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Construction of a Xylanase-Producing Strain of *Brevibacterium lactofermentum* by Stable Integration of an Engineered *xysA* Gene from *Streptomyces halstedii* JM8

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A xylanolytic strain of *Brevibacterium lactofermentum* containing the *Streptomyces halstedii* His-tagged *xysA* gene was generated. The new strain contains DNA derived from *S. halstedii*, expresses xylanolytic activity, and was obtained by an integrative process mediated by a conjugative plasmid targeted to a dispensable chromosomal region located downstream from the essential cell division gene *ftsZ*. The His-tagged Xys1 enzyme was constitutively expressed under the control of the *kan* promoter from Tn*5* and was easily purified by use of Ni-nitrilotriacetic acid-agarose. The new strain is stable for more than 200 generations, lacks any known antibiotic resistance gene, and does not need any selective pressure to maintain the integrated gene. This strategy can be used to integrate any gene into the *B. lactofermentum* chromosome and to maintain it stably without the use of antibiotics for selection.

Coryneform bacteria are industrially used for amino acid production and, due to the lack of detectable extracellular hydrolytic enzymes (cellulases, glucanases, xylanases, proteases, and so forth), the production medium in the fermentation industry is mainly composed of beet molasses and protein hydrolysates. However, the use of recombinant DNA technology has led to the construction of *Brevibacterium lactofermentum* strains able to degrade starch (3, 23), xylan, and cellulose (1) by cloning of the corresponding genes in corynebacterial or *Escherichia coli*-corynebacterial plasmids. The induction of such abilities is a marked improvement for any industrial strain because of the lower cost of the substrates and the production of extracellular enzymes by these microorganisms. However, the above-mentioned strains carry plasmids and antibiotic resistance genes and thus might not be useful in the near future because of stringent regulations on genetically manipulated microorganisms, especially when the fermentation product is to be used in human or animal food (food-grade microorganisms) (5, 14).

To overcome this problem, we designed a method that can be used to integrate any gene in the genome of *B. lactofermentum* by double recombination. There are several descriptions of plasmids that can be used to integrate genes in the genome of corynebacteria based on the presence of a chromosomal gene in the plasmid (18, 19, 22) or very short homologous DNA segments (8 to 12 bp) in the vector and in the host DNA (15). However, all of them require the presence of antibiotics for selection. The generation of a drug-resistant recombinant strain can both reduce the in vivo applicability of the strain and preclude the use of recombinant vectors that use the same drug resistance marker.

Here we describe the construction of a suicide conjugative plasmid, pK18-3, which allows the recombination of any exogenous gene onto the *B. lactofermentum* chromosome. The exogenous gene integrates by double recombination in one of the three nonessential open reading frames (ORFs) located downstream from *ftsZ* in the genome of *B. lactofermentum* (8, 9), and the resulting strain lacks any exogenous drug resistance marker. The method was checked by the integration of the His-tagged *xysA* gene from *Streptomyces halstedii* JM8 (20).

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1.

Culture conditions and transformation procedures. The culture media for *B. lactofermentum* were tryptic soy broth (TSB) (21) and S2 (16). The plasmids to be transferred by conjugation from *E. coli* to *B. lactofermentum* were introduced by transformation into donor strain *E. coli* S17-1 (Table 1). *B. lactofermentum* R31 was used as the recipient strain. Conjugation between *E. coli* and *B. lactofermentum* was performed as described by Fernández-González et al. (4). For direct selection of antibiotic-resistant transconjugants, tryptic soy agar (TSA) plates (21) were supplemented with kanamycin at a final concentration of 30 μg ml^{-1}.

E. coli cells were grown in Luria broth medium (17) and transformed as described by Hanahan (6). Transformants were selected on Luria agar plates containing 100 μg of kanamycin ml^{-1}.

DNA isolation, manipulation, and characterization. Plasmid preparations were obtained from *E. coli* as described by Holmes and Quigley (7). Total DNA from corynebacteria was isolated using a method described for *Streptomyces* (10), but cells were treated with lysozyme for 3 h at 30°C. Total DNA from *B. lactofermentum* transconjugants was digested with different restriction enzymes and hybridized with the *kan* gene, with the *xysA* gene, or with the 3.3-kb *Bam*HI fragment of *B. lactofermentum* containing the three ORFs (YFIH, ORF5, and ORF6) (probe 3) labeled with digoxigenin according to the manufacturer's instructions (Boerhinger Mannhein). The rest of the DNA manipulations were performed using standard procedures (10).

Xylanase assays. The production of xylanase by *B. lactofermentum* transconjugants was assayed on TSA containing 0.3% Remazol brilliant blue R–D-xylan (Sigma) or on minimal medium or TSA supplemented with 0.4% xylan. After 2 days of growth, the plates were flooded with Congo red (0.1%) and washed with NaCl (1 M), and clear zones around the colonies due to xylan degradation were observed.

Xylanase activity in the culture broth was calculated by measuring the amount of reducing sugars released from 0.4% (wt/vol) oat spelts xylan at 60°C for 10 min

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or description	Reference or source
Strains		
E. coli DH5α	r^-m^-; used for general cloning experiments	6
E. coli S17-1	Used for conjugation experiments	22
B. lactofermentum 13869	Wild type	American Type Culture Collection
B. lactofermentum R31	13869 derivative used as a host for transformation, electroporation, or conjugation experiments	21
B. lactofermentum X1	$XylA^+$ Kan^r R31 derivative containing plasmid pK18-3X integrated in the chromosome by a single recombination event	This work
B. lactofermentum X2	$XylA^+$ Kan^s R31 derivative containing the six-His-tagged Δ*xysA* gene integrated in the chromosome by double recombination	This work
Plasmids		
pK18mob	Mobilizable plasmid containing an *E. coli* origin of replication and *kan*	22
pK18-3	pK18mob derivative carrying a 3.3-kb *Bam*HI fragment from the *B. lactofermentum* chromosome containing part of *fts*Z and the ORFs YFIH, ORF5, and ORF6	This work
pIJ2925	pUC18 derivative with *Bgl*II sites flanking a modified multiple cloning site	11
pXHis-Npro	pIJ2925 derivative containing the DNA fragment six-His-tagged Δ*xysA* (encodes the catalytic domain of xylanase Xys1 with a tag of six histidines) under the control of the promoter of the *kan* gene from Tn*5* and flanked by the terminator of the methylenomycin resistance gene (T1) and the terminator of phage fd (T2)	
pK18-3X	pK18-3 derivative carrying His-tagged xylanase from plasmid pXHis-Npro	This work
pUL880M	Bifunctional *E. coli-B. lactofermentum* promoter-probe vector with *bla* and *hyg* genes as selective markers and the promoterless *kan* gene as a reporter gene	13
p880X2A	pUL880M derivative containing six-His-tagged P*kan*-Δ*xysA* in the same orientation as the *kan* gene; the *kan* gene is expressed in *E. coli* and *B. lactofermentum* transformed with this plasmid	

as described previously (1a). Protein concentrations in the culture broth were determined as described by Bradford (2) using bovine serum albumin as a standard.

PAGE and Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) of the culture broth was carried out essentially as described by Laemmli (12). Electrophoresis was performed at room temperature with a vertical slab gel (170 by 130 by 1.5 mm) and 10% (wt/vol) polyacrylamide at 100 V and 60 mA. After electrophoresis, proteins were stained with Coomassie blue or electroblotted on polyvinylidene difluoride membranes (Millipore) and immunostained with rabbit polyclonal antiserum raised against purified Xys1 from *S. halstedii*.

Purification of His-tagged xylanase from *B. lactofermentum*. Purification of the His-tagged xylanase from the culture medium of *B. lactofermentum* X1 or *B. lactofermentum* X2 by using Ni-NTA agarose was performed according to the standard procedures of the manufacturer (Qiagen).

RESULTS AND DISCUSSION

Construction of a conjugative plasmid designed to insert any gene into the chromosome of *B. lactofermentum*. A method for inserting any DNA fragment into the chromosome of *B. lactofermentum* has been developed. The method relies on the suicide conjugative plasmid pK18-3, a pK18mob derivative containing (i) a gene conferring resistance to kanamycin as a selectable marker, (ii) a chromosomal region necessary for its integration into the *B. lactofermentum* chromosome, and (iii) a unique *Nde*I restriction site useful for cloning. Any gene that we wish to integrate (under the control of a promoter capable of functioning in *B. lactofermentum*) is cloned into the unique restriction site of the plasmid, transformed into *E. coli*, and transferred by conjugation to *B. lactofermentum*.

The initial plasmid used was pK18mob, an *E. coli* plasmid described by Schäfer et al. (22) as a suicide vector for *B. lactofermentum* carrying a kanamycin resistance gene that is expressed in both *E. coli* and *B. lactofermentum*. It contains the origin of replication of ColE1, the broad-host-range transfer machinery of plasmid RP4, the *lacZ*α gene, and a multiple cloning site. This plasmid cannot replicate in *B. lactofermentum* but can be transferred by conjugation from *E. coli* S17-1. This plasmid has been used to interrupt several genes in *B. lactofermentum* or *Corynebacterium glutamicum* (4, 8, 22).

The chromosomal region used for integration of the exogenous DNA is that corresponding to three ORFs of unknown function located downstream from *ftsZ* in the *B. lactofermentum* chromosome (9). It has previously been demonstrated that interruption of any of the three ORFs is not deleterious for *B. lactofermentum* growth or cell division (8).

The 3.3-kb *Bam*HI fragment containing the three ORFs was cloned into the unique *Bam*HI restriction site of pK18mob, giving rise to plasmid pK18-3; as indicated in Fig. 1, there is a single restriction site for *Nde*I in pK18-3 that can be used for the cloning of any exogenous DNA fragment.

The six-His-tagged Δ*xysA* gene from *S. halstedii* under the control of the promoter of the *kan* gene from Tn*5* and flanked by terminators was obtained from plasmid pXHis-Npro (Table 1) and subcloned as a 2.6-kb *Bgl*II fragment (Klenow filled) into the unique *Nde*I site (Klenow filled) of pK18-3. The ligation mixture was transformed into *E. coli* DH5α competent cells, and selection was made for kanamycin resistance and xylanase production. Plasmid DNA was isolated from a Kan^r Xyl^+ transformant and named pK18-3X. In *E. coli*, xylanase expression takes place from the P*kan* promoter because of the presence of the terminators (1).

Integration of pK18-3X in the chromosome of *B. lactofermentum* by single recombination. Because chromosomal inte-

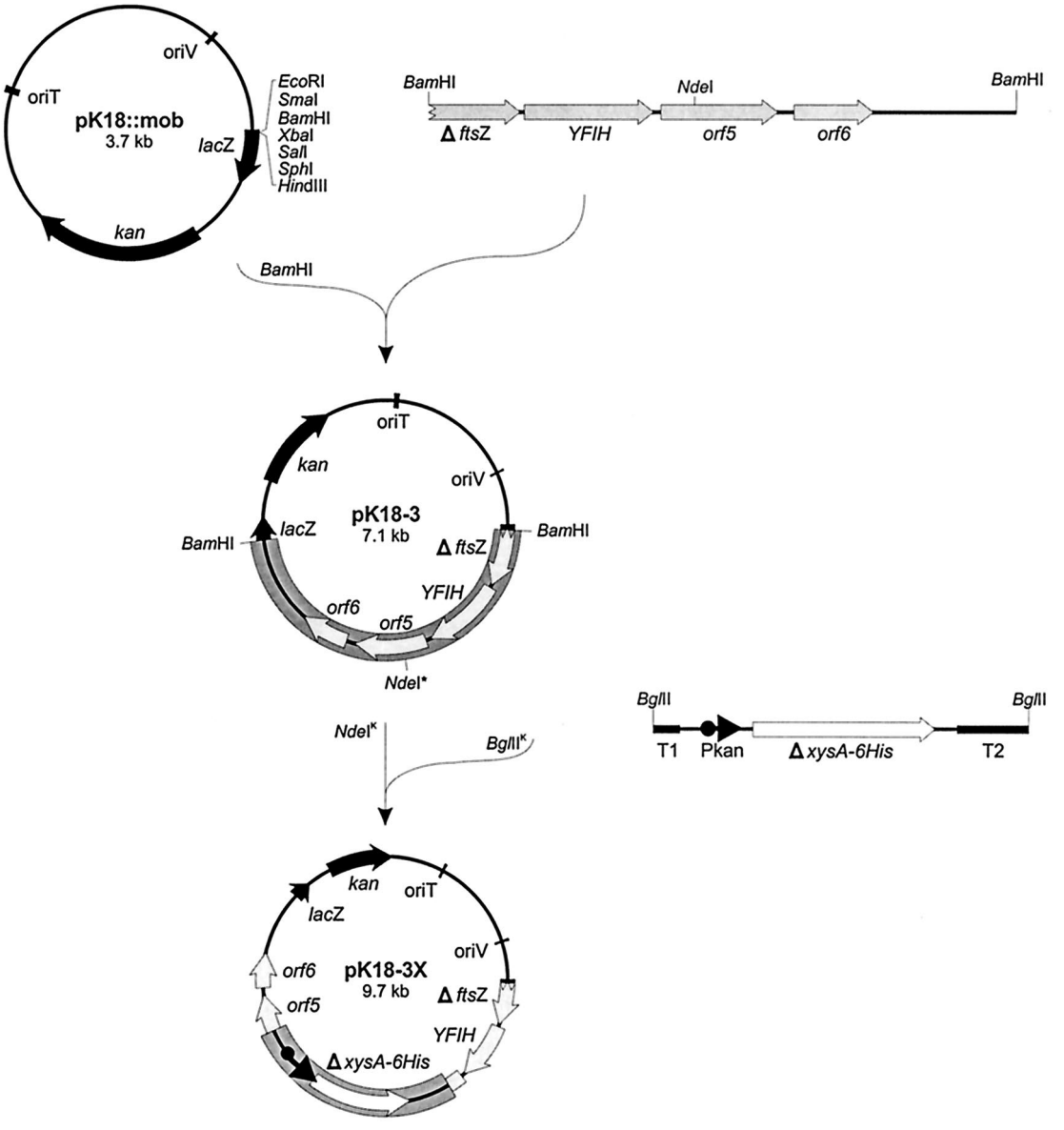

FIG. 1. Schematic representation of the construction of the suicide plasmid pK18-3 designed to deliver any gene into the *B. lactofermentum* chromosome and subcloning of the six-His-tagged xylanase gene (Δ*xysA-6His*) into the unique *Nde*I site (*Nde*I*). For details, see the text. *Nde*I^K and *Bgl*II^K, Klenow-filled *Nde*I and *Bgl*II; P*kan*, promoter of the *kan* gene from Tn*5*; T1, terminator of the methylenomycin resistance gene from *Streptomyces coelicolor* A3 (2); T2, terminator of phage fd.

gration events occur rarely, plasmids used for integration do require the concomitant integration of a drug resistance marker in order to identify colonies of recombinant cells. The conjugative suicide plasmid pK18-3X was transferred by conjugation from *E. coli* S17-1(pK18-3X) cells to *B. lactofermentum* R31, and *B. lactofermentum* kanamycin-resistant transconjugants were readily obtained (10^{-2} to 10^{-3} transconjugants per recipient colony). They expressed xylanolytic activity on 0.3% Remazol brilliant blue R–D-xylan plates, in contrast to the lack of xylanolytic activity of *B. lactofermentum* R31. Because of the relative position of the xylanase gene inside ORF5 (Fig. 2) and the presence of terminators, the *xysA* gene should be expressed in *B. lactofermentum* from the P*kan* promoter. Total DNA isolated from 10 transconjugants did hybridize with the *kan* gene, with the *xysA* gene, and with probe 3 (Fig. 2). Two *Bam*HI chromosomal DNA fragments, one corresponding to the original fragment in the chromosome (3.3 kb) and a second one (5.9 kb) corresponding to the sum of the original fragment (3.3 kb) plus six-His-tagged Δ*xysA* (2.6 kb), hybridized with probe 3 (Fig. 2); the 5.9-kb *Bam*HI DNA band also hybridized with the *xysA* gene, as expected. The integrated plasmid is stable, and the xylanase is stably expressed without continued selection for kanamycin. One of the 10 *B. lactofermentum* transconjugants was named *B. lactofermentum* X1.

Because kanamycin-resistant strain X1 has reduced applicability in vivo, we attempted to construct a double-recombinant

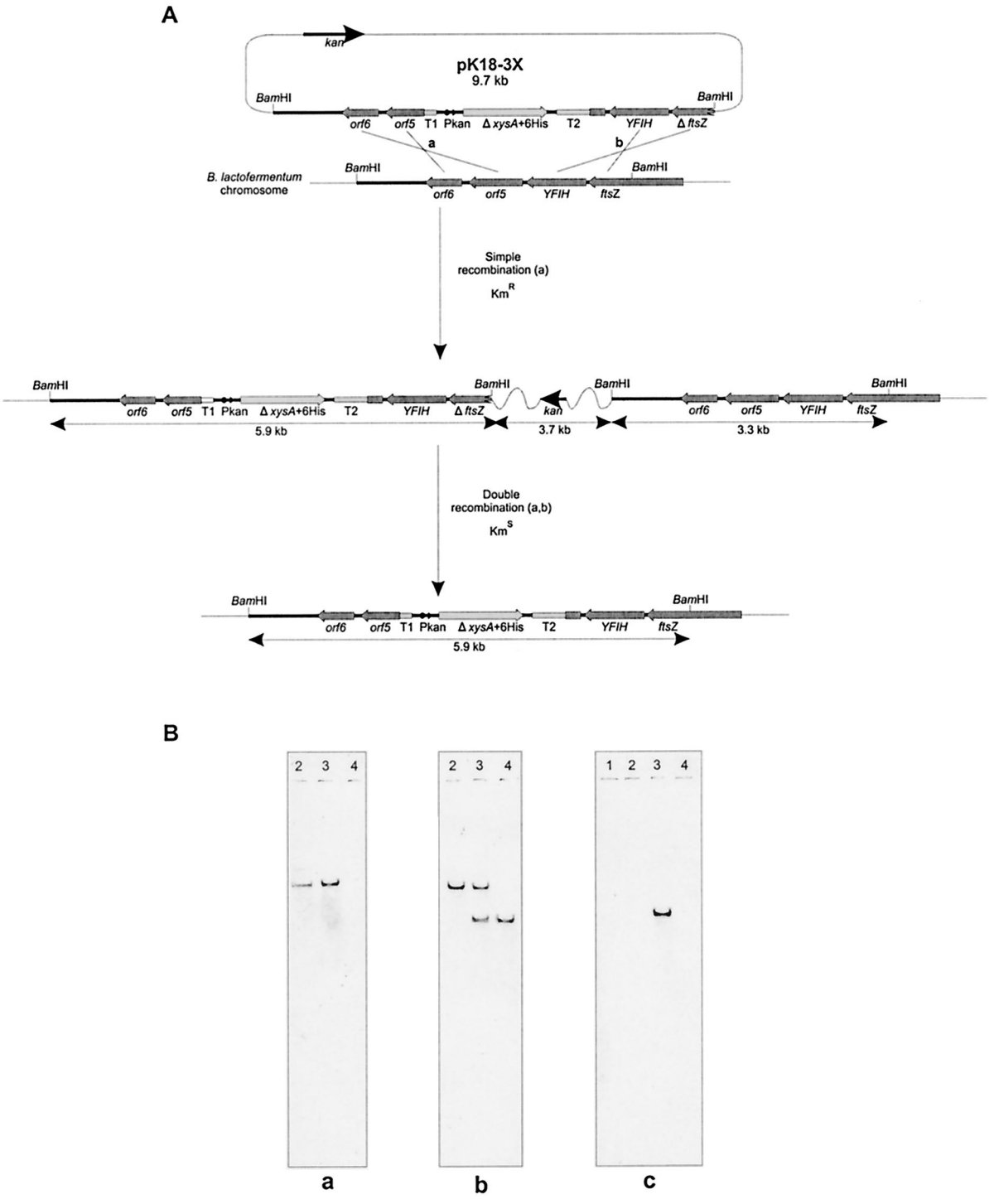

FIG. 2. Integration of the six-His-tagged Δ*xysA* gene in the chromosome of *B. lactofermentum.* (A) Schematic representation of the relevant part of the recipient strain *B. lactofermentum* chromosome and interpretation of the possible integration results. (B) Southern hybridization analysis. Lambda DNA digested with *Hin*dIII (lane 1) and chromosomal DNAs of *B. lactofermentum* X2 (lane 2), *B. lactofermentum* X1 (lane 3), and *B. lactofermentum* R31 (lane 4) digested with *Bam*HI were transferred to nitrocellulose membranes and hybridized separately with the *xysA* gene (a), with probe 3 (b), and with the *kan* cassette (c).

strain expressing the xylanase gene and lacking any exogenous drug resistance marker.

Screening for double recombination. Since the simple recombination strain was stable, screening for a double crossover was difficult because of the lack of a positive selection marker. A single colony of *B. lactofermentum* X1 (XylA$^+$ Kan^r) was incubated in TSB medium without kanamycin at 30°C for 200 generations in order to allow the second recombination event, which would excise the plasmid, rendering XylA$^+$ Kan^s colonies. Almost 50,000 isolated colonies were replica plated from

TSA to TSA supplemented with kanamycin. Many false kanamycin-sensitive colonies were checked (probably because of the lack of inoculum in the transfer), but four of them were clearly $XylA^+$ Kan^s. Therefore, the frequency of appearance was 1 out of ca. 10,000 viable colonies assayed. The second recombination event was confirmed by Southern blot hybridization, and no signal was obtained when the *kan* gene was used as a probe. There was a positive signal of the expected size (5.9 kb) when the *xysA* gene was used as a probe and with probe 3. The integrated *xysA* gene is stably maintained without the need for selective pressure, as confirmed after 20 successive cultures using as an inoculum 1 ml from the previous culture and assaying 20 different single colonies for xylanase activity on solid media. One of these $XylA^+$ Kan^s colonies was named *B. lactofermentum* X2.

Gene expression and secretion of xylanase by *B. lactofermentum*. The integration of foreign genes into the genome constitutes an interesting option for stably maintaining cloned genes without the need for selective markers. However, the level of expression of a given gene is expected to be lower than in a multicopy plasmid.

To compare xylanase production by *B. lactofermentum* X1 and *B. lactofermentum* X2 with that by *B. lactofermentum* R31 containing plasmid p880X2A, cells were grown in TSB medium for 24 h and then transferred to S2 medium. The growth curves of the three strains were similar, indicating that the integration of any exogenous DNA in ORF5 was not deleterious for growth and viability, as previously described (8). As indicated in Fig. 3, the production of xylanase increased during fermentation, but the production of six-His–ΔXys1 by *B. lactofermentum*(p880X2A) was always higher than the production of six-His–ΔXys1 by *B. lactofermentum* X1 or X2 in all the conditions assayed. For these strains, xylanase activity was found in the culture supernatant, could be easily purified by use of Ni-nitrilotriacetic acid, and did react with anti-Xys1 antibodies (Fig. 3).

Conclusions. Several vectors have been developed to express genes and to secrete proteins in *Brevibacterium*. However, if these vectors are to be considered safe for humans, animals, or the environment, only DNA from organisms generally regarded as safe should be used, and no antibiotic resistance markers should remain after genetic manipulation. In this work, we report the construction of a conjugative plasmid designed to integrate any gene into the genome of *B. lactofermentum* and the construction of a strain intended to produce extracellular xylanase containing the *xysA* gene from *S. halstedii* and lacking antibiotic resistance genes. This new strain can be considered a food-grade microorganism for the production of extracellular enzymes.

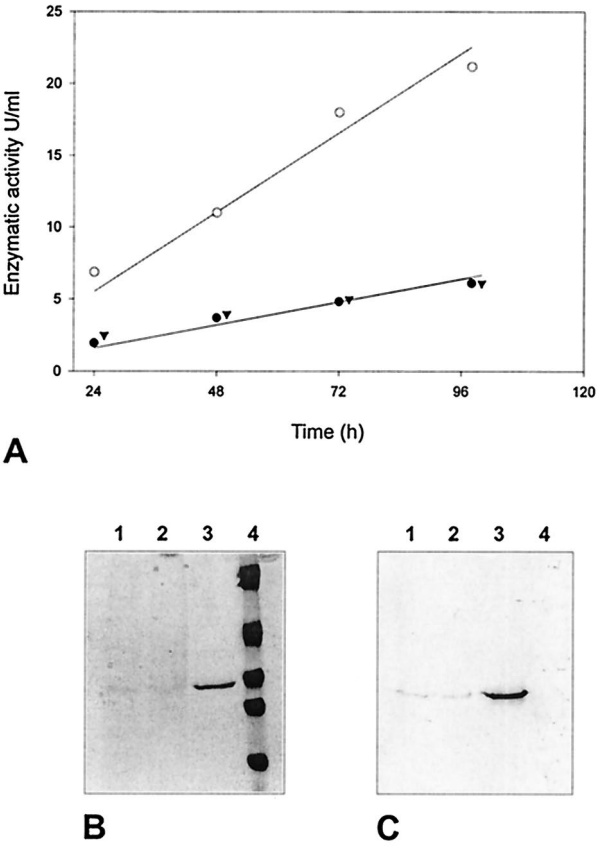

FIG. 3. Production of xylanase by *B. lactofermentum*. (A) Time course of extracellular xylanase activity in cultures of *B. lactofermentum* carrying p880X2A (○), *B. lactofermentum* X1 (●), and *B. lactofermentum* X2 (▼) grown in S2 medium. (B) Sodium dodecyl sulfate-PAGE of culture broth supernatants of *B. lactofermentum* grown in S2 medium. (C) Western blot probed with anti-Xys1 antibodies. Supernatants of cultures were taken at 72 h from *B. lactofermentum* X2 (lane 1), *B. lactofermentum* X1 (lane 2), and *B. lactofermentum* (p880X2A) (lane 3). Lane 4, Molecular mass markers.

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