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Catabolism of *N*-Acetylneuraminic Acid, a Fitness Function of the Food-Borne Lactic Acid Bacterium *Lactobacillus sakei*, Involves Two Newly Characterized Proteins

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In silico **analysis of the genome sequence of the meat-borne lactic acid bacterium (LAB)** ***Lactobacillus sakei*** **23K has revealed a repertoire of potential functions related to the adaptation of this bacterium to the meat environment. Among these functions, the ability to use *N*-acetyl-neuraminic acid (NANA) as a carbon source could provide a competitive advantage for growth on meat in which this amino sugar is present. In this work, we proposed to analyze the functionality of a gene cluster encompassing** ***nanTEAR*** **and** ***nanK*** **(*nanTEAR-nanK*). We established that this cluster encoded a pathway allowing transport and early steps of the catabolism of NANA in this genome. We also demonstrated that this cluster was absent from the genome of other *L. sakei* strains that were shown to be unable to grow on NANA. Moreover, *L. sakei* 23K *nanA*, *nanT*, *nanK*, and *nanE* genes were able to complement *Escherichia coli* mutants. Construction of different mutants in *L. sakei* 23K Δ*nanR*, Δ*nanT*, and Δ*nanK* and the double mutant *L. sakei* 23K Δ(*nanA-nanE*) made it possible to show that all were impaired for growth on NANA. In addition, two genes located downstream from *nanK*, *lsa1644* and *lsa1645*, are involved in the catabolism of sialic acid in *L. sakei* 23K, as a *L. sakei* 23K Δ*lsa1645* mutant was no longer able to grow on NANA. All these results demonstrate that the gene cluster *nanTEAR-nanK-lsa1644-lsa1645* is indeed involved in the use of NANA as an energy source by *L. sakei*.**

Lactobacillus sakei is a lactic acid bacterium (LAB) first isolated from rice wine, but is commonly associated with the food environment. It belongs to the natural microbiota of raw meat and seafood stuffs and is also added as a starter culture for elaboration of fermented meat and fish products, notably raw fermented sausage in Western countries (1, 2). It is generally observed that the presence of *L. sakei* in meat limits the presence of spoilage bacteria (3). *L. sakei* is detected in the human digestive tract, albeit in the subdominant microbiota, and is considered to be a commensal bacterium (4, 5). The genome sequence of a strain isolated from dry sausage, *L. sakei* 23K, has been previously established (3), revealing several unexpected properties. These functions involved the transport and the use of alternative carbon sources such as *N*-acetylneuraminic acid, an efficient catabolism of nucleosides like inosine providing an additional energy source and conversion of arginine through the arginine deaminase pathway (reference 6 and references therein). Some of these functions have been shown to protect this species from acid stress and provide it a competitive advantage in meat (6). Not only one function but the combination of several functions would explain the adaptation of this species to the meat environment. In this work, we were interested in the ability of *L. sakei* to metabolize the nine-carbon amino sugar called *N*-acetyl-neuraminic acid (NANA) which is present in meat. *L. sakei* has the privilege to be generally recognized as safe (GRAS). Our group is interested in characterizing various functions with the aim to use several strains of *L. sakei* with an objective of biopreservation of the fresh meat.

Sialic acid is the generic name for a family of more than 40 naturally existing nine-carbon keto sugars derived from 2-keto-3-deoxy-5-acetamido-D-glycero-D-galacto-nonulosonic acid. *N*-acetylneuraminic acid, or Neu-5Ac (NANA), is the most common such sugar in the living world. Indeed, it is found in mammalian cells as a component of glycoproteins, and most abundantly in mucins, which are glycoproteins of very high molecular weight secreted by mucosa and some exocrine glands into the lumen of the respiratory, gastrointestinal, and reproductive tracts (7–10). It plays a crucial role in cellular interactions in eukaryotes and is also described as a key molecule in host/pathogen adhesion (7, 10–12). This major component, present in the mucous layer of the human digestive tract, can also be used as an energy source by the commensal bacteria of the human microbiota (8). In several pathogenic bacteria, sialic acid or its use is associated with virulence (8, 10, 13). Previous studies, mainly on human-pathogenic bacteria, showed that, after its internalization, sialic acid is either catabolized as a nutrient source or incorporated into polymers for display at the bacterial cell surface, in order to escape the bactericidal effect of serum, subsequently allowing bacterial proliferation within the host (12, 14–16). This phenomenon was particularly studied for the human-pathogenic bacterial species *Neisseria meningitidis*, *Escherichia coli*, *Haemophilus influenzae*, *Haemophilus ducreyi*, *Pasteurella haemolytica*, *Pasteurella multocida*, and the streptococci of group B (10, 12, 15, 17–20). The studies carried out on these pathogenic bacteria focused on the mechanisms of *in vivo* biosynthesis or ability to salvage sialic acid. In *E. coli* K1, it was shown that this strain has all the necessary functions for the uptake of NANA from its cellular environment and for its further metabolism both as a carbon source (by the two operons *nanRATEK* and

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nagAB) and for its polymerization at the surface to produce polysialic acid (PSA) (19, 21).

The NANA metabolism was first described in the nonpathogenic strain *E. coli* K-12 (22). Briefly, NANA from the surrounding environment is transported into the cytoplasm by a symporter (NanT) through the inner membrane. The *N*-acetylneuraminic acid lyase (NanA) first removes a pyruvate group, yielding *N*-acetylmannosamine (NAM), and NAM kinase (NanK) subsequently adds a phosphate group, which yields NAM-6P. The NAM-6P epimerase (NanE) transforms NAM-6P into *N*-acetylglucosamine-6P (NAG-6P), and the acetyl group and the amino moiety are cleaved, respectively, by NAG-6P deacetylase (NagA) and glucosamine-6P deaminase (NagB), generating fructose-6P, which may then enter the glycolysis pathway. In *E. coli* K-12, the expression of the genes involved in the catabolism of sialic acid is regulated by a repressor, encoded by *nanR*. NanR inactivation induces constitutive expression of the sialic acid catabolic genes (22, 23).

In *H. influenzae*, the transport, catabolism, and polymerization of NANA have been shown to be tightly controlled. Transcriptional analysis of the mutant *siaR* indicated that both the *nan-nag* and *siaPT* operons were upregulated (23). Regulatory mechanisms that determine routing of sialic acid into the transport, the catabolic, or the polymerization pathways have been described (13, 24, 25). Recently, it was shown that the repressor SiaR and CRP (Cyclic AMP [cAMP] Receptor Protein) cooperatively regulate the expression of the operons encoding the NANA tripartite ATP-independent periplasmic (TRAP) transporter SiaPT, also called SiaPQM, and the catabolic genes *nanEKA* and *nagBA* (10, 13, 24, 25). NANA utilization, whether to escape the bactericidal effect of serum or as an energy source, has also been described in bacteria such as *Vibrio cholerae*, *Bacteroides fragilis*, *E. coli*, *H. influenzae*, and *Clostridium perfringens* (21, 22, 26–29). Little is known regarding Gram-positive bacteria with respect to NANA metabolism except for *C. perfringens* (29).

Recently, the evolution and the sequence similarities of the genes involved in sialic acid metabolism among bacteria, based on *in silico* analysis of finished or unfinished genomes, have been described (14, 30). In this study, 1,902 genomes were investigated; the Nan cluster present in 46 species is predominantly confined to pathogens (33 species) and commensal bacteria (9 species). Several types of *nan* operons were found in many bacteria; while the NANA transporters and repressors were shown to vary, the core metabolic enzymes encoded by *nanAEK* were well conserved (30).

So far, no functional analysis of sialic acid metabolism has been reported in Gram-positive bacteria, including lactic acid bacteria. However, with regard to *L. sakei*, it is likely that NANA is an abundant source of energy, as it is found in the sialylated glycoforms of many glycoproteins present in meat, both in connective tissues and decorating the red blood cell membrane (11). The aim of the present study was an initial characterization of the Nan cluster found in *L. sakei* 23K and to explore its role in NANA metabolism.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacterial strains and plasmids used in this study are described in Table 1. *L. sakei* strains were routinely grown at 30°C on MRS medium (37). For *L. sakei*, the chemically defined medium MCD (38) supplemented with 25 mM concentrations of various carbon sources was used. When necessary, erythromycin was used at 5 $\mu g \cdot ml^{-1}$.

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
L. sakei strains		
23K	*nan*$^+$ wild-type plasmid-cured strain	3
LV5	*nan*$^+$ wild type	31 (and references therein)
300	*nan*$^+$ wild type	31 (and references therein)
LTH675	Wild-type plasmid-free strain lacking *nan*	31 (and references therein)
LTH677	Wild-type strain lacking *nan*	31 (and references therein)
64	Wild-type strain lacking *nan*	31 (and references therein)
RV6030	23K Δ*lsa1645*	This study
RV6031	23K Δ(*nanA-nanE*)	This study
RV6032	23K Δ*nanK*	This study
RV6033	23K Δ*nanT*	This study
RV6034	23K Δ*nanR*	This study
E. coli strains		
DH5α	Recipient strain for plasmid construction	Invitrogen
MG1655	*nan*$^+$ wild-type *E. coli* K-12 strain	32
JW25113	*nan*$^+$ strain	33
JW3192	JW25113 Δ*nanE*, Kan^r	34
JW3193	JW25113 Δ*nanT*, Kan^r	34
JW3194	JW25113 Δ*nanA*, Kan^r	34
JW3195	JW25113 Δ*nanR*, Kan^r	34
JW5538	JW25113 Δ*nanK*, Kan^r	34
Plasmids		
pRV610	Shuttle vector, Ap^r, Erm^r	35
pRV386	pRV610::*nanRAETK*	This study
pRV392	pRV610::*nanRAETK lsa1644 lsa1645*	This study
pRV300	Integrative vector for *L. sakei*, Ap^r, Erm^r	36
pRV398	pRV300::Δ(*nanA-nanE*), Ap^r, Erm^r	This study
pRV400	pRV300::Δ*lsa1645*, Ap^r, Erm^r	This study
pRV716	pRV300::Δ*nanR*, Ap^r, Erm^r	This study
pRV717	pRV300::Δ*nanK*, Ap^r, Erm^r	This study
pRV718	pRV300::Δ*nanT*, Ap^r, Erm^r	This study

[a] Ap, ampicillin; Erm, erythromycin; Kan, kanamycin.

E. coli strains were grown at 37°C under conditions of agitation on Luria broth (LB) or M63 or M9 minimal medium (39) supplemented with the appropriate carbon source at a concentration of 25 mM. The main difference between M63 medium and M9 medium is that M63 does not contain a sodium source. When mentioned, ampicillin and kanamycin were used at 100 $\mu g \cdot ml^{-1}$ and 30 $\mu g \cdot ml^{-1}$ in *E. coli*, respectively.

Growth on NANA. NANA was purchased from Sigma. As this product is very expensive, we evaluated growth on NANA as a sole carbon source on small plates (5-ml diameter) on MCD agar for *L. sakei* and on M9 or M63 agar for *E. coli* strains. Overnight cultures on minimal media_glucose were harvested and washed. A drop of 5 μl was spotted on the plates and then incubated.

DNA isolation and amplification. PCR was carried out using *Taq* DNA polymerase purchased from Fermentas by using a conventional protocol comprising an initial denaturation step (4 min at 94°C), followed by 25 amplification cycles (30 s at 95°C; 1 min at 55°C; 2 min at 72°C) and a final elongation step (5 min at 72°C). Isolation of plasmid DNA from *E. coli* or *L. sakei* using commercially available reagents (Qiagen) and chro-

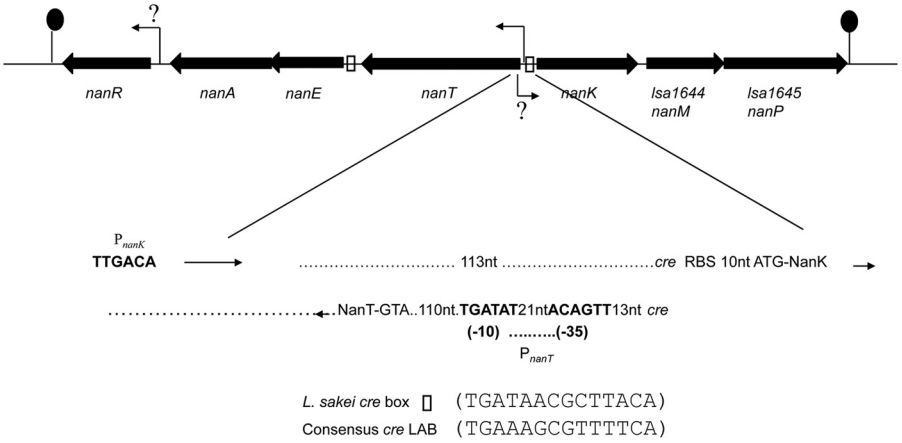

FIG 1 Genetic organization of the *nan* gene cluster of *L. sakei* 23K. Putative promoters (arrows) and putative terminators (vertical lines with circles) are indicated. The genes are represented by large dark arrows oriented according to their transcriptions. The extended part of the figure is intended for visualizing the sequence corresponding to the putative promoter sequence located between *nanT* and *nanK*. Empty rectangles represent the localizations of the potential *cre* boxes.

mosomal DNA extraction from *L. sakei* were performed as previously described (40).

Plasmid construction. The shuttle vector pRV610 (35) is able to replicate in both *E. coli* and *L. sakei*, and it was used to clone the gene cluster encompassing *nanTEAR* and *nanK* (*nanTEAR-nanK*). A 5,659-bp fragment was obtained by PCR amplification of strain 23K chromosomal DNA with primers olsnan1 and olsnan3 (see Table S1 in the supplemental material), thereby introducing an XbaI and a SacI restriction site at the two extremities of the amplification product. The PCR product was then cloned at the XbaI and SacI sites of pRV610, generating the plasmid pRV386 (Table 1).

A PCR fragment encompassing *lsa1644* and *lsa1645* (*lsa1644-lsa1645*) was obtained by amplification of 23K chromosomal DNA using primers olsnan12 and olsnan13 (see Table S1 in the supplemental material), which both bore an extension with a SacI site. The PCR product was cloned downstream from *nanK* at the SacI site of pRV386, generating pRV392 (Table 1). The pRV386 and pRV392 plasmids were transformed in *E. coli* DHα, extracted, and verified by restriction digestion and sequence analysis.

Transformation of *E. coli* and *L. sakei*. *E. coli* mutants from the Keio collection (34) were made chemically competent (39), and electrocompetent cells of *E. coli* DH5α were obtained as previously described (41). Transformants were selected on an LB agar plate containing 100 $\mu g \cdot ml^{-1}$ ampicillin with or without 30 $\mu g \cdot ml^{-1}$ kanamycin depending on the strain used. Transformation of *L. sakei* was carried out by electroporation as previously described (42). Transformants were selected on MRS agar containing 5 $\mu g \cdot ml^{-1}$ of erythromycin. Screening of the potential transformants was routinely done by PCR amplification on total DNA.

Construction of the *L. sakei* mutants. For the construction of *L. sakei* 23K Δ*nanR*, Δ(*nanA-nanE*), Δ*nanT*, Δ*nanK*, and Δ*lsa1645*, the method described previously involving two successive crossovers was performed (36, 43). Briefly, two PCR fragments were first amplified using specific primers (see Table S1 in the supplemental material) to generate the upstream and downstream regions of the gene to be deleted. The primers used were carefully designed in order to keep the frame in the final product with extensions bearing restriction enzyme sites. The two PCR fragments were digested, and then ligated, and a second run of PCR amplification using the ligated DNA as a template was performed using the two external primers to produce a fragment with the expected internal deletion. This fragment was then introduced into the *L. sakei* integrative plasmid pRV300 (36). Five plasmids, pRV398[Δ(*nanA-nanE*)], pRV400(Δ*lsa1645*), pRV716(Δ*nanR*), pRV717(Δ*nanK*), and pRV718(Δ*nanT*), were obtained in this way. These plasmids were used to transform *L. sakei* 23K, and correct insertion at the targeted locus was checked by PCR amplification of genomic DNA with appropriate primers. The second event of homologous recombination was performed as previously described (43) by screening for erythromycin-sensitive bacteria, which yielded around 50% of appropriately deleted strains. All potential deletion mutants were verified in comparison to the wild-type strain by PCR on genomic DNA using appropriate primers (see Table S1 in the supplemental material).

RESULTS

The gene repertoire of *L. sakei* 23K may allow NANA utilization. The genome sequence of *L. sakei* 23K (3) revealed three loci comprising genes putatively involved in NANA catabolism: a first locus that encompassed two divergent operons, one on the lagging strand annotated *nanTEAR* and a *nanK* gene on the leading strand (Fig. 1), and two loci comprising *nagA* (*lsa1588*) and *nagB* (*lsa0417*) genes, located elsewhere on the chromosome. Neither the genes putatively involved in NANA polymerization nor the putative sialidase was detected in the genome. Three potential promoters that closely matched the consensus sequence deduced from previously characterized *L. sakei* genes were identified. The first one is likely to initiate transcription of the *nanR* gene. Two potential −10 boxes (TATAAT) were present at, respectively, 39 bp and 139 bp upstream from the *nanR* start codon, although no obvious −35 box was found. The second promoter, comprising −35 (TTGACA) and −10 (TATAGT) consensus boxes, was identified 110 bp upstream from the *nanT* gene. The third promoter, potentially directing the expression of the *nanK* gene, was localized 362 bp upstream from *nanK* start codon. In addition, a bidirectional putative consensus *cre* (catabolite repression element)-like box was present 17 bp upstream from the start codon of the *nanK* gene (TGATAACGCTTACA) and 154 bp upstream from the *nanT* start codon (TGTAAGCGTTATCA). These *cre*-like boxes were similar to those described previously in *L. sakei* (6, 44). A second *cre*-like box was also present 62 bp upstream from the *nanE* start codon gene (TGTAAGCGGTTAATACA). Such elements are generally present upstream from genes regulated by the carbon catabolite protein A (CcpA) as described previously (6, 44, 45). Another striking observation is the presence of a conserved

TABLE 2 Complementation and growth on M63 or M9 media of *E. coli* mutants by *L. sakei nan* genes

	Growth[a] with indicated carbon source (25 mM) when complimented with plasmid:							
			pRV386 (pRV610::*nanTEARK*)			pRV392 (pRV610::*nanTEARKlsa1644lsa1645*)		
	pRV610			NANA			NANA	
E. coli strain	Glucose	NANA	Glucose	M63	M9	Glucose	M63	M9
MG1655 (wild type)	+	+	+	+	+	NT	NT	NT
JW3192 (Δ*nanE*)	+	−	+	+	+	+	+	+
JW3193 (Δ*nanT*)	+	−	+	−	+	+	−	−
JW3194 (Δ*nanA*)	+	−	+	−	+	+	−	−
JW3195 (Δ*nanR*)	+	+	+	+	+	+	+	+
JW5538 (Δ*nanK*)	+	−	+	+	+	+	+	+

[a] +, growth; −, absence of growth; NT, not tested.

box of 14 bp [(A/C)TAATTTTTAATT(A/T)] found 136, 90, and 112 bp upstream from the start codon of *nanR*, *nanT*, and *nanK* genes, respectively. Moreover, this 14-bp box was not found elsewhere on the chromosome. In addition to these genes, which are commonly found for the catabolism of sialic acid in bacteria, we also investigated the two genes located downstream from *nanK*, *lsa1644* and *lsa1645* (Fig. 1). Sequence analysis revealed that the *L. sakei* NanR belongs to the RpiR family. These proteins are usually involved in regulation of the expression of various genes. While most are repressors, these regulators can also activate expression of genes (46). NanR was also found in other species in association with *nan* gene clusters, such as in *Pediococcus pentosaceus* ATCC 25745, in two strains of *Lactobacillus salivarius* (UCC118 and NIAS840), in *Carnobacterium* sp. AT7, and in three staphylococcal strains, *Staphylococcus aureus* strain Newman, *S. saprophyticus* ATCC 15305, and *S. lugdunensis* HKU09-01. Like the *L. sakei* NanR protein, all share the same two domains, that is, a helix turn helix (HTH) motif, which suggests a capacity to bind to DNA, and a sugar isomerase domain in the carboxy-terminal region. *L. sakei* NanT presents high similarity to members of the sodium solute symporter family, which is mostly found in lactobacilli. The LSA1644 protein is similar to a putative sugar-phosphate isomerase/epimerase also present in two *Lactobacillus plantarum* strains (Lp_3566 in WFCS1 and JDM1_2850 in JDM1) within *nan* gene clusters and in *Carnobacterium* sp. AT7, though with no link to the *nan* genetic cluster (CAT7_09150:86511 . . . 87272). The LSA1645 protein has several transmembrane domains, and has been identified as a putative Na^+/H^+ antiporter. This protein is similar to many Na^+/H^+ antiporters found in Gram-positive bacteria, though two *L. plantarum* species have these homologs located in association with *nan* cluster genes, and presents 59% identity and 76% similarity to the *L. plantarum* WCFS1 and JDM1 *napA4* gene products. As neither a putative sialidase nor polymerizing genes have been identified in the genome of *L. sakei* 23K, it is likely that only free NANA can be used as an energy source during growth on this sugar alone.

In order to demonstrate the function of this cluster of *nan* genes, several strategies were employed. First, using *L. sakei* strains that were able to grow on NANA as the sole carbon source and strains that were not, we searched to establish a link between the NANA-positive ($NANA^+$) phenotype and the presence of the *nan* cluster. Second, we used *E. coli* mutants from the Keio collection bearing deletions in various components of the *nan* gene cluster and tested the capacity of the *L. sakei* genes to complement these mutations. Finally, we constructed *L. sakei* mutants deficient in each gene belonging to this cluster and studied their phenotype.

The presence of the *nan* cluster in *L. sakei* strains is associated with the ability to grow on NANA. The ability to grow on NANA as the sole carbon source was investigated on chemically defined medium supplemented with NANA (MCD_NANA) for different *L. sakei* strains from our collection. Among 73 strains of *L. sakei* analyzed for intraspecies genomic diversity using 57 individual genes (31), 53 strains were $NANA^+$ and encoded *nanA*, *nanE*, and *lsa1645*. Three $NANA^-$ strains (LTH675, LTH677, and 64) and three $NANA^+$ strains (23K, LV5, and 300) were selected for further characterization. The presence or absence of each of the above-mentioned *nan* genes was ascertained in the six strains by PCR. All of the $NANA^+$ strains harbored the *nanTEAR-nanK* genes as well as *lsa1644-lsa1645*, whereas all the wild-type-mutant strains lacking all the essential genes cannot grow on NANA. Thus, a good correlation was observed between the ability to grow on MCD_NANA and the presence of *nan* genes, including *lsa1644* and *lsa1645*.

***L. sakei nan* genes can complement *E. coli nan* mutants.** As no information on transcriptional regulation was available, we chose to use the complete clusters to test *L. sakei* gene complementation in *E. coli*. Plasmids pRV386 and pRV392, encompassing *nanTEAR-nanK* and *nanTEAR-nanK-lsa1644-lsa1645*, respectively, were introduced into each of the five *E. coli* strains deficient in NanA, NanE, NanK, and NanT or NanR (34). The five *E. coli* deletion mutants were first checked for growth on M63 minimal solid medium supplemented with NANA as the sole carbon source (M63_NANA). As already described (22), only the *E. coli* Δ*nanR* mutant strain was able to grow on minimal medium with NANA, whereas each of the *nanA*, *nanE*, *nanT*, and *nanK* knockout strains was unable to use NANA. Surprisingly, after complementation with pRV386 or pRV392, growth on M63_NANA was restored only for the *E. coli* Δ*nanE* and Δ*nanK* strains (Table 2). The *L. sakei* NanA protein did not complement the *E. coli* NanA deficiency when grown on M63_NANA. Similarly, the *L. sakei* NanT protein did not allow growth of *E. coli* on M63_NANA medium. Since *L. sakei* NanT belongs to the sodium solute symporter family, and because there is no sodium source in the M63 medium, we carried out complementation on the M9 minimal medium containing NANA. Under these conditions, growth on NANA was restored in all *E. coli* mutants by complementation with pRV386 (Table 2). However, when pRV392 containing the two additional genes *lsa1644* and *lsa1645* was used, NANA growth

TABLE 3 Growth of *L. sakei* strains on MCD medium supplemented with various carbon sources (25 mM)

Strain	Carbon source (25 mM)[a]			
	Glucose	NAG	NAM	NANA
23K	+	+	+	+
RV6030 (Δ*lsa1645*)	+	+	+	−
RV6031 [Δ(*nanA-nanE*)]	+	+	−	−
RV6032 (Δ*nanK*)	+	+	+	−
RV6034 (Δ*nanR*)	+	+	−	−
RV6035 (Δ*nanT*)	+	+	+	−

[a] NAG, *N*-acetylglucosamine; NAM, *N*-acetylmannosamine; NANA, *N*-acetylneuraminic acid.

was not restored in *nanA* and *nanT* mutants. As LSA1645 is similar to a Na^+/H^+ antiporter found only in Firmicutes, its presence in *E. coli* might be toxic when complementation of *nanT* occurs. From these heterocomplementation experiments, we conclude that the *L. sakei* cluster studied is indeed involved in NANA catabolism. Moreover, the complementation of *E. coli* deleted for *nanE* and *nanK* is observed independently of the presence of sodium. Since there are structural differences in the *nan* gene cluster between *E. coli* and *L. sakei*, we decided to test the role of the *nan* cluster by inactivating each gene of the *L. sakei* 23K cluster using in-frame deletions.

As shown using isogenic mutants, the *nan* gene cluster of *L. sakei* 23K plays a role in the utilization of NANA. In order to determine the role of the *nan* gene cluster, in-frame deletions of *lsa1645*, (*nanA-nanE*), *nanK*, *nanT*, and *nanR* genes were constructed in *L. sakei* 23K, giving rise to strains RV6030, RV6031, RV6032, RV6033, and RV6034, respectively. The wild-type and knockout strains exhibited similar growth rates on MCD_glucose and MCD_NAG, but while none of the knockout strains were able to metabolize NANA on MCD_NANA, the wild-type strain grew (Table 3). These results indicated that each gene, including *lsa1645*, is involved in NANA catabolism. On NAM, the two mutant strains RV6031 [Δ(*nanA-nanE*)] and RV6034 (Δ*nanR*) were deficient for growth, whereas the other mutant strains grew (Table 3). As the first step in the use of NAM involves the NanE protein, we can conclude that in *L. sakei*, NanE is the NAM-6P epimerase and that NanR probably regulates *nanE* gene expression. It is noteworthy that the absence of growth on NANA of the RV6030 (Δ*lsa1645* mutant) strain suggests that a newly identified protein is involved in catabolism of sialic acid in *L. sakei* (Table 3). As *lsa1645* is the distal gene of the operon *nanK-lsa1644-lsa1645*, we suggest that LSA1644 may be involved in NANA utilization too.

DISCUSSION

The present paper is the first known initial report on the NANA catabolic pathway in *L. sakei*. These genetic approaches allowed us to establish that *L. sakei* NanA, NanE, NanT, NanK, NanM, and NanP play a role in the transport and catabolism of sialic acid. Regulation of the expression of this gene cluster is likely to be different from that described for other bacteria since deletion of the putative regulator *nanR* in *L. sakei* 23K did not lead to the $NANA^+$ phenotype. The *L. sakei* NanR protein and *H. influenzae* SiaR belong to the same RpiR family, but their deletion did not lead to the same phenotype. In our case, *L. sakei* NanR does not seem to be the repressor alone, as its inactivation impaired growth on NANA. Regulation of gene expression for this catabolic pathway should therefore be investigated. A second possibility is that deletion of *nanR* is toxic in the presence of NANA, as has previously been observed for *E. coli* (47). Our results suggest that NanR is likely to activate expression of at least the *nanE* gene, but perhaps also *nanA*, which seems to be structurally cotranscribed with *nanE*. Since we only found three putative promoters upstream from *nanR*, *nanT*, and *nanK*, it is possible that the genes spanning *nanT* to *nanA* constitute an operon whose expression might be activated by NanR. Between the *nanT* and *nanK* start codons, a potential bidirectional catabolic-responsive element (*cre*) site (T GATAACGCTTACA) is present. This box is also present upstream from the *nanR* and *nanE* genes, suggesting potential regulation by the carbon catabolic response. Whether transcription of *nanK*, *lsa1644*, and *lsa1645* is under the control of NanR and/or carbon catabolite protein A (CcpA) repression, as recently described for their counterparts in the Gram-negative *H. influenzae* and *Vibrio vulnificus* species (25, 48), has to be investigated. Moreover, the presence of an additional conserved putative "*nan* box" upstream from *nanR*, *nanT*, and *nanK* suggests that regulation of this system is complex. Whether NanR and/or CcpA could separately or cooperatively be involved in such a regulation remains to be clarified.

The five knockout mutants constructed in *L. sakei* 23K allowed us to demonstrate that the cluster of *nan* genes is functional and is involved in NANA catabolism. To our knowledge, two newly identified proteins not yet described for such a metabolism were first identified in this work, and their functions still remained to be identified. The protein LSA1644 is similar to a putative sugar isomerase/epimerase found in several lactobacilli. We suggest that this protein could play a role in determining the conformation of the NANA molecule that is taken up and propose to name it NanM. In solution, the NANA molecule is mostly found in the α-anomer form whereas the physiological form is a β-anomer (49). Recently, it has been shown in *E. coli* that a periplasmic protein called YjhT, which is well conserved among pathogens, is a mutarotase which facilitates the NANA uptake *in vivo* (49). However, genome analysis seeking such a function in *L. sakei* 23K did not find evidence of any potential gene encoding an eventual NANA-specific mutarotase, and further studies must be carried out in order to define the function of NanM protein. The LSA1645 protein resembles a sodium/H^+ antiporter. We postulate that this protein is involved in the regulation of the intracellular pH or the sodium gradient in *L. sakei*. In fact, internalization and metabolism of sialic acid as a carbon source led to acidification of the medium. Specialized machinery must be present for excreting protons, and such a function is of utmost importance in lactic acid bacteria. Indeed, fermentation of NANA, which has a $pK_a = 2.6$, by lactic acid bacteria could be stressful due to the high rate of lactic acid production during glycolysis. These fermentative bacteria may therefore require highly tuned intracellular pH. In LAB, the observation that similar Na^+/H^+ antiporters (LSA1645 and the *L. plantarum* LP3565, NapA4) are found at a position close to the cluster containing the *nan* genes must be of great importance. The LSA1645 protein might have just such a regulatory role in maintaining internal pH, and we propose to name it NanP. It has recently been reported that in *Vibrio cholerae*, the NhaP1 described to be an $K^+(Na^+)/H^+$ antiporter is required for growth and internal pH homeostasis (50). Although LSA1645 presents no homology with NhaP1, they could play similar roles.

The NANA catabolic pathway, as proposed in Fig. 2, is likely to

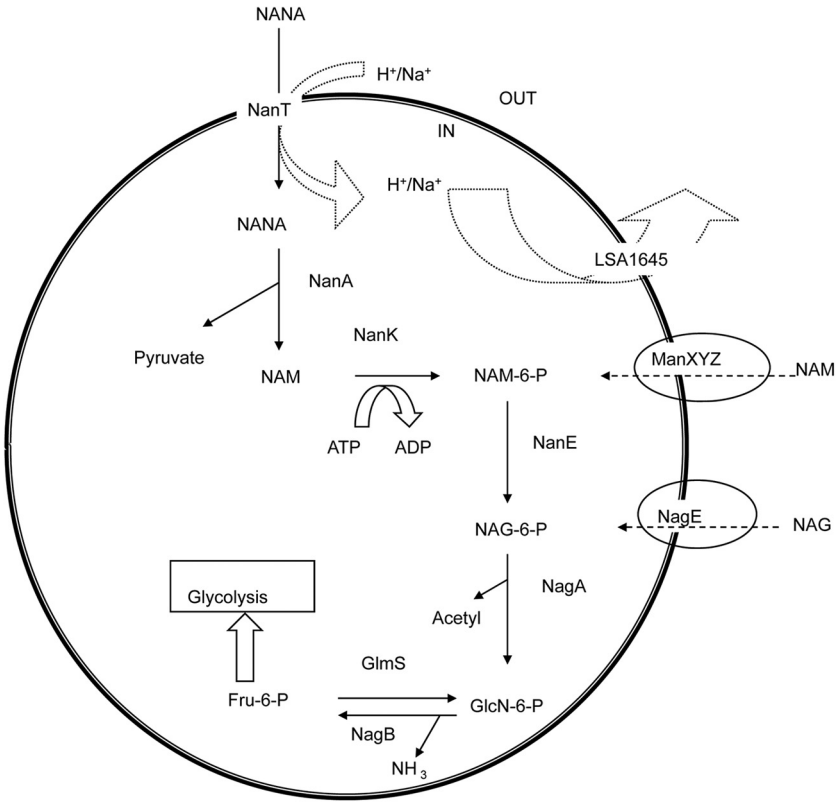

FIG 2 Model proposed for the metabolism of the NANA in *L. sakei*. NANA is transported through NanT, a sodium/proton symporter. Intracellular NANA molecule is cleaved by NanA into pyruvate and NAM. Nam is phosphorylated by NanK into NAM-6-P. NanE epimerase transforms NAM-6-P into NAG-6-P and is deacetylated by NagA into GlcN-6-P which enters the glycolysis pathway. NanP (LSA1645) is supposed to export protons and sodium excess. NAM and NAG pathways are convergent to the NANA metabolism, respectively, transported by ManXYZ and NagE.

be similar to the pathway described in *E. coli* (22) and other bacteria (28), although the regulation and the transport system are specific to *L. sakei*. This means that when grown on NANA, *L. sakei* is able to use it only as an energy source. We suggest that in *L. sakei* 23K, transport of NANA into the cytoplasm by NanT would be concomitant with transport of a sodium cation as previously described (51). The catabolism of NANA into fructose-6P is identical to that described for *E. coli* (22), and NanP would be involved in exporting protons and Na^+ excess from the cytoplasm (Fig. 2) (50).

Taken all together, these data demonstrate that the *L. sakei* 23K cluster of *nan* genes is involved in NANA catabolism and that regulation of the expression of the genes involved this catabolism is unlike that in other bacteria so far described. Transcriptional analysis is under way. This is the first known study describing the NANA catabolic pathway in a Gram-positive lactic acid bacterium.

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