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Development of a Versatile Procedure Based on Natural Transformation for Marker-Free Targeted Genetic Modification in *Streptococcus thermophilus*▽

Laetitia Fontaine,[1] Damien Dandoy,[1] Céline Boutry,[1] Brigitte Delplace,[1] Marie Henry de Frahan,[1] Christophe Fremaux,[2] Philippe Horvath,[2] Patrick Boyaval,[2] and Pascal Hols[1]*

Biochimie et Génétique Moléculaire Bactérienne, Institut des Sciences de la Vie, Université Catholique de Louvain, Place Croix du Sud 5/6, B-1348 Louvain-la-Neuve, Belgium,[1] and Danisco France SAS, BP 10, 86220 Dangé-Saint-Romain, France[2]

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A versatile natural transformation protocol was established for and successfully applied to 18 of the 19 *Streptococcus thermophilus* strains tested. The efficiency of the protocol enables the use of *in vitro*-amplified mutagenesis fragments to perform deletion or insertion of large genetic fragments. Depending on the phenotype linked to the mutation, markerless mutants can be selected either in two steps, i.e., resistance marker insertion and excision using an adapted Cre-*loxP* system, or in one step using a powerful positive screening procedure as illustrated here for histidine prototrophy.

Streptococcus thermophilus is of major importance for the food market. The improvement of its industrially relevant properties is thus a main concern for dairy manufacturers. Since this step necessarily requires a deeper knowledge of the genetics of *S. thermophilus* in relation to its metabolism, there is a need to develop versatile and efficient tools for mutagenesis (genetic insertion/deletion and point mutation) for this species. Until now, they have classically included a DNA electrotransformation procedure (21, 24) and the use of thermosensitive pG$^+$host-derivative plasmids that enable targeted mutagenesis by double homologous recombination (4, 9, 26). However, the low and variable electrotransformation frequencies obtained with existing protocols is a limiting factor in the manipulation of *S. thermophilus* (21, 24). This has been attributed to the existence of strain-specific host defense systems such as DNA restriction/modification (13, 22) and active CRISPR systems (2, 16). In addition, plasmid-based mutagenesis is time-consuming: it requires the cloning of recombination fragments, steps for double recombination with the target chromosome, and a tedious screening procedure to select the expected double recombination events (4). The latter steps can be quicker if a selectable marker is inserted between the recombination fragments, but this can lead to unwanted polar effects on downstream genes.

Recently, *S. thermophilus* was shown to be able to naturally acquire exogenous DNA and to stably maintain it in its genome by entering a physiological state known as "competence for transformation" (5, 8, 11). Natural transformation has been shown previously to facilitate the mutagenesis of streptococcal species since it allows the use of PCR products as recombinant DNA, avoiding the time-consuming steps of cloning into vectors (17, 27). The method used to select the mutant after natural transformation depends on the efficiency of competence development. For example, almost the entire *S. pneumoniae* population is transformable, which allows a simple PCR screening approach for the selection of transformants (17). For *S. mutans*, the lower competence rate (1%) led to the development of a two-step procedure based on the insertion and subsequent excision of a resistance marker using the Cre-*loxP* system (1). For *S. thermophilus*, some transformation and selection procedures have been proposed previously (6, 8). However, they were tested on a limited number of strains, i.e., strains LMD-9, LMG18311, and CNRZ1066, making them difficult to be retained as the most efficient and versatile techniques.

The objective of our study was to use natural transformation as a tool for the development of a versatile marker-free procedure for site-directed genetic modification in *S. thermophilus*. In a first step, we evaluated the efficiencies of different procedures to induce natural transformation with a large sample of strains. Nineteen strains were selected to represent the diversity existing within the *S. thermophilus* species: they exhibit unique combinations of industrially relevant properties (texturing and fast acidification) and can be divided into 11 clusters based on their CRISPR1 genetic contents (16). In a second step, we validated the use of PCR products as mutagenic DNA. Two-step and one-step strategies for genetic deletion and insertion, respectively, were tested with the model strains LMD-9 and LMG18311. The chosen genetic target was the histidine biosynthesis locus (*his*). The main reasons are (i) the relatively large size of the locus (10 genes; 7.348 kb), (ii) the strain-specific occurrence of the locus, and (iii) its associated phenotype, which allows the use of either positive (histidine prototrophy) or negative (histidine auxotrophy) screening procedures.

* Corresponding author. Mailing address: Institut des Sciences de la Vie, Université Catholique de Louvain, Place Croix du Sud 5, Box 6, B-1348 Louvain-la-Neuve, Belgium. Phone: 32-10-478896. Fax: 32-10-473109. E-mail: pascal.hols@uclouvain.be.

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TABLE 1. Primers and plasmids used in this study

Plasmid or primer	Relevant features or sequence[a] (5′ to 3′)	Reference(s) or target
Plasmids		
pXL	Em^r; pTRKH2 derivative containing a P_{blpU}-$comX_{LMG18311}$ fusion	5
pMGX	Em^r; pMG36eT derivative containing a P32-$comX_{LMG18311}$ fusion; *comX* was amplified with primers MX1/MX2 and cloned between the SmaI and SalI sites of pMG36eT	This study and reference 10
pGIUD0855cat	Cm^r Ap^r; pUC18 derivative containing P32-*cat* flanked by 1-kb fragments corresponding to the upstream and downstream regions of *stu0855*	This study
pComECcat	Cm^r Ap^r; pCR2.1-TOPO derivative allowing the insertion of P32-*cat* at the *comEC* locus	This study and reference 5
pGhostcre	Em^r RepA(Ts); pG$^+$host9 derivative containing a P1144-*cre* fusion and the *pepN* transcriptional terminator from pNZ5348 between HindIII and KpnI sites	This study and reference 19
Primers for construction of mutagenesis PCR products		
Uplox66	TAAGGAAGATAAATCCCATAAGG	*lox66*-P32-*cat*-*lox71*[b]
DNlox71	TTCACGTTACTAAAGGGAATGTA	*lox66*-P32-*cat*-*lox71*[b]
UpHis1	TTATGTCTTGGCCCTTGTCAAGGATTTGGG	*his* operon
UpHis2	CCTTATGGGATTTATCTTCCTTATTCAATCTTTCGTAATCCTTT	*his* operon
DNHis1	TACATTCCCTTTAGTAACGTGAAAAAGCAATGTTCATGACC	*his* operon
DNHis2	TTTACTAGTCCCAGATGCACGCATACGACG	*his* operon
Primers for validation of recombinant strains		
ChHis3	TAAGTTGGAGTATGCTGTTGGTCGTGTGGACGC	*his* operon
ChHis4	TTATCTTTAGCCTTGACCAATTCTTGTGAGGCC	*his* operon
IDHis1	CTACCGAGTTAACAGCGGTTGGTTGGGC	*his* operon
IDHis2	TTGGTCCTCAAAACCAGCCTCCAACTG	*his* operon
Chstu0855A	GGTATTGATCCCGAATTCAGATGTTTGTAG	*stu0855*
Chstu0855B	GGCTGGATGGCATAACCGAGCTTTTGTTTC	*stu0855*
Primers for sequence amplification for plasmid construction		
Up0855KA	GGGGTACCATCCGTAAAACCATCAAATCTT	*stu0855*
Up0855XB	CCGCTCGAGAACCCCAAGCAAAAAGTGGA	*stu0855*
P32catXA	CCGCTCGAGGTACCATGCAGTTTAAATTCG	P32-*cat*[c]
P32catXB	CCGCTCGAGTACAGTCGGCATTATCTCATA	P32-*cat*[c]
DN0855XA	CCGCTCGAGTAATGTTTGGTGTAGGTAATA	*stu0855*
DN0855SB	TCCCCCGGGTTAAACCAATCTGTCCTCGGG	*stu0855*
MX1	TATCCCGGGTATTATTGGAGGTTGAATGGAACAA	*comX*
MX2	ACGCGTCGACTTCCTTGTATACTGGCTTCTA	*comX*

[a] Restriction sites introduced in the primers are underlined. Em^r, Cm^r, and Ap^r, erythromycin, chloramphenicol, and ampicillin resistance; Ts, temperature sensitive.
[b] The PCR product obtained with primer pair Uplox66/DNlox71 was used to create all chimeric PCR fragments required for mutant construction. The Uplox66/DNlox71 primer set hybridized on pNZ5319 (19).
[c] The P32catXA/P32catXB primers were used to amplify P32-*cat* from pGIZ850 (12).

Plasmid-based systems for competence induction are restricted to a narrow range of strains. For streptococcal species, the key step of competence development is the transcriptional induction of *comX*. It encodes the alternative sigma factor σ^X, which positively regulates genes required for DNA transformation and homologous recombination with the recipient genome (18). We compared the abilities of two *comX* expression plasmids to turn on competence in *S. thermophilus*. The pXL plasmid contains a fusion between the BlpCHR-regulated promoter P_{blpU} and the *comX* gene from strain LMG18311 ($comX_{LMG18311}$), which enables the inducible expression of $comX_{LMG18311}$. This plasmid was shown previously to induce competence for natural transformation in strain LMG18311 grown in a complex culture medium (5). The constitutive expression system pMGX was constructed by cloning $comX_{LMG18311}$ and its translation signals under the control of the constitutive P32 promoter of *Lactococcus lactis* into pMG36eT (10), yielding plasmid pMGX (Table 1).

The first step consisted of the introduction of both *comX* expression systems into the 17 selected strains and laboratory strains LMD-9 and LMG18311 by electroporation as described previously (5). We managed to transform 15 and 7 of 19 strains with pXL and pMGX, respectively (Table 2). Seven strains were permissive of electrotransformation with both pXL and pMGX, while four were recalcitrant. However, the pG$^+$host9 vector was successfully introduced into the latter strains, indicating that electroporation failure was not due to poor quality of competent cells. The narrower host range of pMGX than of pXL could be explained by the different nature of its replication origin (pWV01 instead of pAMβ1), by the lack of target sequences for endogenous defense systems, or by a possible toxic effect due to strong and constitutive ComX production.

TABLE 2. Electroporation test results and natural transformation rates for *S. thermophilus* strains

Strain[a]	Phenotype[b]	Electroporation test result for plasmid[c]:			Natural transformation rate[d] for *comX* overexpression strain carrying plasmid:		Natural transformation rate[d] for strain in the presence of:	
		pG$^+$host9	pXL	pMGX	pXL	pMGX	0 μM $ComS_{17–24}$	1 μM $ComS_{17–24}$
LMG18311	F^-, T^+	NT	+	+	8.8×10^{-4} (-3.5 ± 0.8)	7.7×10^{-3} (-2.7 ± 1.1)	3.4×10^{-6} (-5.5 ± 0.3)	1.3×10^{-1} (-0.9 ± 0.3)
LMD-9	F^+, T^-	NT	+	+	8.8×10^{-6} (-5.1 ± 0.2)	5.9×10^{-5} (-4.4 ± 0.5)	1.7×10^{-3} (-3.0 ± 0.5)	5.4×10^{-3} (-2.5 ± 0.6)
DGCC7790	F^-, T^+	NT	+	+	2.7×10^{-2} (-2.6 ± 1.3)	5.9×10^{-5} (-5.0 ± 1.5)	ND	4.3×10^{-2} (-2.1 ± 1.5)
DGCC7710	F^+, T^+	NT	+	+	ND[e]	6.1×10^{-3} (-2.2 ± 0.2)	6.7×10^{-7} (-6.3 ± 0.6)	1.0×10^{-2} (-2.2 ± 0.5)
DGCC7853	F^+, T^-	NT	+	+	ND	8.4×10^{-4} (-3.6 ± 1.2)	2.2×10^{-4} (-3.7 ± 0.3)	4.9×10^{-4} (-3.3 ± 0.1)
DGCC7879	F^+, T^-	NT	+	+	ND	2.3×10^{-3} (-2.6 ± 0.4)	8.1×10^{-7} (-6.3 ± 0.5)	8.6×10^{-3} (-2.1 ± 0.2)
DGCC715	F^+, T^-	NT	+	+	ND	1.6×10^{-2} (-1.9 ± 0.3)	ND	1.2×10^{-2} (-1.9 ± 0.2)
DGCC7773	F^-, T^+	NT	+	−	1.5×10^{-2} (-1.9 ± 0.3)		ND	1.5×10^{-1} (-1.0 ± 0.4)
DGCC7785	F^-, T^+	NT	+	−	2.5×10^{-4} (-3.7 ± 0.4)		ND	4.1×10^{-5} (-4.5 ± 0.4)
DGCC7796	F^+, T^-	NT	+	−	2.5×10^{-2} (-1.9 ± 0.7)		3.1×10^{-6} (-5.5 ± 0.2)	6.9×10^{-2} (-1.3 ± 0.4)
DGCC7854	F^+, T^-	NT	+	−	6.9×10^{-5} (-4.5 ± 0.7)		1.1×10^{-7} (-7.2 ± 0.6)	5.3×10^{-3} (-2.5 ± 0.7)
DGCC7891	F^-, T^+	NT	+	−	1.4×10^{-4} (-4.1 ± 0.5)		ND	1.4×10^{-4} (-4.2 ± 0.7)
DGCC782	F^+, T^-	NT	+	−	ND		ND	2.0×10^{-3} (-2.8 ± 0.4)
DGCC7666	F^-, T^-	NT	+	−	ND		1.8×10^{-6} (-5.9 ± 0.4)	4.2×10^{-2} (-1.4 ± 0.2)
DGCC7694	F^-, T^-	NT	+	−	ND		ND	3.9×10^{-2} (-1.5 ± 0.3)
DGCC3367	F^+, T^-	+	−	−			4.7×10^{-7} (-6.5 ± 0.5)	1.8×10^{-3} (-2.8 ± 0.3)
DGCC7809	F^+, T^-	+	−	−			ND	5.8×10^{-4} (-3.5 ± 0.6)
DGCC7909	F^-, T^+	+	−	−			ND	4.5×10^{-3} (-2.4 ± 0.1)
DGCC7984	F^+, T^+	+	−	−			ND	ND

[a] All strains are from the Danisco collection, except strains LMD-9 (from the ATCC) and LMG18311 (from the LMG Collection).

[b] F^+ and F^- refer to fast and slow acidification, respectively, by strains grown in pasteurized milk. T^+ and T^- refer to the presence and absence, respectively, of texturing properties of strains grown in pasteurized milk according to sensory analyses.

[c] NT, not tested. + indicates that electrotransformants could be obtained under a range of conditions. − indicates that no electrotransformants could be obtained under a range of conditions.

[d] Calculated as the ratio of transformants (chloramphenicol-resistant CFU) to the total CFU count per 1 μg of DNA (arithmetic mean of three independent experiments). Geometric means ± standard deviations expressed in $\log_{10}$ are provided in parentheses.

[e] ND, not detected (the transformation rate was below the detection limit of the test [$<1 \times 10^{-8}$]).

Natural transformation was then assessed with the following protocol. Each pXL- or pMGX-containing strain was grown for 6 h at 37°C in 300 μl of Todd-Hewitt broth (THB) medium supplemented with 1% glucose, 2.5 μg/ml erythromycin, and 1 μg plasmid (pGIUD0855cat) DNA. Mature BlpC from strain LMD-9 ($BlpC_{LMD-9}$; 250 ng/ml of the D9C19 form) (9) was added to cultures of pXL-containing strains to induce $comX_{LMG18311}$ expression. Plasmid pGIUD0855cat is a pUC18 derivative containing a chloramphenicol resistance cassette flanked by 1-kb fragments corresponding to the upstream and downstream regions of *stu0855* (Table 1). This plasmid is unable to replicate in *S. thermophilus*, and upon natural transformation, it promotes the genetic replacement of *stu0855* by P32-*cat* through homologous recombination. The presence of this locus in all selected strains was checked by PCR prior to further experiments. Transformation rates were calculated as the ratio of transformants (chloramphenicol-resistant CFU) to the total CFU count (Table 2). Constitutive and inducible *comX* expression systems yielded transformants for 7 of 7 and 8 of 15 strains, respectively, which indicates that pMGX is a more versatile system than pXL to induce competence. This was particularly clear when we compared efficiencies in the same genetic background: 4 of 7 of pXL-carrying strains were not transformable. We also found that transformation rates were highly strain dependent: between 10^{-2} and 10^{-5} with pMGX and between 10^{-2} and 10^{-6} with pXL. For pXL-bearing strains, poor or no transformability could be directly correlated with the poor level of *comX* expression. Indeed, we measured no or low luminescence driven from the P_{comGA}-*luc* reporter fusion carried by pXL, whose activity is dependent on the amount of ComX (*comGA* is a late competence gene) (5) (data not shown). This finding suggests that endogenous Blp regulatory systems that control *comX* expression are impaired in these strains.

Altogether, these results demonstrate that plasmid systems are not appropriate candidates to develop a universal and efficient competence induction procedure for *S. thermophilus*; their versatility is limited by (i) the electroporation efficiency (for the pMGX system) and (ii) the genetic background of the host strain (for the pXL system).

Addition of mature ComS induces competence in 18 of 19 *S. thermophilus* strains tested. In *S. thermophilus*, induction of *comX* was shown recently to rely on the activation of a quorum-sensing system involving the transcriptional regulator ComR and its dedicated pheromone ComS. The current model proposes that ComS is secreted, matured, and reimported into the cells to interact with and activate ComR, leading to the binding of ComR to the *comX* promoter (8). Strain LMD-9 was shown previously to be naturally transformable in chemically defined medium (CDM) (11), while addition of synthetic heptapeptides corresponding to the C-terminal part of ComS ($ComS_{18-24}$) was necessary to activate the ComRS systems of strains LMG18311 and CNRZ1066 (8). The same experiment was performed using the selected sample of strains with addition of the octapeptide $ComS_{17-24}$ (8). This peptide has inducing properties equivalent to those of $ComS_{18-24}$, but its activity is more stable over time (data not shown). Strains were grown at 37°C in 300 μl CDM, and after 1.5 h, 1 μM $ComS_{17-24}$ was added to half of each culture. Plasmid pGIUD0855cat (1 μg) was used as donor DNA. Strains LMD-9 and LMG18311 were used as controls. The results presented in Table 2 show that in the absence of $ComS_{17-24}$, most strains are either not competent (10 of 19 strains) or poorly competent (7 of 19 strains) (Table 2). Remarkably, supplementation with $ComS_{17-24}$ renders 18 of the 19 strains tested highly transformable: rates between 10^{-2} and 10^{-4} were measured for most strains. Furthermore, strains LMG18311 and DGCC7773 developed rates of 10^{-1}, which is comparable to that for *Bacillus subtilis* (14). These results show that production of a sufficient amount of ComS is critical to develop the competence state. Only one strain, DGCC7984, yielded no chloramphenicol-resistant transformants. The same result was obtained with plasmids pComECcat, which promotes the insertion of P32-*cat* at the *comEC* locus (data not shown) (Table 1). The sequences of ComR and ComS from strain DGCC7984 differ only at positions 239 (K239N) and 15 (A15P), respectively, from their counterparts from strain LMG18311. However, the substitutions do not account for the competence-negative phenotype of strain DGCC7984 since they were also found in the transformable strain DGCC7710. The presence of additional mutations that impaired natural transformation, at the level of *comX* induction and/or DNA processing, cannot be excluded. In conclusion, ComS supplementation can be regarded as an efficient procedure to transform *S. thermophilus*.

Two-step procedure for genetic modification without acquisition of a positively screenable phenotype. The choice of a markerless mutagenesis and screening procedure does not depend only on the natural transformation efficiency; it can also be influenced by the nature of the phenotype associated with the mutation. To create mutant phenotypes that cannot be positively selected, which is typical of genetic deletions, we developed a two-step mutagenesis procedure. For this purpose, we tested the possibility of performing allelic exchange of the *his* locus with a resistance marker by using chimeric PCR products as transforming DNA. The functionality of the improved Cre-*loxP* site-specific recombination system for marker excision (19) was then evaluated. For this purpose, a *cre* expression vector for *S. thermophilus*, pGhostcre, was constructed (Table 1). A HindIII-KpnI fragment from pNZ5348 (19) that encompasses the P1144-*cre* fusion and the *pepN* transcriptional terminator was cloned into the thermosensitive and erythromycin-selectable pG^{+}host9 vector (20). Strain LMD-9 contains all histidine biosynthesis genes (Fig. 1) (15). They are organized into two loci: the putative histidinol phosphatase gene *hisK* (*ster_1212*) and the *ster_1198-ster_1207* cluster (Fig. 1A). To create a His^{-} strain, we decided to delete the *ster_1198-ster_1207* cluster (10 genes; 7.348 kb).

The cassette allowing in-frame exchange of the *his* locus with the chloramphenicol marker was assembled *in vitro* by overlapping PCR as described previously (8). This cassette consists of a central *lox66*-P32-*cat*-*lox71* fragment flanked by sequences corresponding to the upstream (Up) and downstream (DN) regions of the *his* locus (Fig. 1A and Fig. 2). Primers UpHis1/UpHis2 and DNHis1/DNHis2 (Fig. 1A) used to amplify the recombination regions are listed in Table 1. *lox66* and *lox71* are mutated *loxP* sites that create the doubly mutated *lox72* site, which has a reduced affinity for the Cre recombinase, after site-specific recombination (Fig. 2 illustrates the general strategy) (1, 19). To obtain the marker-free

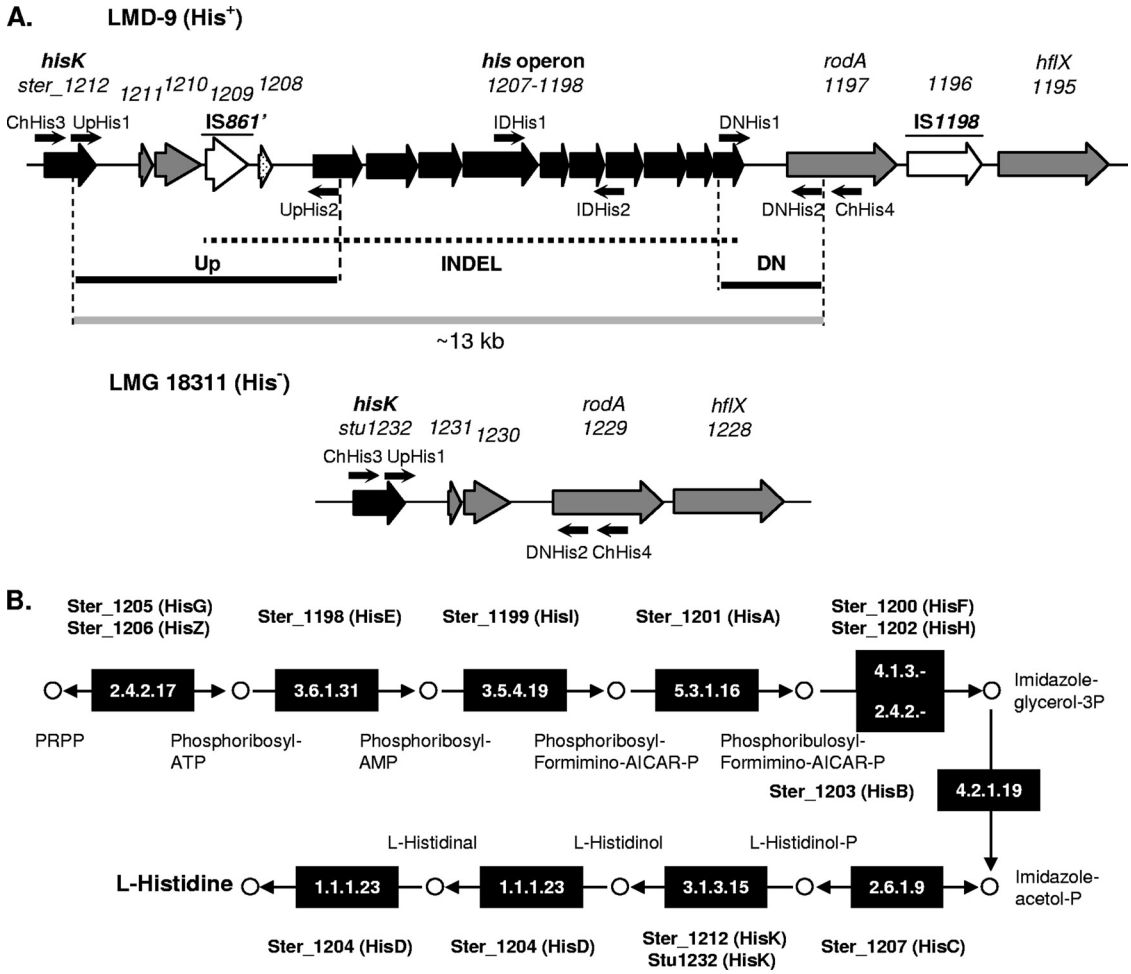

FIG. 1. Histidine biosynthesis in *S. thermophilus*. (A) Schematic representations of the *hisK-hflX* gene cluster of *S. thermophilus* LMD-9 (upper panel) and that of LMG18311 (lower panel). His^+ and His^- (in parentheses) indicate the histidine prototrophy and auxotrophy of strains LMD-9 and LMG18311, respectively. Genes encoding proteins are represented by thick arrows as follows: arrows for genes involved in histidine biosynthesis (the *his* operon and the putative histidinol phosphatase gene *ster_1212* or *stu1232*) are black, arrows for insertion sequences are white, arrows for genes conserved between LMD-9 and LMG18311 are gray, and the arrow for a gene of unknown function unique to LMD-9 (*ster_1208*) is dotted. The dotted line in the upper panel delimits a potential insertion/deletion event (INDEL). The following elements are indicated: the names of the insertion sequences (underlined), the region amplified by PCR to transfer the histidine prototrophy from LMD-9 to LMG18311 (thick gray line), the Up and DN recombination fragments used to delete the *his* operon of LMD-9 (thick black lines), and the primers used to amplify the Up/DN regions and to validate the identities of His^+ and His^- recombinant strains (thin black arrows). (B) L-Histidine biosynthesis pathway and respective contributions of *S. thermophilus his* genes. The EC numbers of the enzymes catalyzing the reactions from phosphoribosyl pyrophosphate (PRPP) to L-histidine are black boxed. The corresponding gene products from strains LMD-9 and LMG18311 are indicated in bold at the top or bottom of each box. The His nomenclature of the enzymes is indicated in parentheses. The intermediate metabolites are indicated and represented by open circles. AICAR, aminoimidazole carboxamide ribonucleotide.

ster_1198-ster_1207::*lox72* strain, LMD-9 underwent two steps of natural transformation in CDM supplemented with $ComS_{17-24}$. First, 25 ng of the chimeric PCR products was used as donor DNA, which yielded the Cm^r *ster_1198-ster_1207*::*lox66*-P32-*cat-lox71* strain (transformation rate, 2.6×10^{-5}). The allelic substitution was confirmed by PCR analysis (using primers ChHis3/ChHis4 [Table 1]) and NcoI restriction of PCR products. Second, the strain was transformed with 1 μg of pGhostcre. Em^r colonies were recovered after 48 h of incubation at 29°C (mean transformation rate, 1×10^{-4}) and transferred onto solid medium in the presence or absence of chloramphenicol. Cre recombinase was expressed during incubation at 29°C since 100% of pGhostcre-containing His^- colonies were Cm^s (50 colonies were tested). Recombination and loss of P32-*cat* was also confirmed by PCR (using primers Uplox66 and DNlox71). As a final step, Em^r Cm^s colonies were cured of the pGhostcre plasmid by growth at 37°C (nonpermissive for pGhostcre replication) in the absence of antibiotic as described previously (1). After 16-h culture, 100% of *ster_1198-ster_1207*::*lox72* colonies were Em^s (50 colonies were tested). Finally, phenotypic tests were performed to validate the biological effect of the mutagenesis performed. To check histidine auxotrophy, we compared the growth kinetics of wild-type (LMD-9) and *ster_1198-ster_1207*::*lox72* (LMD-9 His^-) strains in the presence and absence of histidine. Results showed that they grow similarly in complete CDM (Fig. 3A)

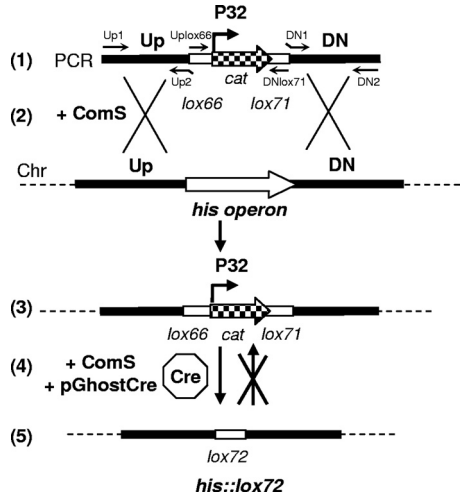

FIG. 2. Schematic representation of the markerless deletion procedure developed for *S. thermophilus*. (1) *In vitro* construction of mutagenesis PCR products. They consist of 3 individual PCR fragments joined together by overlapping extension: the upstream region (obtained using primers generically named Up1/Up2) and the downstream region (obtained using primers generically named DN1/DN2) of the target gene/locus, separated by a *lox66*-P32-*cat*-*lox71* fragment (obtained using primers Uplox66/DNlox71). (2) *S. thermophilus* cells growing in ComS-supplemented CDM are naturally transformed with chimeric PCR fragments. (3) The target gene/locus is replaced by the *lox66*-P32-*cat*-*lox71* cassette through double homologous recombination. The transformants are selected on chloramphenicol-containing M17 medium. (4) Mutants from step 3 are naturally transformed with plasmid pGhostcre by growing cells in ComS-supplemented CDM. Site-specific recombination between *lox66* and *lox71* by Cre recombinase (represented by a polygon) promotes P32-*cat* excision. This step is performed by growing cells at 29°C in the absence of chloramphenicol. (5) The *lox72* site created has reduced affinity for Cre, which prevents the reintroduction of the cassette. The following elements are represented: primer pairs used to separately amplify the Up, DN, and *lox66*-P32-*cat*-*lox71* fragments (thin black arrows); the target gene/locus (white arrow); *cat* (checkered arrows); the chromosome (Chr; dotted lines); *lox* sites (thick white lines); recombination fragments (thick black lines); and recombination events (crossed lines).

and that the *ster_1198-ster_1207*::*lox72* (LMD-9 His$^-$) strain, solely, has a clear growth defect in CDM lacking histidine (Fig. 3B).

In conclusion, the efficiency of our natural transformation protocol allows the combined use of chimeric PCR products and the Cre-*loxP* system to produce large markerless deletions (7.278 kb in the case of the *his* locus). This strategy was successfully applied to other loci, such as *lacZ* and *comRS* (data not shown). The efficiency of the first step, i.e., allelic exchange, depends mostly on the yield of the overlapping PCR extension rather than on the sizes of the target loci (data not shown).

One-step procedure for genetic modification that leads to the acquisition of a positively screenable phenotype. Compared to strain LMD-9, strain LMG18311 lacks homologues of *ster_1198-ster_1207* (Fig. 1A), and it was shown previously to be a histidine auxotroph (25). Since insertion of the *ster_1198-ster_1207* genes into strain LMG18311 should lead to the acquisition of a positively selectable phenotype, i.e., histidine prototrophy, a one-step mutagenesis protocol was attempted.

Two types of DNA were used as transforming DNA: a 13-kb PCR fragment amplified from strain LMD-9 (by using primer pair UpHis1/DNHis2) (Fig. 1A) and the whole LMD-9 chromosome. The PCR fragment contains the *ster_1198-ster_1207* genes flanked by the Up and DN sequences, used previously to create strain LMD-9 His$^-$ (Fig. 1A). The 5′ end of the Up sequence and the 3′ end of the DN sequence will mediate double homologous recombination events between the PCR fragment and the LMG18311 chromosome since these sequences contain an ~1-kb region that is homologous (with 99% identity) to *stu1229* and *stu1230-stu1231*, respectively (Fig. 1A). Regarding the LMD-9 chromosome, the potentially homologous sequences (with 99% identity to LMG18311) allowing insertion of the *his* locus into the LMG18311 chromosome are longer than 10 kb (data not shown). The competence experiments were performed with $ComS_{17-24}$-supplemented CDM in the presence of 200 ng unpurified PCR product or 45 μg total chromosomal DNA prepared as described previously (7). For both experiments, transformants having acquired the histidine prototrophy were selected on CDM lacking histidine. After 48 h of incubation at 37°C, we obtained 30 CFU/ml with PCR fragments and 3,840 CFU/ml with chromosomal DNA. The higher number of transformants obtained with the chromosomal DNA may be related to the longer regions available for homologous recombination (see above). The respective strains were designated LMG18311 His$^+$ PCR and LMG18311 His$^+$ Ch. The insertion of the *ster_1198-ster_1207* locus between *stu1299* and *stu1230* in three His$^+$ colonies from both strains was validated by PCR (using primer pairs IDHis1/IDHis2, ChHis3/ChHis4, ChHis3/IDHis2, and ChHis4/IDHis1) (Fig. 1A and Table 1). In addition, the identities of the transformants were also checked with primers that are specific to strain LMG18311. To study the functionality of *his* transfer, we measured the growth kinetics of strains LMG18311 His$^+$ PCR and LMG18311 His$^+$ Ch in complete CDM and in CDM lacking histidine. The results are presented in Fig. 3. In the presence of histidine, we measured no significant growth difference between the wild-type strain and the three LMG18311 His$^+$ PCR clones (Fig. 3A). However, in the absence of histidine, the latter displayed growth similar to that in the presence of histidine while the wild-type strain was clearly growth deficient, which confirmed the histidine prototrophy of the LMG18311 His$^+$ PCR clones (Fig. 3B). Similar results were obtained for strain LMG18311 His$^+$ Ch, except that some growth disparity among clones of strain LMG18311 His$^+$ Ch was observed (Fig. 3C and D and data not shown). This could indicate that their genetic contents are different. Indeed, we cannot exclude the possibility that, besides the *his* locus, LMG18311 His$^+$ Ch isolates have acquired other LMD-9-specific loci. The corollary is that they could also have lost LMG18311-specific clusters. Genetic cotransfer during natural transformation has already been observed in *S. mutans* (3).

With the example illustrated above, we showed that an efficient natural transformation procedure combined with a powerful positive screen allows the construction of recombinant strains in one step without using any antibiotic resistance marker.

Concluding remarks. The transformation protocol developed in this work is applicable to all *S. thermophilus* strains tested except one recalcitrant strain, and its efficiency for most strains is close to that for *S. mutans* (transformation rate, 1%).

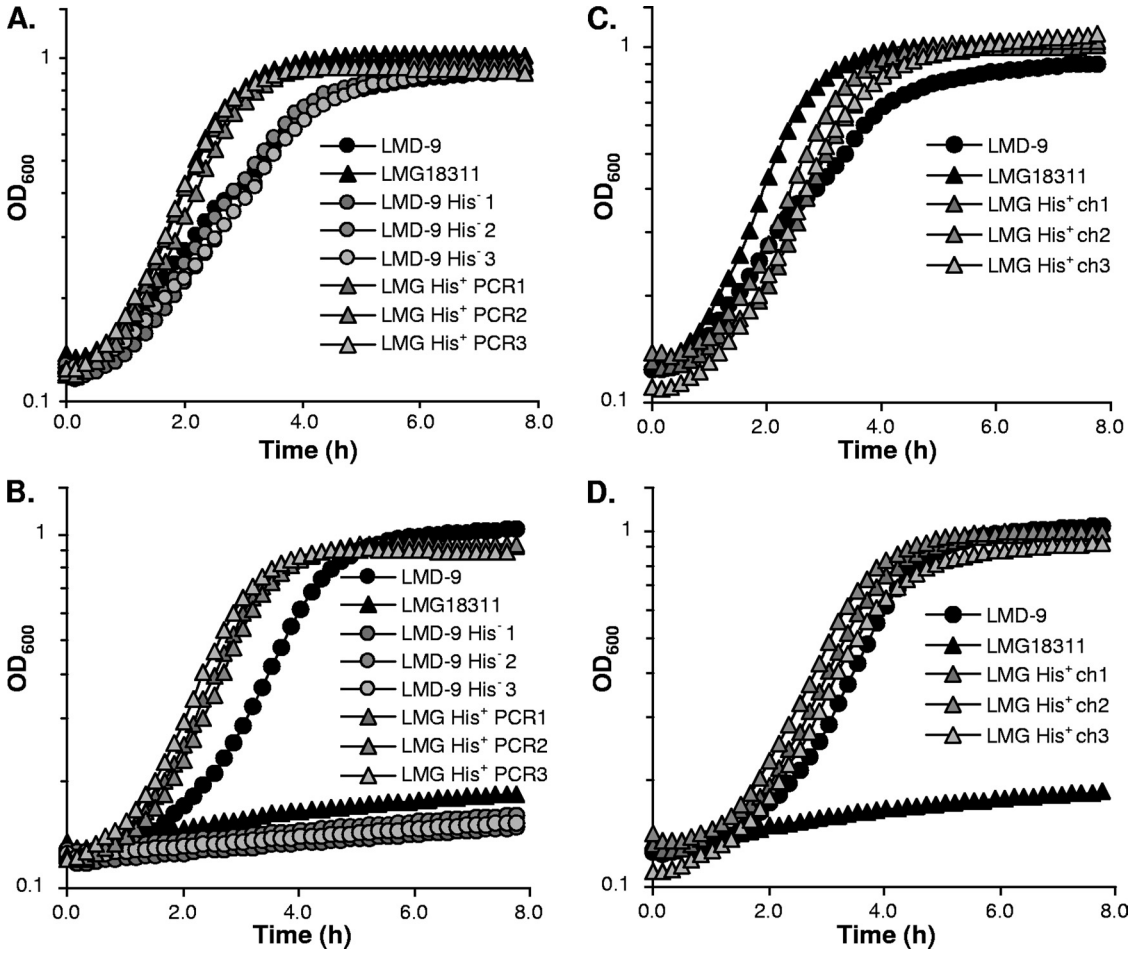

FIG. 3. Growth (expressed as the optical density at 600 nm [OD_{600}]) of wild-type (WT) strains LMD-9 and LMG18311 and their respective His^- and His^+ derivatives. (A and B) Growth in CDM (A) and CDM lacking histidine (B) of the LMD-9 WT, LMD-9 His^- (*ster_1198-ster_1207*::*lox72*) (3 clones), the LMG18311 WT, and LMG18311 His^+ PCR (3 clones). (C and D) Growth in CDM (C) and CDM lacking histidine (D) of the LMD-9 WT, the LMG18311 WT, and LMG18311 His^+ Ch (3 clones). Growth was monitored at 10-min intervals with the Varioskan Flash multimode reader (Thermo Fisher Scientific, Zellic, Belgium) as described previously (8). Mean values calculated from triplicates are presented for WT strains.

Consequently, quick and effective selection of transformants depends on the use of a powerful phenotypic screen. We showed that it can be based on either a two-step procedure consisting of the insertion and subsequent deletion of an antibiotic marker, similar to the procedure for *S. mutans* (1), or the acquisition/loss of metabolic properties. Blomqvist and coworkers recently proposed a markerless strategy based on colony hybridization with digoxigenin-labeled probes to select small genetic changes in *S. thermophilus* (6). However, it could turn out to be labor-intensive in the case of strains displaying a transformation rate of $\leq 10^{-4}$, which applies to 4 of 18 strains in our sample of competent strains. The reason for their poor transformability remains to be elucidated. We found no correlation with some metabolic properties (fast acidification and texturing). We cannot exclude that these strains lack some important competence determinants located either upstream or downstream of *comX* induction. Demonstration of competence in *S. thermophilus* is recent, and a better study of the factors involved and their regulation is thus a prerequisite to improve transformation protocols. In addition, it is interesting that our procedure for genetic modification is potentially applicable to *S. salivarius*, since this species also uses the ComRS system to control competence (8).

Natural transformation in a microorganism of importance for the food sector, like *S. thermophilus*, represents a unique opportunity for the industrial market. Indeed, natural transformation could be considered a self-cloning strategy as long as the DNA used, such as a total chromosome or a PCR product containing no intentionally introduced mutations, includes no recombinant DNA. This approach is excluded from the European legislation on genetically modified microbes (GMM) (23). The transfer of the histidine prototrophy between strains showed for the first time that it is possible to use natural transformation to insert large genetic fragments without altering the sequence of the donor DNA and without using any foreign non-*S. thermophilus* genes. This opens interesting perspectives to engineer novel non-GMM starter strains by combining industrially relevant traits such as fast acidifica-

tion (corresponding to the *prtS* locus), texture production (corresponding to the *eps* locus), or phage resistance (corresponding to CRISPR spacers).

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