-Lactose	+	+	+	+	+	−
D-Sucrose	+	+	−	−	+	+
D-Trehalose	+	+	+	−	−	−
Amidon (starch)	(+)d	(+)d	−	−	−	−
Gentiobiose	+	+	−	−	+	+

[a] All strains were Gram-positive, catalase-negative, homofermentative cocci which grew at 15°C. None grew at 45°C, in 6.5% salt, and on kanamycin esculin azide (KAA) agar. None survived heating at 60°C for 30 min. All strains fermented D-glucose, D-fructose, D-mannose, and *N*-acetylglucosamine. None fermented glycerol, erythritol, D-arabinose, L-arabinose, L-xylose, adonitol, α-methyl-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, α-methyl-D-mannopyranoside, α-methyl-D-glucopyranoside, D-melibiose, inulin, D-melezitose, D-raffinose, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate, or 5-ketogluconate. +, positive reaction; −, negative reaction; (+), weak reaction; (+)d, delayed reaction.

RESULTS

Phenotypic identification of the wild raw-milk isolates as *L. lactis* subsp. *lactis*. All phenotypic/biochemical tests conducted (Table 2) confirmed previous results of the Samelis et al. (35) study which first reported that the wild Nis$^+$ raw-milk isolates M78 and M104 were phenotypically identified as *L. lactis* subsp. *lactis*. Indeed, both strains are Gram-positive, catalase-negative, homofermentative, arginine-positive cocci which grow at 15°C and in 4% salt but not at 45°C and in 6.5% salt, while neither grows on kanamycin esculin azide agar or survives heating at 60°C for 30 min; thus, they are not enterococci. As expected, *L. lactis* subsp. *lactis* LMG 6890^T and the Nis$^+$ *L. lactis* subsp. *lactis* strains NCFB 1402 and F1 shared those phenotypic properties with strains M78 and M104, whereas *L. lactis* subsp. *cremoris* LMG 6897^T differed in being arginine negative and unable to grow in 4% salt (Table 2). Moreover, strain LMG 6897^T was confirmed to possess a very restricted sugar fermentation profile, since it gave positive results with glucose, fructose, mannose, lactose, and *N*-acetylglucosamine only. In contrast, strains M78 and M104 had identical sugar fermentation profiles which were much more enriched than those of the *cremoris* type strain and similar to the profiles of the *L. lactis* subsp. *lactis* strains tested (Table 2). Specifically, compared with the *lactis* LMG 6890^T strain, strains M78 and M104 were superior in fermenting mannitol and sucrose plus amygdalin, gentiobiose, and, with a delay, starch. In contrast, the main differ-

TABLE 3 Enzymatic activity reactions of the wild Nis$^+$ *Lactococcus lactis* M78 and M104 in comparison with reference strains determined by the API ZYM method[a]

Enzyme assayed for	*Lactococcus lactis* strain reaction color grade					
	M78	M104	LMG 6890^T	LMG 6897^T	NCFB 1402	F1
Alkaline phosphatase	5	5	5	3	4	4
Esterase (C_4)	3	3	3	4	4	4
Esterase lipase (C_8)	4	4	4	5	4	4
Lipase (C_{14})	−	−	−	−	−	−
Leucine arylamidase	5	5	5	5	5	5
Valine arylamidase	4	4	4	2	4	4
Cystine arylamidase	4	4	4	2	4	4
Trypsin	−	−	−	−	−	−
α-Chymotrypsin	3	3	2	3	3	3
Acid phosphatase	5	5	5	5	5	5
Naphthol-AS-BI-phosphohydrolase	5	5	5	5	5	5
α-Galactosidase	−	−	−	−	−	−
β-Galactosidase	−	−	−	−	−	−
β-Glucuronidase	−	−	−	−	−	−
α-Glucosidase	5	5	5	−	−	−
β-Glucosidase	−	−	−	−	−	−
N-Acetyl-β-glucosaminidase	−	−	−	−	−	−
α-Mannosidase	−	−	−	−	−	−
α-Fucosidase	−	−	−	−	−	−

[a] Numbers indicate a positive reaction based on the change in color intensity in each API ZYM cupule. According to the manufacturer's instructions, the color change was graded using a scale from 1 (extremely weak reaction) to 5 (very strong positive reaction). −, negative reaction similar to that seen with the control. Reactions with a grade of 3 to 5 are considered as clearly positive.

ence in sugar fermentation between our wild Nis$^+$ strains M78 and M104 and Nis$^+$ strains NCFB 1402 and F1 was the inability of the latter to ferment D-xylose and, of course, the known inability of strain F1 to ferment lactose (see Table 1) plus maltose (Table 2).

Strains M78 and M104 had identical enzymatic activities profiles, which also were identical with the API ZYM profile of *L. lactis* subsp. *lactis* LMG 6890^T (Table 3). A strong positive reaction with the substrate for α-glucosidase was actually the only major enzymatic difference between the three strains discussed above and the *L. lactis* subsp. *cremoris* LMG 6897^T and Nis$^+$ NCFB 1402 and F1 strains, which all gave negative results; all other differences were semiquantitative (based on the change in color intensity corresponding to how the strain utilized the same API ZYM substrates in the cupules) rather than qualitative (based on the ability of each strain to utilize a given substrate). On this basis, the *cremoris* type strain differed from the *lactis* type strain and the wild Nis$^+$ strains M78 and M104 in displaying weaker valine and cystine arylamidase and alkaline phosphatase reactions and stronger esterase/lipase reactions. The API ZYM profiles of the Nis$^+$ NCFB 1402 and F1 strains were intermediate overall but clearly were more similar to the subspecies *lactis* profile than to the subspecies *cremoris* profile with regard to their proteolytic reactions (Table 3).

Differentiation of *L. lactis* strains by SDS-PAGE. The wild Nis$^+$ *L. lactis* M78 and M104 strains had identical whole-cell protein profiles, which macroscopically shared an overall higher similarity to the profile of the subspecies *cremoris* LMG 6897^T strain than to that of the subspecies *lactis* LMG 6890^T strain (Fig. 1). Indeed, while their profiles were clearly distinct macroscopically from the profile of the LMG 6890^T strain, they showed only two differences in certain protein bands from the profiles of the LMG 6897^T strain; in specific, whereas strains M78 and M104 exhibited a protein band of approximately 25 kDa that is not present in the protein profile of LMG 6897^T strain, the latter possesses a protein below 14.4 kDa that strains M78 and M104 lack (Fig. 1).

Molecular identification of the wild raw-milk isolates as *L. lactis* subsp. *cremoris*. In contrast to the biochemical criteria (Tables 2 and 3) which confirmed that the Nis$^+$ *L. lactis* M78 and M104 strains are phenotypically representative of subspecies *lactis*, specific molecular identification tools revealed that the two strains cluster genotypically within subspecies *cremoris*. Indeed, M78 and M104 strains produced the same rep-PCR (Fig. 2) and RAPD (Fig. 3) patterns; their rep-PCR pattern had a higher similarity with that of subspecies *cremoris* than with that of subspecies *lactis* (Fig. 2). Specifically, strains M78 and M104 clustered apart from all five representative *L. lactis* subsp. *lactis* strains, including the type strain LMG 6890^T, while they clustered relatively closely with the *L. lactis* subsp. *cremoris* type strain LMG 6897^T and together with subspecies *cremoris* strain 1L8 (Fig. 2). On the other hand, the RAPD profile of strains M78 and 104 was different from the RAPD profiles of the *L. lactis* subsp. *lactis* LMG 6890^T and *L. lactis* subsp. *cremoris* LMG 6897^T strains, which, as expected, were also different from each other, whereas it also was different from the RAPD profiles of Nis$^+$ *L. lactis* subsp. *lactis* strains NCFB 1402 and F1, which were identical (Fig. 3).

Plasmid DNA analysis results were, in general, consistent with the rep-PCR and RAPD analysis results reported above. As shown in Fig. 4, the plasmid restriction patterns of strains M78 and M104 were identical, exhibiting a unique profile in comparison with those of all the other *L. lactis* strains; existing bands in each profile were dependent on the restriction endo-

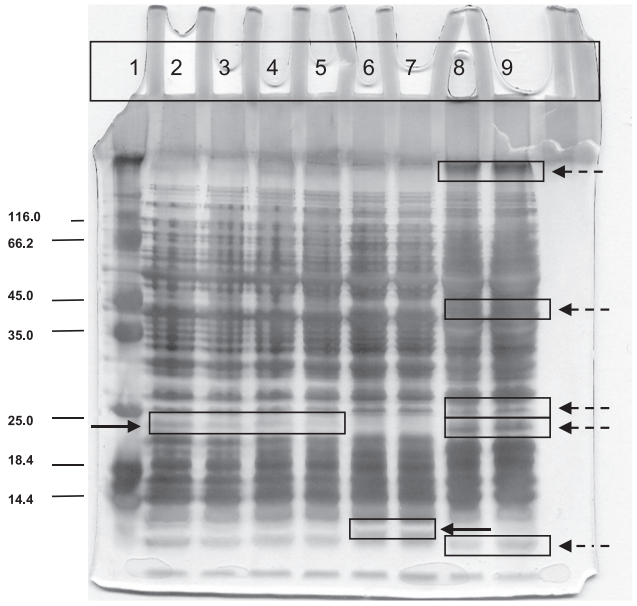

FIG 1 Polyacrylamide gel electrophoresis of crude whole-cell protein extracts of *Lactococcus lactis* spp. Lane 1, protein molecular mass (kDa) marker (Fermentas, Germany); lanes 2 and 3, M78 (wild Nis$^+$ strain); lanes 4 and 5, M104 (wild Nis$^+$ strain); lanes 6 and 7, *L. lactis* subsp. *cremoris* LMG 6897^T; lanes 8 and 9, *L. lactis* subsp. *lactis* LMG 6890^T. Solid/compact arrows indicate the positions of major protein differences between the wild Nis$^+$ strains M78 and M104 and *L. lactis* subsp. *cremoris* LMG 6897^T strain. Dashed arrows indicate the positions of major protein differences between the wild Nis$^+$ strains M78 and M104 and *L. lactis* subsp. *lactis* LMG 6890^T strain.

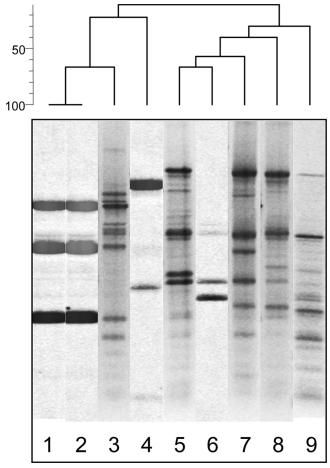

FIG 2 rep-PCR patterns of *Lactococcus lactis* wild and reference strains. Lane 1, M78 (wild Nis$^+$ strain); lane 2, M104 (wild Nis$^+$ strain); lane 3, *L. lactis* subsp. *cremoris* 1L8; lane 4, *L. lactis* subsp. *cremoris* LMG 6897^T; lane 5, *L. lactis* subsp. *lactis* CNRZ 1075; lane 6, *L. lactis* subsp. *lactis* LMG 6890^T; lane 7, *L. lactis* subsp. *lactis* CNRZ 301; lane 8, *L. lactis* subsp. *lactis* CNRZ 125; lane 9, *L. lactis* subsp. *lactis* CNRZ 258.

nuclease used for the plasmid DNA digestion. However, with all four restriction enzymes used, strains M78 and M104 were found to share common bands with the *L. lactis* subsp. *cremoris* LMG 6897^T strain whereas their plasmid profiles were greatly distinct from those of the subspecies *lactis* strains, including the LMG 6890^T strain (Fig. 4).

The differentiation of isolates M78 and M104 at the subspecies level was further evaluated by 16S rRNA gene polymorphism analyses employing primer pair CreF and LacreR and primer pair LacF and LacreR; both isolates were identified as *L. lactis* subsp. *cremoris*, exhibiting a 163-bp amplicon using primers CreF and LacreR (compact/solid square in Fig. 5), while no PCR product was amplified with primers LacF and LacreR (dashed square in Fig. 5).

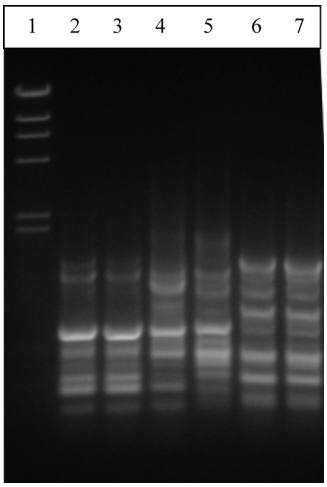

FIG 3 RAPD profiles of *Lactococcus lactis* wild and reference strains determined using primer M13. Lane 1, molecular size marker λDNA/HindIII; lane 2, M78 (wild Nis$^+$ strain); lane 3, M104 (wild Nis$^+$ strain); lane 4, *L. lactis* subsp. *cremoris* LMG 6897^T; lane 5, *L. lactis* subsp. *lactis* LMG 6890^T; lane 6, *L. lactis* subsp. *lactis* F1 Nis$^+$ strain; lane 7, *L. lactis* subsp. *lactis* NCFB 1402 Nis$^+$ strain.

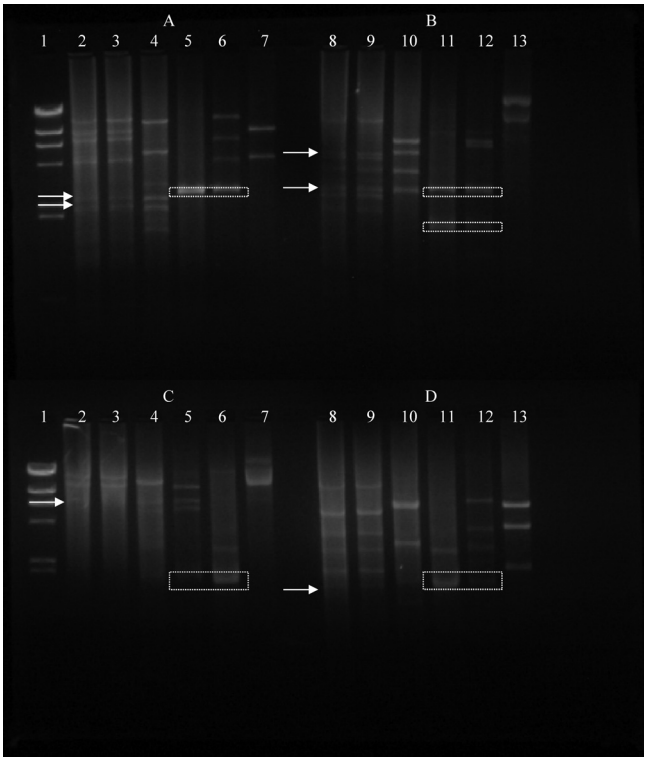

FIG 4 Plasmid DNA patterns of *Lactococcus lactis* strains determined using the restriction endonucleases EcoRI (A), EcoRV (B), BamHI (C), and HindIII (D). (A and C) Lanes 1, marker λDNA/HindIII; lanes 2, M78; lanes 3, M104; lanes 4, *L. lactis* subsp. *cremoris* LMG 6897^T; lanes 5, *L. lactis* subsp. *lactis* NCFB 1402 Nis$^+$ strain; lanes 6, *L. lactis* subsp. *lactis* F1 Nis$^+$ strain; lanes 7, *L. lactis* subsp. *lactis* LMG 6890^T. (B and D) Lanes 8, M78; lanes 9, M104; lanes 10, *L. lactis* subsp. *cremoris* LMG 6897^T; lanes 11, *L. lactis* subsp. *lactis* NCFB 1402 Nis$^+$ strain; lanes 12, *L. lactis* subsp. *lactis* F1 Nis$^+$ strain; lanes 13, *L. lactis* subsp. *lactis* LMG 6890^T. Arrows indicate common bands between the *L. lactis* subsp. *cremoris* LMG 6897^T strain and the wild Nis$^+$ M78 and M104 strains. Squares indicate common bands between the *L. lactis* subsp. *lactis* NCFB 1402 Nis$^+$ strain and the *L. lactis* subsp. *lactis* F1 Nis$^+$ strain.

These results were confirmed by the size polymorphism of the histidine biosynthesis operon determined using primers Lhis5F and Lhis6R (32); the size of the latter PCR product was that expected for the *L. lactis* subsp. *cremoris* and not that expected for *L. lactis* subsp. *lactis* (Fig. 6). The expected sizes of amplification products for *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* were 1,149 bp and 934 bp, respectively (32). In contrast, *acm* polymorphisms failed to discriminate between all tested *L. lactis* strains on the subspecies level (data not shown).

The *ldh* and 16S rRNA gene sequencing analyses further confirmed that strains M78 and M104 cluster genotypically with *L. lactis* subsp. *cremoris* whereas they are close relatives of *L. lactis* subsp. *lactis* and, of course, are of low genotypic relatedness to *L. garviae* (Fig. 7 and 8). The *ldh* sequences of isolates M78 and M104 share 100% homology (GenBank accession number JX402633), whereas their 16S rRNA sequences are nearly identical (99.9% homology), differing in two nucleotides only (GenBank accession numbers JX402634 and JX402635).

DISCUSSION

Based on the results of the analysis of polymorphisms in a combination of selected genes previously reported as specific for the

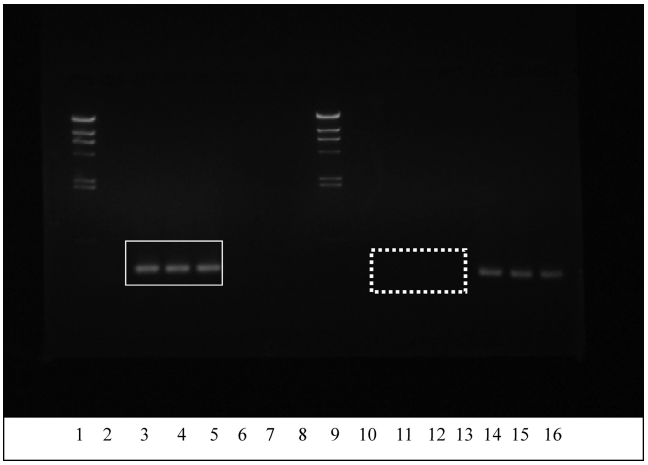

FIG 5 PCR identification of *Lactococcus lactis*. PCR amplification products in lanes 2 to 8 were determined using primers CreF and LacreR. Lane 1, marker λDNA/HindIII; lane 2, no-DNA control; lane 3, *L. lactis* subsp. *cremoris* LMG 6897^T; lane 4, M78; lane 5, M104; lane 6, *L. lactis* subsp. *lactis* LMG 6890^T; lane 7, *L. lactis* subsp. *lactis* F1 Nis$^+$ strain; lane 8, *L. lactis* subsp. *lactis* NCFB 1402 Nis$^+$ strain. PCR amplification products in lanes 10 to 16 were determined using primers LacF and LacreR. Lane 9, marker λDNA/HindIII; lane 10, no-DNA control; lane 11, *L. lactis* subsp. *cremoris* LMG 6897^T; lane 12, M78; lane 13, M104; lane 14, *L. lactis* subsp. *lactis* LMG 6890^T; lane 15, *L. lactis* subsp. *lactis* F1 Nis$^+$ strain; lane 16, *L. lactis* subsp. *lactis* NCFB 1402 Nis$^+$ strain. The compact/solid square indicates the presence of amplified PCR product bands for the *L. lactis* subsp. *cremoris* LMG 6897^T strain and the wild Nis$^+$ M78 and M104 isolates when the *cremoris*-specific primer pair CreF and LacreR was used, whereas the dashed square indicates the absence of amplified PCR product bands for the same strains when the subspecies *lactis*-specific primer pair LacF and LacreR was used.

differentiation of *L. lactis* subsp. *lactis* from subsp. *cremoris* (24, 28, 32, 33), this study showed that the wild Nis$^+$ *L. lactis* M78 and M104 isolates from Greek raw-milk cluster genotypically within subspecies *cremoris* although their phenotypical-biochemical properties are highly representative of subspecies *lactis*. In fact, according to this study, M78 and M104 may represent duplicate isolates of a novel *L. lactis* subsp. *cremoris* NisA$^+$ strain given that isolate M78 was originally picked from M-17 agar plates incubated

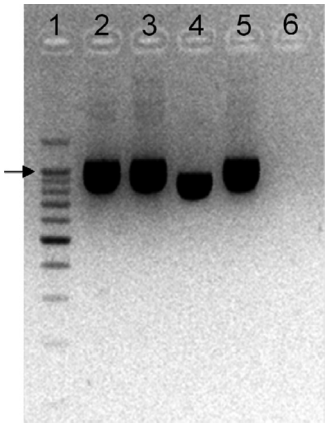

FIG 6 Size polymorphism in the histidine biosynthesis operon of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*. Lane 1, molecular size marker; lane 2, M78; lane 3, M104; lane 4, *L. lactis* subsp. *lactis* LMG 6890^T; lane 5, *L. lactis* subsp. *cremoris* LMG 6897^T; lane 6, no-DNA control. The arrow indicates the position of the 1,000-bp band in the molecular size marker.

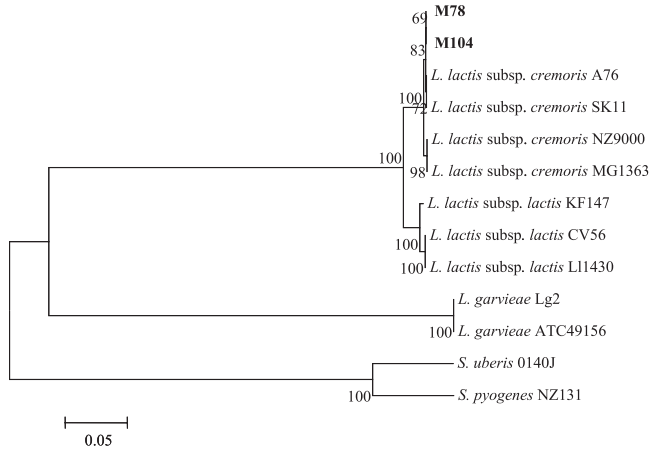

FIG 7 Phylogenetic location of the wild Nis$^+$ *Lactococcus lactis* strains M78 and M104 among closely related species of bacteria based on the lactate dehydrogenase (*ldh*) gene sequence. The scale bar indicates 0.05 substitutions per nucleotide position. *S. uberis*, *Streptococcus uberis*; *S. pyogenes*, *Streptococcus pyogenes*.

at 42°C whereas isolate M104 was from the respective MRS agar plates at 30°C of the same sample of raw bulk milk (35). In that study (35), the ability of isolate M104 to grow in MRS broth at 45°C, and in MRS broth with added 6.5% NaCl at 30°C, was the only phenotypic difference between the two isolates. Growth of isolate M104 at 45°C and in 6.5% salt, generally considered atypical phenotypic properties in *L. lactis* (8), was not confirmed in this study (Table 2). This discrepancy observed in these physiological tests/assays at the growth boundaries of *L. lactis* leads us to the logical conclusion that those initial results were a technical artifact. Conversely, the same basic biochemical—sugar fermentation—reactions of isolates M78 and M104 in miniplates reported by Samelis et al. (35) were fully confirmed in this study by the API 50CHL method (Table 2). Results further showed that the two isolates share their entire sugar profiles, including a delayed-positive reaction with starch; the later is quite surprising and, in our opinion, worthy of noting because *L. lactis* is typically starch negative (20, 21). In fact, very few species among the total number in all LAB genera ferment starch, and those are mostly plant-associated lactobacilli. So, the delayed, even weak starch fermentation, along with the pronounced ability of isolates M78 and M104 to ferment, all together, xylose, maltose, sucrose, mannitol,

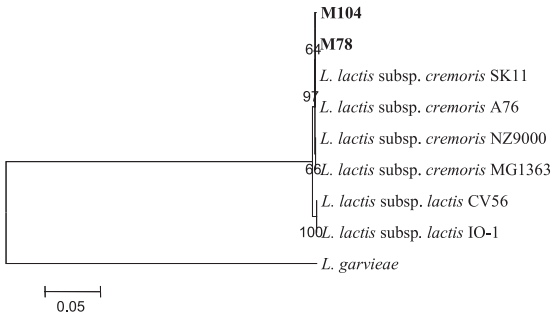

FIG 8 Phylogenetic location of the wild Nis$^+$ *Lactococcus lactis* strains M78 and M104 among closely related species of bacteria based on the 16S rRNA gene sequence. The scale bar indicates 0.05 substitutions per nucleotide position.

and amygdalin plus its derivative gentiobiose, unlike the LMG 6890^T strain and other *L. lactis* strains (Table 2), suggests that these wild raw-milk isolates may actually have a plant-based origin. That several *L. lactis* strains were isolated directly from plants, or from milk and cheese while originating from plants, has been well established in recent studies (27, 56), supporting the concept of the plant-milk transition for wild *Lactococcus* strains (12, 25).

In addition to their sugar fermentation patterns, this study showed that isolates M78 and M104 exhibit identical enzymatic, whole-cell protein, rep-PCR, RAPD, and plasmid restriction profiles, facts that further support the hypothesis that M78 and M104 may actually represent isolates of a single novel wild Nis$^+$ *L. lactis* strain/genotype, given that, as previously reported (35), they were randomly selected colonies grown on different agar media of a raw-milk sample. In regard to their enzymatic activities, the negative reactions of both isolates and all other *L. lactis* strains for lipase (C14), trypsin, α-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase are in complete agreement with the API ZYM results of Nomura et al. (27), who tested 41 natural *L. lactis* strains from milk and plants. The remaining enzymatic reactions in Table 3 also are in general agreement with those of the Japanese *L. lactis* subsp. *lactis* biovar diacetylactis or *L. lactis* subsp. *cremoris* strains mentioned above (27). The most prominent differences are that several Japanese strains were positive for β-galactosidase and β-glucosidase (27) whereas, in this study, none of the six *L. lactis* strains tested positive (Table 3). Nomura et al. (27) showed that 63% to 100% of the subsp. *lactis* biovar diacetylactis plant or milk strains compared to 38% of the subspecies *cremoris* milk strains were positive for α-glucosidase, which was the most clearly differentiating API ZYM reaction for our strains, as shown in Table 3. This further supports the notion that M78 and M104 isolates belong to less-frequent *cremoris* genotypes which possess enzymatic and biochemical properties that are highly representative of subspecies *lactis*.

Interestingly, the SDS-PAGE of whole-cell proteins was the only biochemical analysis of this study which indicated a closer genotypic relatedness of M78 and M104 isolates with subspecies *cremoris* than with subspecies *lactis*. Considering, however, that several species or subspecies of lactic acid bacteria are known to be very diverse in their whole-cell protein profiles at the strain level (30), it is necessary to apply SDS-PAGE analysis of isolates M78 and M104 in comparison with analyses of large numbers of reference or wild *L. lactis* subsp. *lactis* or *cremoris* strains. Nevertheless, the different protein profile of isolates M78 and M104 compared to the subspecies *lactis* LMG 6890^T strain plus the distinct differences from the subspecies *cremoris* LMG 6897^T strain (Fig. 1) are the first results underlining on the protein level the uniqueness of *L. lactis* strains presenting genotypic subspecies *cremoris* and phenotypic subspecies *lactis* properties. However, a future comparative proteomic study would draw useful conclusions on the technological and bioprotective potential of these strains.

The uniqueness of M78 and M104 and their closer relationship to subspecies *cremoris* above discussed were further confirmed by the results of rep-PCR analysis (Fig. 2). In addition, RAPD (Fig. 3) and plasmid DNA (Fig. 4) profiling clearly showed the genetic relationship of the two isolates under study but failed to discriminate them on the subspecies level. At this point, it should be noted that the two Nis$^+$ NCFB 1402 and F1 strains share the same RAPD profile (Fig. 3), whereas they also share certain plasmid DNA restriction bands (Fig. 4). Thus, strain F1 may indeed represent a lactose-negative variant of strain NCFB 1402 (see Table 1).

The first molecular identification tools that clearly delineated the taxonomy of isolates M78 and M104 on the subspecies level were 16S rRNA gene and histidine biosynthesis operon size polymorphism analyses. As presented in Fig. 5, both isolates exhibited a 163-bp amplicon using primers CreF and LacreR (compact/solid square) whereas no PCR product was amplified with primers LacF and LacreR (dashed square). As reported by Pu et al. (28), primer pair CreF and LacreR and primer pair LacF and LacreR can differentiate representatives of species *L. lactis* on the subspecies level: with primers CreF and LacreR, a 163-bp amplicon is obtained for strains *L. lactis* subsp. *cremoris*, while a 163-bp product is obtained for *L. lactis* subsp. *lactis* strains using primers LacF and LacreR. Regarding histidine biosynthesis operon size polymorphisms, in analyses performed using primer pair Lhis5F and Lhis6R, strains M78 and M104 exhibited the PCR product expected for subspecies *cremoris* representatives (Fig. 6), as reported by Beimfohr et al. (32). On the subspecies level, in contrast, *acm* polymorphisms failed to discriminate the isolates under study. According to Garde et al. (34), with primer pair PALA 4 and PALA 14, a 1,131-bp product was obtained with a *L. lactis* subsp. *cremoris* template whereas an additional product of 700 bp was amplified for a *L. lactis* subsp. *lactis* template. In this study, however, all *L. lactis* strains exhibited a one-band profile (data not shown), a finding contradictory to that of the Garde et al. (34) approach that has also been previously reported by Prodelalová et al. (57).

In consistency with the findings presented above, *ldh* and 16S rRNA gene phylogenies also successfully taxonomized both isolates as representatives of the *L. lactis* subsp. *cremoris* genotype (Fig. 7 and 8). Their high sequence homologies, 100% for the *ldh* gene and 99.9% for the 16S rRNA gene, further suggest that isolates M78 and M104 may actually represent different raw-milk isolates of the same wild Nis$^+$ strain/genotype.

In conclusion, the wild isolates M78 and M104 from Greek raw milk should be considered to represent a novel *L. lactis* subsp. *cremoris* strain or genotype. Their actual novelty is not supported by their genotypic subspecies identification, which contradicts their phenotypic identification, since many previous dairy ecology studies have shown similar results for several wild *L. lactis* strains (3, 5, 12, 23, 24, 25). *L. lactis* subsp. *cremoris* M78 and M104 are novel because they are strong nisin (Nis$^+$) producers (35, 36) capable of inactivating or inhibiting growth of *Listeria monocytogenes* and *Staphylococcus aureus in vitro* as well as *in situ* in various dairy foods (35, 38, 39). Other wild Nis$^+$ *L. lactis* subsp. *cremoris* strains that have been reported so far are raw-milk or cheese isolates which, however, produce nisin Z and not nisin A (44). Regardless of genotype, however, NisZ$^+$ *L. lactis* strains generally are of lower inhibitory efficacy against pathogens than typical NisA$^+$ *L. lactis* subsp. *lactis* strains (45), including strain ATCC 11454 used to create the nisin-positive transconjugant *L. lactis* subsp. *cremoris* JS102 strain (14).

To our knowledge, there is only one very recent technological study, by Dal Bello et al. (45), which described the effective use of 40FEL3, an Italian wild NisA$^+$ *L. lactis* subsp. *cremoris* strain, to control *L. monocytogenes* in cottage cheese. This strain, originally isolated and described by Dal Bello et al. (44) as the producer of a nonidentified bacteriocin, was later found to be most inhibitory against *L. monocytogenes in vitro* and in cottage cheese (45). Likewise, our wild NisA$^+$ *L. lactis* subsp. *cremoris* M78 and M104 iso-

lates (37) have been shown to show high nisin production rates (36) and strong nisin-mediated antilisterial and/or antistaphylococcal activity *in vitro* in culture broth, skim milk, and/or model cheese, as well as *in situ* in thermized milk and traditional Greek fresh whey cheese or fermented hard cheese curds; their activity was higher than that of *L. lactis* subsp. *lactis* NCFB 1402 and other Nis^+ *L. lactis* strains (35, 38, 39). Additional studies are therefore required to genotypically and technologically compare our wild $NisA^+$ M78 and M104 isolates with other *L. lactis* raw-milk or cheese strains, including the Italian strain 40FEL3 and several nisin-negative Greek lactococcal isolates available in our laboratory (13, 35). Such comparisons have been out of the scope of this study, which focused on elucidating the subspecies identity of the M78 and M104 isolates. This is the first complete report on wild *L. lactis* subsp. *cremoris* strains of the subspecies *lactis* phenotype which produce nisin A and thus have high potential for use as a novel dairy starter, costarter, and/or protective adjunct cultures.

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