

## Development of Genetic Tools for *Lactobacillus sakei*: Disruption of the $\beta$ -Galactosidase Gene and Use of *lacZ* as a Reporter Gene To Study Regulation of the Putative Copper ATPase, AtkB

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Received 8 May 2000/Accepted 10 July 2000

Downstream from the *ptsHI* operon of *Lactobacillus sakei*, the genes *atkY* and *atkB*, organized in an operon, were observed. The two putative proteins, AtkB and AtkY, show sequence similarity to the *Enterococcus hirae* copper P-type ATPase, responsible for copper efflux, and its negative regulator. Characterization of AtkB as a copper P-type ATPase could not be demonstrated since an *atkB* mutant did not show any phenotype. Thus, another strategy was followed in order to investigate the transcriptional regulation of the *atkYB* locus, leading to the development of new genetic tools for *L. sakei*. A plasmid was constructed, the use of which allowed gene replacement at the *lacLM* locus in *L. sakei* by two successive crossovers. A strain deleted of the *lacLM* operon encoding the  $\beta$ -galactosidase of *L. sakei* was constructed by this method, and the *Escherichia coli lacZ* gene could then be used as a reporter gene to investigate the regulation of *atkYB*. Results show that the *atkYB* operon is induced by small concentrations of  $\text{CuSO}_4$  (30 to 40  $\mu\text{M}$ ) but not when  $\text{CuSO}_4$  is omitted or added at higher concentrations.

The P-type ATPases are a large family of enzymes so named because of a phospho-aspartate intermediate in the ATP-driven cation transport cycle. A wide range of different cations has been demonstrated to serve as substrates for P-type ATPases of prokaryotes (39). All P-type ATPases function as cation pumps, either for uptake, efflux, or exchange. Bacteria have developed specific genes for resistance to the toxic ions of heavy metal elements (see reference 40 for a review). In the chromosome of *Lactobacillus sakei*, downstream from the 3' end of *ptsI* encoding enzyme I of the phosphotransferase system, two open reading frames (ORFs) were observed (41). They showed sequence similarity to a copper efflux P-type ATPase and its negative regulator, which have been cloned from *Enterococcus hirae* (30, 31). Expression of these proteins is regulated by copper in *E. hirae* (29, 42). *L. sakei* is naturally found on meat and meat products, but little information is known about the requirement and sensitivity of this species for metal ions. The requirement for manganese by some bacteria, including lactic acid bacteria, is known (34). It was previously shown that the L-lactate dehydrogenase of *L. sakei* is activated by manganese and cadmium salts (15), and a dipeptidase activated by cobalt and manganese has been purified (25).

Although several genes have now been cloned from *L. sakei* (14) and some genetic tools are emerging (2, 3, 18, 22), the molecular biology techniques specific for this species are still poorly developed. For example, no straightforward reporter gene system has been developed that would help in the analysis of gene regulation. The aim of the present work was to use the

promoter of the *atkYB* locus to identify its possible regulation by heavy metals in order to develop a reporter gene system for *L. sakei*. Several genes have been used as reporter genes in lactic acid bacteria, such as the luciferase genes in *Lactococcus lactis* (5); the *gusA* gene in *L. lactis*, *Leuconostoc lactis*, *Lactobacillus plantarum*, and *Lactobacillus casei* (33); the *nuc* gene in *L. lactis* (21); or the *gfp* gene in *L. plantarum* (12) and *L. lactis* (38). The *lacZ* gene of *Escherichia coli*, encoding  $\beta$ -galactosidase, and the chloramphenicol acetyltransferase gene have also been widely used in bacteria. Among these genes *nuc*, *gusA*, and *lacZ* are very convenient as reporter genes since their expression can be very easily monitored on plates with a chromogenic substrate (17, 21, 24). The activity of  $\beta$ -glucuronidase (*gusA*) and  $\beta$ -galactosidase (*lacZ*) can also be very easily and rapidly quantified on bacterial extracts. *lux* genes encoding luciferase or *gfp* encoding the green fluorescent protein require more expensive equipment, and the luciferase activity might depend on the energetic state of bacteria. The small size of *gusA* might render its use easier than *lacZ*; however, the difficulty of expressing *gusA* in some species of lactic acid bacteria has been reported (33). In *L. sakei*, the presence of a  $\beta$ -galactosidase encoding operon (*lacLM*) in the chromosome (28) enabled the use of *lacZ* since a  $\beta$ -galactosidase activity is already present in this species. For these reasons, several genes could be expressed experimentally in *L. sakei*, but no reporter gene was obviously ideal. Preliminary experiments showed that the luciferase genes could not be expressed at a satisfactory level, and the expression of *gusA* could not be detected. Finally, we decided to use the most widely used reporter gene *lacZ*. First, a vector was constructed that allows gene replacement by two successive crossovers. The *lacLM* operon, encoding *L. sakei*  $\beta$ -galactosidase, was deleted by the use of this vector. Second, we used *lacZ* to construct a chromosomal fusion with the promoter of the *atkYB* operon in the  $\Delta$ *lacLM* mutant. The transcription level of the *atkY::lacZ* fusion in different conditions suggested that AtkB is a copper ATPase operating at low levels of Cu.

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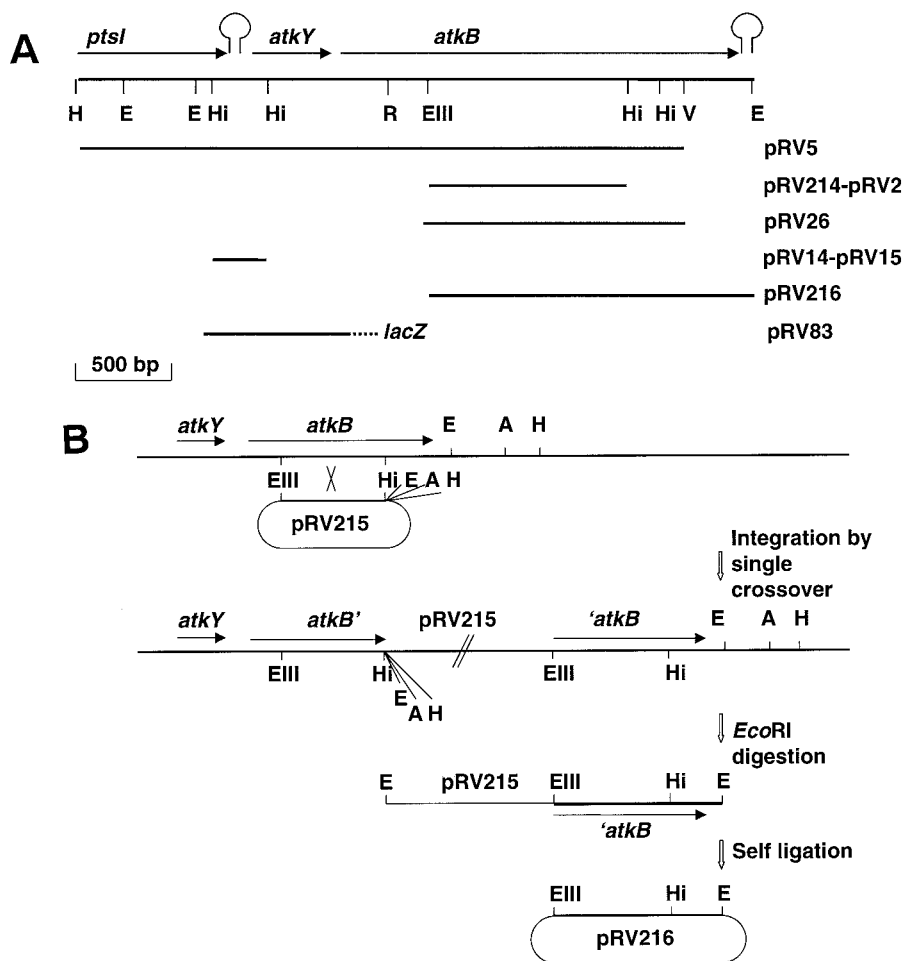


FIG. 1. Organization and restriction map of the *atkYB* region and plasmids. (A) Restriction map of the *atkYB* region and plasmids. The arrows represent the genes. The transcription terminators of *ptsI* and *atkB* are shown. Restriction sites for *EcoRI* (E), *EcoRV* (V), *Eco47III* (EIII), *HindIII* (H), and *HincII* (Hi) are shown. The *RsaI* site (R), upstream from which the DNA sequence was previously determined, is shown. The inserts cloned in various plasmids used in this study are presented on the lower part of the figure. pRV5, pRV14, and pRV15 contain inserts cloned in pBluescript. pRV214, pRV215, pRV27, and pRV83 contain inserts cloned in the integrative vector pRV300. In pRV83, the *lacZ* gene is fused downstream from the *atkYB* promoter. (B) Cloning of the 3' end of *atkB* in pRV216 by chromosome walking. Plasmid pRV215 was introduced into the chromosome of *L. sakei* 23K by single crossover. In the resulting transformant, the 3' end of *atkB* plus the part of plasmid which is replicative in *E. coli* and including selective markers, were surrounded by two *EcoRI* (E), *AccI* (A), and *HindIII* (H) sites. After digestion of chromosomal DNA, self-ligation, and transformation of *E. coli* to ampicillin resistance, plasmid pRV216 carrying the 3' end of *atkB* was isolated.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** *E. coli* strains TG1 (37), TG90 (13), and DH5 $\alpha$  (37) were used for cloning and subcloning experiments and for plasmid propagation. The *E. coli* strain GM2929 carrying a *dem* mutation was used for the propagation of plasmids prior to digestions with the *BalI* restriction enzyme. *E. coli* XL1 Blue (Stratagene) was used for single-strand DNA preparation. *L. sakei* 23K, a laboratory strain originally isolated from sausage and cured of plasmids, was used as the recipient strain for cloning experiments and for the construction and analysis of mutants. Fragments cloned into plasmids and their subsequent names are shown in Fig. 1A. The *HincII* fragment from pRV5 (41) was cloned in both orientations in the phagemid pBluescript (Stratagene), yielding to pRV14 and pRV15. The *Eco47III/HincII* fragment was cloned in both orientations in the integrative vector pRV300 (22), yielding pRV214 and pRV215. The plasmid pRV216 was obtained by chromosome walking (see the text and Fig. 1B), and pRV27 contains an internal fragment of *atkB* resulting from a deletion in pRV5 generated by exonuclease III, followed by the cloning of the erythromycin resistance gene of pAM $\beta$ 1 in the pBluescript moiety. The plasmid pRV80 was obtained by the cloning in pRV300 of two PCR-amplified DNA fragments containing, respectively, the 5' and 3' ends of *lacLM* with a large internal deletion. pRV81 was obtained by the cloning of the *atkYB* promoter region upstream from *lacZ* in pJM783 (32). pRV83 was obtained by cloning the *EcoRI/BalI* fragment of pRV81, containing the *atkY* operon and the *lacZ* gene, into the integrative plasmid pRV300.

**Media, growth conditions, and transformations.** *E. coli* strains were grown in Luria-Bertani medium (37) at 37°C. *L. sakei* strains were grown at 30°C in the

complex medium MRS (6) or in the defined medium MCD (19). *E. coli* and *L. sakei* electrocompetent cells were prepared and transformed by the methods of Dower et al. (8) and Berthier et al. (3), respectively. *E. coli* transformants were selected on ampicillin at 100  $\mu\text{g} \cdot \text{ml}^{-1}$  or erythromycin at 200  $\mu\text{g} \cdot \text{ml}^{-1}$ . *L. sakei* transformants were selected on erythromycin at 5  $\mu\text{g} \cdot \text{ml}^{-1}$ .

**Oligonucleotides and PCR.** Primers lac1 (5'-GATCAAGCTTATGCTTTAA GGGTACTGG), lac2 (5'-ACGTGAATTCCTGTTCATCGGACGTTGAA), lac4 (5'-GATCGAGCTCGCGCTTGAACAATAGCT), and lac6 (5'-ACGTGAAT TCCGGTGCTGGATAATTGTT) were used for the PCR amplification of two DNA fragments corresponding, respectively, to the 5' and 3' ends of the *L. sakei* 23K *lacLM* operon. Restriction sites added at the 5' end of each primer are underlined. Primers ATK1 (5'-GATCGAATTCCTAGTCGAAGATTTTATGA) and ATK3 (5'-GATCGGATCCCATCGTGTTCATCGTTA) were used to generate a fragment containing the promoter and regulatory region of *atkYB*, plus the upstream transcription terminator of *ptsI*. PCR experiments were performed on a Perkin-Elmer 9600 apparatus, with *Taq* DNA polymerase from Boehringer. Reactions were carried out in 100- $\mu\text{l}$  mixtures containing 0.2 mM concentrations of each deoxynucleoside triphosphate, 1  $\mu\text{g}$  of chromosomal DNA template, and 2.5  $\mu\text{M}$  concentrations of each primer. Amplification was performed for 30 cycles (94°C for 1 min, 55°C for 2 min, and 72°C for 3 min). The PCR reactions on chromosomal DNA of mutants, to verify the integration of recombinant plasmids, were performed in the same conditions, but the volume of the reaction mixtures was 10  $\mu\text{l}$ .

**Nucleic acid manipulation.** DNA and RNA were prepared by standard methods as previously described (41).



was also found in AtkB (positions 328 to 330). Among the known P-type ATPases, only CopB, Hra1, and Hra2 contain a CPH instead of the CPC motif usually found in other P-type ATPases and which might be part of the cation channel. Another feature of P-type ATPases is the DKTGTLLT motif, with the D residue being phosphorylated and dephosphorylated during transport (1). This motif is conserved in AtkB (positions 372 to 378). The GDGVND motif, which is believed to be part of the nucleotide-binding domain (4), was also observed in AtkB from positions 569 to 574. The putative transmembrane helices of AtkB were investigated by the TM-pred of BCM launcher and the SAPS program. The compilation of the two methods predicted eight transmembrane helices for AtkB.

**Construction and analysis of *atkB* mutants.** The sequence of *atkB* suggests that it encodes a P-type ATPase. Since the identity of AtkB is higher to the CopB of *E. hirae*, responsible for copper efflux, than to the CopA responsible for copper uptake in *E. hirae*, one could expect that AtkB encodes a protein also responsible for copper efflux. Mutation of such efflux P-type ATPases might lead to a higher sensitivity to the transported ion salt, which should be accumulated in the mutant, as has been described in *E. hirae*, *Synechococcus* sp., and *Helicobacter pylori* (11, 20, 31). Two *atkB* mutants were constructed by insertional mutagenesis in *L. sakei* 23K. For this purpose, two plasmids were used. First, a clone derived from pRV5 was obtained by exonuclease III deletion. It contained an internal part of *atkB* missing its 5' and 3' ends. The erythromycin resistance gene of pAM $\beta$ 1 was then cloned at the *Ssp*I site of the pBluescript moiety, leading to pRV27 (Fig. 1A). Second, the plasmid pRV215 was constructed, which contained an internal fragment of *atkB* cloned in pRV300. These plasmids were used to transform the strain 23K for erythromycin resistance. Transformants obtained by single crossover integration of pRV27 or pRV215 were grown in MCD medium containing increasing concentrations of various heavy metal ions [ $\text{CuSO}_4$ , 0.1 to 1.2 mM;  $\text{Cd}(\text{CH}_3\text{COO})_2$ , 1 to 7  $\mu\text{M}$ ;  $\text{CoCl}_2$ , 0.125 to 1.5 mM] or NaCl (3 to 5%). The wild-type strain was grown under the same conditions, and the OD was measured after 16 h of growth. No significant difference was observed between the parent strain and the two *atkB* mutants (data not shown). The copper sensitivity of the mutants was also tested either by streaking or by depositing a drop of a fresh culture on MCD plates containing 10 to 500  $\mu\text{M}$   $\text{CuSO}_4$ . No difference was observed between the parent strain and the two *atkB* mutants. Exponential growth was also followed on cultures in MCD medium containing  $\text{CuSO}_4$ ,  $\text{Cd}(\text{CH}_3\text{COO})_2$ ,  $\text{CoCl}_2$ , or NaCl. Concentrations affecting the growth of the wild-type strain were chosen, but no significant differences could be observed between the three strains 23K (data not shown). Thus, although sequence similarities suggested that AtkB was a copper P-type ATPase, the analysis of two *atkB* mutants could not confirm the function of AtkB. In order to obtain more information on the putative role of AtkB, we investigated its regulation. For that purpose, an approach using a reporter gene was chosen.

**Construction of a *lacLM* mutant by double crossover for use as recipient strain of *lacZ* fusions.** No reporter gene is yet available for *L. sakei*. The plasmid described by Platteeuw et al. (33) carrying the  $\beta$ -glucuronidase encoding gene (*gusA*) designed for lactic acid bacteria was used to construct an *atkYB::gusA* fusion. However, as was previously mentioned by these authors for other lactobacilli, *gusA* was not expressed in *L. sakei*. Although the fusion was expressed in *E. coli*, no activity could be detected in *L. sakei*. This prompted us to try other genes. Among several genes usually used as reporter genes, the *lacZ* gene of *E. coli* has several advantages since the

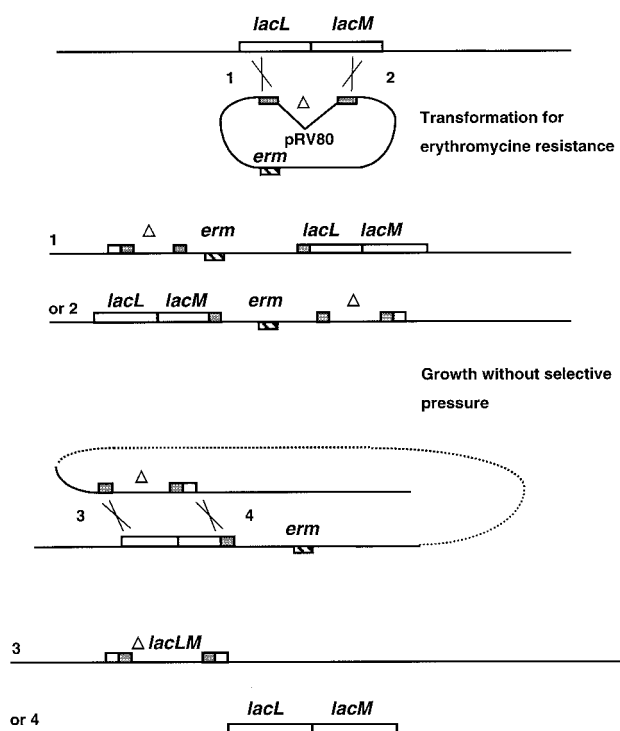


FIG. 3. Construction of an internal deletion in the *lacLM* operon. Plasmid pRV80 contains the 5' end of *lacL* and the 3' end of *lacM*, with a deletion of an internal part of *lacLM*. pRV80 was introduced in the chromosome of *L. sakei*. Transformants, selected on erythromycin, can result from a crossover at position 1 or position 2. In each case, one copy of the WT *lacLM* operon and one copy of the  $\Delta lacLM$  operon are present. The duplication of part of the *lacLM* operon is unstable. By growing one transformant without erythromycin, strains resulting from a second crossover can be isolated. The plasmid can excise at position 3, leading to replacement of *lacLM* by  $\Delta lacLM$ , or at position 4, restoring a wild-type genotype.

$\beta$ -galactosidase activity can easily be detected both on plates and in bacterial extracts. Some *L. sakei* strains possess a  $\beta$ -galactosidase or a phospho- $\beta$ -galactosidase activity (27). The strain 23K, used as the recipient strain, has a  $\beta$ -galactosidase. In order to use *lacZ* as a reporter gene, it was therefore necessary to first delete the *lacLM* operon of *L. sakei* 23K encoding the *L. sakei*  $\beta$ -galactosidase. The *lacLM* operon of *L. sakei* DSM 20017 was previously cloned and sequenced (28). Oligonucleotides deduced from this sequence were designed in order to amplify two fragments corresponding to the 5' end and the 3' end of *lacLM*. A first PCR fragment, obtained with the *lac1* and *lac2* primers, was 513 bp long and contained the *lacLM* promoter and the 5' end of *lacL*. The second fragment, obtained with the *lac4* and *lac6* primers, was 522 bp long and contained the 3' end of *lacM* and the downstream region. A restriction site was present at the 5' extremity of each primer. The two resulting PCR fragments were cloned in the pRV300 integrative vector. In the resulting plasmid pRV80, the *lacLM* operon contains a 2,193-bp internal deletion. pRV80 was then used to transform *L. sakei* 23K for erythromycin resistance (Fig. 3). The correct insertion of pRV80, by a Campbell-like recombination, at the *lacLM* locus was checked by a PCR experiment using the *lac1* and *lac4* primers. One transformant, RV2001, was then kept for further experiments. Since pRV80 contains the 5' end and the 3' end of the *lacLM* operon, its insertion in the chromosome by a single crossover in *lacLM* restored one copy of the wild-type operon and was therefore

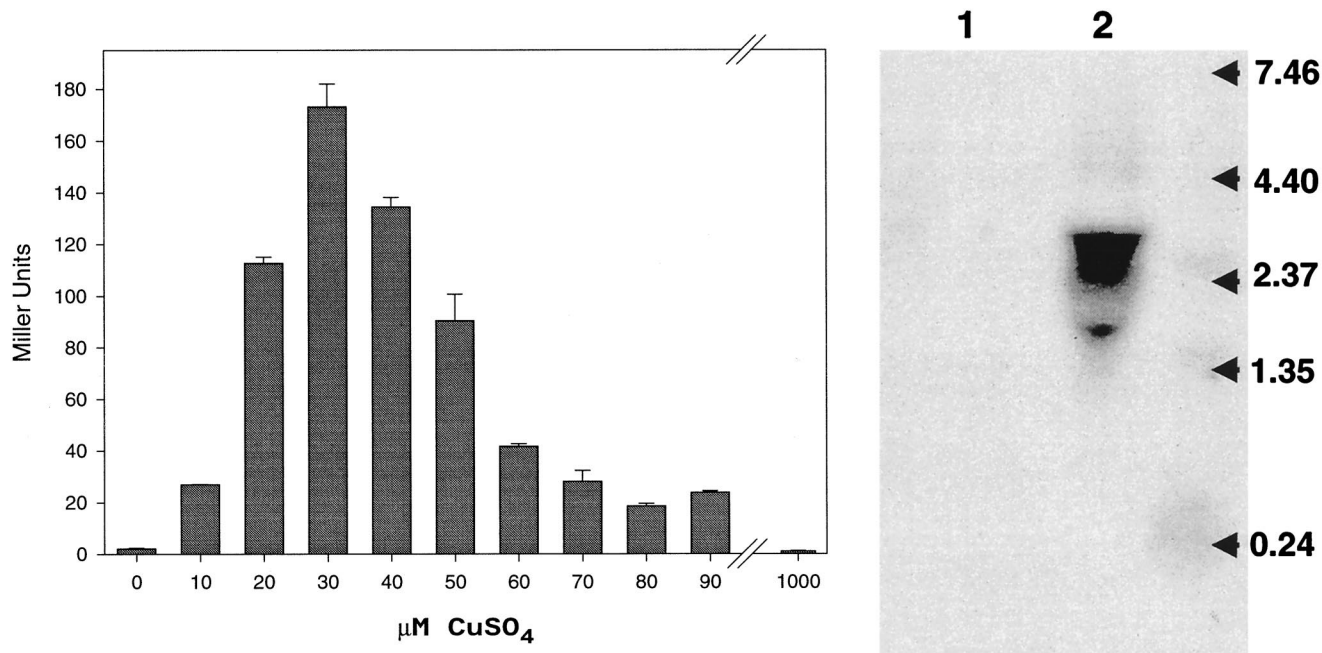


FIG. 4. Expression of the *atkYB* operon. (Left) Induction of  $\beta$ -galactosidase activity of the *atkYB::lacZ* fusion in RV1023 by increasing  $\text{CuSO}_4$  concentration in the medium. (Right) Northern blot of RNA isolated from 23K induced with 40  $\mu\text{M}$   $\text{CuSO}_4$  (lane 2) or noninduced (lane 1) and hybridized with *atkB*. The size of the molecular weight ladder is indicated.

not mutagenic. Additionally, the insertion of pRV80 led to the duplication of part of the *lacLM* operon, a structure which is unstable unless selective pressure is maintained by the addition of erythromycin. In the absence of selective pressure, the plasmid can excise, by a second crossover, and is then lost since pRV300 cannot replicate in *L. sakei*. Since one copy of the wild-type *lacLM* operon and one copy of the mutated *lacLM* operon are present after the first recombination, the excision of the plasmid could lead to the excision either of the wild-type *lacLM* copy or of the  $\Delta$ *lacLM* copy. The excision of the mutated copy would restore a wild-type genotype, whereas the excision of the wild-type copy would lead to a copy of the *lacLM* operon with the internal deletion (Fig. 3). In order to generate the second crossover, the RV2001 transformant was grown in MRS without erythromycin. After 100 generations, diluted culture aliquots were plated on MRS containing X-Gal and no erythromycin. Of 300 clones, two were white. The structure of the *lacLM* operon was verified by PCR on the chromosomal DNA extracted from these two clones, with primers complementary to various parts of *lacLM*. The expected 2.2-kb deletion of the internal part of *lacLM* was demonstrated. PCR experiments performed with combinations of primers complementary to *lacLM* and reverse or universal primers complementary to pRV300 were negative. Moreover, these two clones were erythromycin sensitive and had thus lost the pRV300 moiety. This confirmed that the two strains were resulting from the expected recombination. One of the two clones was named RV2002 and was used for further constructions.

**Construction of an *atkY::lacZ* transcriptional fusion.** Plasmid pJM783 contains the *lacZ* gene of *E. coli* and a chloramphenicol resistance gene (32). This plasmid has already been largely used to construct transcriptional or translational fusions in *Bacillus subtilis*. A 634-bp DNA fragment, containing the transcription terminator of *ptsI*, the promoter region of *atkYB*, and the repressor encoding gene *atkY*, was amplified by

PCR with primers ATK1 and ATK3 and cloned in pJM783, in front of *lacZ*. Then, the *lacZ* gene, fused to the *atkYB* promoter region described above, was cloned in the integrative plasmid pRV300. The resulting plasmid, pRV83 (Fig. 1A) was used to transform RV2002, which is deficient for  $\beta$ -galactosidase activity. The resulting strain, RV1023, contains an insertion of the *atkYB::lacZ* fusion and an intact copy of the *atkYB* operon. The  $\beta$ -galactosidase activity of RV1023 was tested on MCD plates containing X-Gal. Fifty microliters of various solutions of metals or salts ions was added in the center of the plates and allowed to diffuse from the center to the periphery of the plates. Only copper induced the expression of the *atkYB::lacZ* fusion, as seen from the blue color of the clones. However, the color was observed only at a certain distance from the center of the plates, suggesting that the fusion was induced by a precise concentration of  $\text{CuSO}_4$ . Furthermore, the activity was higher when the plates were incubated at room temperature than at 30°C. RV1023 was grown in liquid MCD medium to an  $\text{OD}_{600}$  of 0.3, and then various concentrations of  $\text{CuSO}_4$  were added and the cultures were incubated for 90 min at room temperature. The  $\beta$ -galactosidase activities measured on crude extracts obtained from these cultures are shown in Fig. 4 (left panel). As expected from the observation made on the plates, an optimal concentration of  $\text{CuSO}_4$  which induced expression of the *atkYB::lacZ* fusion was found (30 to 40  $\mu\text{M}$ ), which is much lower than the concentration required to inhibit the growth of *L. sakei*.

In order to confirm copper induction of the *atkB* gene, Northern blot experiments were performed with RNAs isolated from bacteria grown under the same conditions as described for the measurement of  $\beta$ -galactosidase activity. The probe used was the internal part of *atkB* in pRV214. A 2.7-kb mRNA was detected in the 23K strain induced by 40  $\mu\text{M}$   $\text{CuSO}_4$ , which was absent in the noninduced bacteria (Fig. 4, right panel). This size correlates with a transcript initiated at

the putative promoter located upstream from *atkY* and ending at the palindromic sequence, downstream from *atkB*.

**Conclusion.** The two genes found downstream from the *ptsHI* operon of *L. sakei* are homologous to a copper P-type ATPase responsible for copper efflux and its negative regulator. The construction of mutants in the gene encoding the putative copper ATPase did not lead to clear identification of its function, since no sensitivity to copper nor to other ions could be observed. It is possible that a second gene, coding for a protein able to export copper, is present on the *L. sakei* chromosome. The use of *lacZ* as a reporter gene and Northern blot experiments showed that the *atkYB* operon is induced when small concentrations of  $\text{CuSO}_4$  were added to the medium. Although the function of AtkB could not be demonstrated, this study suggests that it is indeed a copper ATPase. Among other known copper P-type ATPases, CopA and CopB from *E. hirae* have been extensively studied, and it was shown that these two ATPases, which are part of the same operon, are induced by high concentrations of copper (1 mM) or when copper is depleted from the medium (29). In *E. hirae*, *copZ* and *copY*, encoding a positive and a negative regulator, respectively, are also present in the operon. In *L. sakei*, only genes similar to *copY* and *copB* are present in the operon. Whether *copZ* and *copA* are present somewhere else on the chromosome is not yet known. The gene organization in *L. sakei*, which is different from what was described for *E. hirae*, might result in a different regulation. Finally, since AtkB was not expressed at a high (1 mM) copper concentration, this suggests that it is not required at this concentration and might explain why no hypersensitivity to a high concentration of copper was observed in the *atkB* mutants.

The strain RV2002 was obtained by two successive crossovers. The construction of such a stable deletion mutation represents a new method for the construction of mutants in *L. sakei*. The use of pRV300 to generate the first crossover, followed by growth without selective pressure to generate the second, mutagenic crossover will allow the construction of a new generation of *L. sakei* mutants with the possibility of generating point mutations, large deletions, or stable insertions. The resulting mutants, containing point mutations or deletions, will not contain any trace of heterologous DNA and might thus be considered as food-grade strains. Plasmid pVR80, derived from pRV300, was used to generate a deletion in *lacLM*. Plasmids derived from pRV80 containing foreign genes should allow gene replacement at the *lacLM* locus by using X-Gal as an indicator to select recombinant clones. The strain RV2002 ( $\Delta lacLM$ ) was then used as a recipient strain to introduce a *atkYB::lacZ* fusion that allowed investigation of the *atkYB* regulation. This study shows that *lacZ* can be used as reporter gene in *L. sakei*. Since genetic tools based on pRV300 designed for *L. sakei* have been successfully used in other lactic acid bacteria, such as *L. casei* and *L. lactis* (7, 9, 35), new pRV300 derived vectors containing *lacZ* will be helpful in studying gene regulation in these species. Furthermore, the PCR-amplified fragment containing the transcription terminator of *ptsI*, the copper-inducible promoter of *atkYB*, *atkY*, and a few codons of *atkB*, might be used to expressed foreign genes in *L. sakei*, with an expression controlled by the addition of small amounts of  $\text{CuSO}_4$ .

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