

Quantitative Analysis of Histidine Decarboxylase Gene (*hdcA*) Transcription and Histamine Production by *Streptococcus thermophilus* PRI60 under Conditions Relevant to Cheese Making^{∇†}

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Received 26 October 2010/Accepted 17 February 2011

This study evaluated the influence of parameters relevant for cheese making on histamine formation by *Streptococcus thermophilus*. Strains possessing a histidine decarboxylase (*hdcA*) gene represented 6% of the dairy isolates screened. The most histaminogenic, *S. thermophilus* PRI60, exhibited in skim milk a high basal level of expression of *hdcA*, upregulation in the presence of free histidine and salt, and repression after thermization. HdcA activity persisted in cell extracts, indicating that histamine might accumulate after cell lysis in cheese.

Histamine is the biogenic amine (BA) most frequently implicated in food poisoning. It interacts with the respiratory, cardiovascular, gastrointestinal, and hematological/immunological systems, causing “histamine intoxication” symptoms like hypotension, headache, diarrhea, vomiting, and skin flushing that, in the case of “scombroid poisoning,” caused by scombroid fish, are particularly severe. Moreover, “histamine intolerance,” i.e., acute toxicity even at relatively low levels of histamine (30), occurs especially in diamine oxidase (DAO)-deficient subjects (2, 17). Histamine is the only BA for which maximum specific levels in food are indicated, though only for some fresh and processed fish products with a high content of free histidine (11) that can be decarboxylated to histamine mainly by Gram-negative bacteria (17). However, some Gram-positive bacteria involved in food production, including lactic acid bacteria (LAB) strains of the species *Lactobacillus buchneri*, *Lactobacillus curvatus*, *Lactobacillus helveticus*, *Lactobacillus hilgardii*, *Lactococcus lactis*, *Oenococcus oeni*, *Pediococcus damnosus*, and *Pediococcus parvulus* (1, 4, 14, 15, 25), also possess this enzymatic activity, encoded by *hdc* gene clusters, and form histamine in cheeses, sausages, and fermented beverages. The presence of histamine is well documented in long-ripened and blue cheeses (19, 25) and is attributed to both nonstarter and starter LAB (4). Though *Streptococcus thermophilus* is the starter LAB species of major importance for the manufacture of yogurt and many cheese varieties, like Grana type, Swiss type, and pasta filata cheeses, and in spite of the wide literature concerning BA production among LAB (1, 3, 8,

28), there are few reports of histamine decarboxylase activity in this bacterial species. The presence in two *S. thermophilus* strains of a complete *hdc* gene cluster was only very recently reported (5).

The *hdc* gene clusters of Gram-positive bacteria usually comprise the gene *hdcA*, encoding a pyruvoyl-dependent decarboxylase, and *hdcP*, encoding a histidine/histamine antiporter, not yet identified in some histamine-producing LAB. Moreover, an *hdcB* gene involved in the conversion of the HdcA proenzyme to the active decarboxylase (29) and *hdcRS*, with functions not essential to the decarboxylation process, can also be found. In the case of *S. thermophilus*, the gene order is *hdcAPB* (5), similarly to *Staphylococcus capitis* and *Clostridium perfringens*, which, however, lack *hdcB* (10).

Conditions that favor histidine decarboxylation are the presence of the specific substrate or high concentrations of free amino acids and hostile environmental conditions that require the maintenance of pH homeostasis (15, 21). The first recognized role of decarboxylases was keeping a high intracellular pH as a consequence of the conversion of an amino acid to the corresponding amine. Their function as secondary energy supply processes was put in evidence by observing that some bacteria, for example *O. oeni* 9204, produce larger amounts of histamine under the poorest nutritional conditions (12). Indeed, a proton motive force is generated by exchange of the amine with the amino acid from the medium. In addition, histamine biosynthesis is modulated by the growth phase. Calles-Enríquez et al. (5) observed an increase of histamine production in *S. thermophilus* during the stationary phase, while Landete et al. (14) found that *hdcA* expression in *L. hilgardii* was higher in the exponential phase. Moreover, histidine induced *hdcA* expression, whereas histamine repressed it (14).

The aims of this study were (i) establishing the occurrence of genetic determinants encoding histidine decarboxylases in *S. thermophilus* strains isolated from natural sources, (ii) studying

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† Supplemental material for this article may be found at <http://aem.asm.org/>.

[∇] Published ahead of print on 4 March 2011.

TABLE 1. Targets and sequences of the primer pairs used in this study

Function	Primer	Sequence (5'→3')	Nucleotide positions	Product size (bp)	Reference
Screening of <i>hdcA</i> presence in Gram-positive bacteria	HIS1-F HIS1-R	GGNATNGTNSNTAYGAYMGNGCNGA ATNGCDATNGCNSWCCANACNCCRTA	172–197 ^a 520–545 ^a	372	9
Screening of <i>hdcA</i> presence in <i>S. thermophilus</i> strains and RT-qPCR	STDEC-F STDEC-R	GAATTACCGATCTATGATGC ACACCTTTGTTAGCACAAAC	349–368 ^a 451–470 ^a	121	This study
RT-qPCR on the <i>S. thermophilus rpoA</i>	RPOST-F RPOST-R	ACTGTCATTGTTGCTTGAATG AGCTGAGGTTACTGCTGGAGAT	521–542 ^b 613–634 ^b	114	13
Random primer for uneven PCR	Coc-1	AGCAGCGTGG			This study
Random primer for uneven PCR	RDW	CCGATCTTCTCGC			This study

^a Positions in the *S. thermophilus* PRI60 *hdcA* sequence.

^b Positions in the *rpoA* ORF of *S. thermophilus* LMD-9 (accession no. CP000419; region 1755506 to 1756444).

hdcA expression and histamine production under conditions relevant for cheese making (presence of the substrate histidine, addition of NaCl, presence of different lactose levels, natural acidification, thermization of the inoculum), to evaluate the risk of histamine formation by *S. thermophilus* adventitious strains in dairy products, and (iii) evaluating the functionality of the HdcA enzyme released after cell lysis.

Screening of *hdcA* gene presence in *S. thermophilus* strains. Eighty-three *S. thermophilus* strains, all (except two) dairy isolates, identified and genotypically differentiated in previous studies (16, 24), were screened by consensus PCR with oligonucleotides HIS1-F and HIS1-R (9) made more stringent by changing the annealing temperature to 58°C for 40 s. They were subcultured in M17 broth (Oxoid, Milan, Italy) added with 0.5% (wt/vol) lactose (LM17) at 37°C for 24 h, and total genomic DNA was extracted and purified from 2-ml cultures as described by Rossi et al. (26).

The strains PRI17, PRI18, and PRI21, all isolated from natural whey starter cultures for Grana-type cheese, PRI60 from mozzarella cheese, and PRI74 from traditional yogurt originated an amplification product of the expected size, close to 372 bp (data not shown). The amplicons were sequenced with primers T7 and SP6 (Promega) after purification by the Wizard SV gel and PCR clean-up system (Promega), ligation with the pGEM-T Easy vector (Promega), transformation in *Escherichia coli* DH5α, and plasmid extraction by the PureYield plasmid purification kit (Promega). Sequencing was done by BMR-Genomics (Padova, Italy). BLASTn (www.ncbi.nlm.nih.gov) was used for sequence similarity searches. The sequences were identical and showed the highest identity with a fragment of the *hdcA* gene.

The results indicated a percentage of *hdcA*-positive strains of 6%, somewhat higher than that reported for industrial strains by Calles-Enríquez et al. (5). Since in this case naturally occurring strains from dairy sources were screened, the frequency of *hdcA* presence can be considered to be more representative of the diffusion of histaminogenic strains potentially able to reach high numbers in products made from raw milk and by spontaneous fermentation.

Histaminogenic potential of *hdcA*-positive *S. thermophilus* strains. The ability of the five *S. thermophilus* strains to produce histamine was confirmed and quantified by high-performance liquid chromatography (HPLC) on cultures grown in LM17 for 24 h. These were centrifuged at 8,000 × g for 10 min at 10°C. Histamine determination was done according to the

method of Martuscelli et al. (18) after derivatization with dansyl chloride (Sigma-Aldrich, Gallarate, Italy) and using a PU-2089 Intelligent HPLC quaternary pump and Intelligent UV-VIS multiwavelength detector UV 2070 Plus (Jasco Corporation, Tokyo, Japan). The amount of histamine was expressed as mg liter⁻¹ by reference to a calibration curve obtained with aqueous histamine standards of concentrations ranging between 10 and 200 mg liter⁻¹.

The amounts of histamine produced by the *S. thermophilus* strains ranged between 13 and 40 mg liter⁻¹. Strain PRI60, which produced the highest quantity of this BA, was chosen for the following experiments.

Sequencing of the *S. thermophilus* PRI60 *hdcA* gene and flanking regions. The *hdcA* gene fragment of *S. thermophilus* PRI60 was used as a target for uneven PCR (6) to obtain the complete sequence of the gene and indications on its neighboring regions. Exact match primers STHDEC-F and STHDEC-R (Table 1) were designed on the available 372-bp *hdcA* gene fragment by the Primer Express 2.0 program (Applied Biosystems, Foster City, CA), also for use in reverse transcription-quantitative PCR (RT-qPCR), and were coupled with arbitrary primers. Primers RDW and Coc1 (Table 1) permitted us to determine the upstream and downstream adjacent regions, respectively. The amplification products from uneven PCR were sequenced either with specific primers or after cloning in the pGEM-T Easy vector (Promega).

The open reading frames (ORFs) encoding the histamine decarboxylase and the amino acid permease were identified by the ORF Finder tool at NCBI. The entire *hdcA* ORF, 1,313 bp preceding its ATG translation start and 466 bp following its stop codon, was determined. The complete *S. thermophilus* PRI60 *hdcA* sequence was found to share a 99% identity with those of *S. thermophilus* CHCC6483 and CHCC1524 (GenBank accession no. FN686790 and FN686789, respectively). In total, eight mutations, at positions 34, 78, 318, 501, 571, 645, 879, and 915, were present. The first five mutations were in common with both strains. Among these, that at position 571 resulted in a missense Asp→Asn at the amino acid level. Of the remaining mutations, two were in *S. thermophilus* CHCC1524 (nucleotides 879 and 915) and the other one was in *S. thermophilus* CHCC6483. Moreover, the *hdcA* gene was 71% identical to its homolog in *Lactobacillus* spp. 30A (GenBank accession no. DQ132891) and shared a slightly lower degree of similarity with *hdcA* genes from *Lactobacillus reuteri* (70%), *Staphylococcus epidermidis*, *S. capitis*, *Lactoba-*

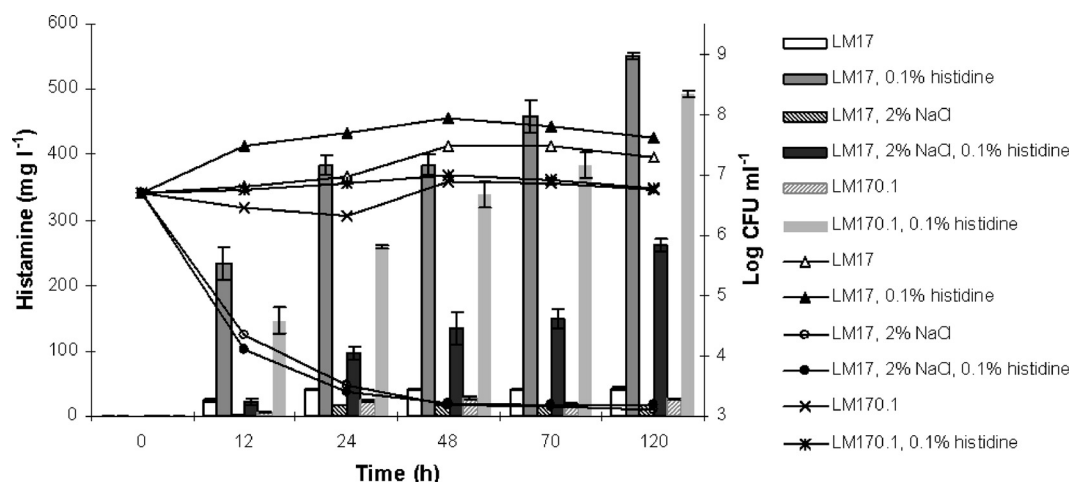


FIG. 1. Production of histamine (bars) and counts (lines) of *S. thermophilus* PRI60 grown in LM17 under the following conditions: LM17, LM17 with 2% (wt/vol) NaCl, LM17_{0.1} (with 0.1% [wt/vol] lactose), all with and without the addition of 0.1% (wt/vol) histidine.

cillus sakei, *O. oeni*, *Tetragenococcus halophilus*, *L. hilgardii* (69%), *Tetragenococcus muriaticus*, and *L. buchneri* (68%).

The 458-bp portion of the *hdcP* ORF was 99% identical to the corresponding region in strains *S. thermophilus* CHCC1524 and CHCC6483. Also in this case, there were different numbers of mutations found in *S. thermophilus* PRI60 with respect to these two strains (three and one, respectively). One of these mutations, at position 207 of the ORF, was the same. The next most similar sequence was that of the *hdcP* gene of *S. capitis* (69% identity).

According to the prediction analysis carried out at http://www.fruitfly.org/seq_tools/promoter.html, a putative promoter (maximum score, 1.00) starting 132 nucleotides upstream of the ATG codon precedes the *hdcA* gene of *S. thermophilus* PRI60. Two and one deletions were found between the putative -35 and -10 sequences compared to those of strains *S. thermophilus* CHCC1524 and CHCC6483, respectively, that could account for different promoter strengths. The ribosome binding site (RBS) is probably a GGAGA motif found at -14 nucleotides from the translation start site.

The 1,313-bp sequence upstream of *hdcA* showed the absence of other genes belonging to the *hdc* cluster. BLASTn analysis revealed two regions of 614 and 220 bp, separated by a 93-bp deletion, sharing, respectively, 98% and 95% identity with portions of the *S. thermophilus* genomic island ΔCIME308, GenBank accession no. AJ586570.1 (20), involved in lateral gene transfer (27). The 614-bp segment comprised the first 283 bp of the *hdsR* gene, encoding the restriction subunit of a type I modification/restriction system. Based on these indications, the *S. thermophilus* *hdc* gene cluster is probably located on the chromosome, but the vicinity of such genetic elements suggests that *hdc*-positive *S. thermophilus* strains might confer these genes to other strains or species.

Histamine production by *S. thermophilus* under different conditions. The levels of histamine produced by *S. thermophilus* PRI60 in different media and growth conditions were assayed. The chosen media were skim milk (Oxoid), skim milk with added 2% (wt/vol) NaCl, LM17, LM17 with 2% (wt/vol)

NaCl, and LM17_{0.1} (i.e., LM17 with a reduced amount of lactose, 0.1% [wt/vol]); when indicated, these media were supplemented after sterilization with 0.1% (wt/vol) histidine. The strain was inoculated in triplicate at 5×10^6 CFU ml⁻¹ and incubated at 37°C for 5 days. Counts of *S. thermophilus* PRI60 during incubation were done by plating in duplicate serial dilutions of the cultures on LM17 agar medium and incubating at 37°C for 48 h.

Histamine was not detected when the strain was grown in skim milk, with or without 2% (wt/vol) NaCl, but it was produced when histidine was supplemented (see Fig. S1 in the supplemental material). In this case, regardless of the presence of NaCl, a maximum level of histamine, approximately 350 mg liter⁻¹, was reached in the first 12 h. This value corresponded to the decarboxylation of about 50% of the histidine added to the medium. The growth of the strain was similar in all the tested conditions in skim milk, both with and without NaCl.

The experiment with synthetic medium LM17 was aimed at verifying the effect of low energy supply by diminishing the amount of lactose. In both LM17 and LM17_{0.1}, histamine was produced at relatively low levels (17 to 42 mg liter⁻¹) after 24 h and remained stable for 5 days also in the samples not supplemented with histidine for the presence of this amino acid or hydrolyzable peptides containing it already in the formulation (Fig. 1). When histidine was added, the production of histamine was considerably higher. However, differences in the medium composition determined the accumulation of various amounts of the BA that are not proportional to the cell number. Particularly, in the presence of NaCl relatively large amounts of histamine were formed notwithstanding the very low count values.

Our experiments highlighted the existence of *S. thermophilus* strains able to produce histamine at concentrations which can cause health concerns. In LM17 medium, after 24 h, *S. thermophilus* PRI60 produced approximately double the amount of histamine of *S. thermophilus* CHCC1524 (5). Moreover, the longer incubation time used in the present study permitted us to observe a further increase of the histamine concentration after 48 h (Fig. 1).

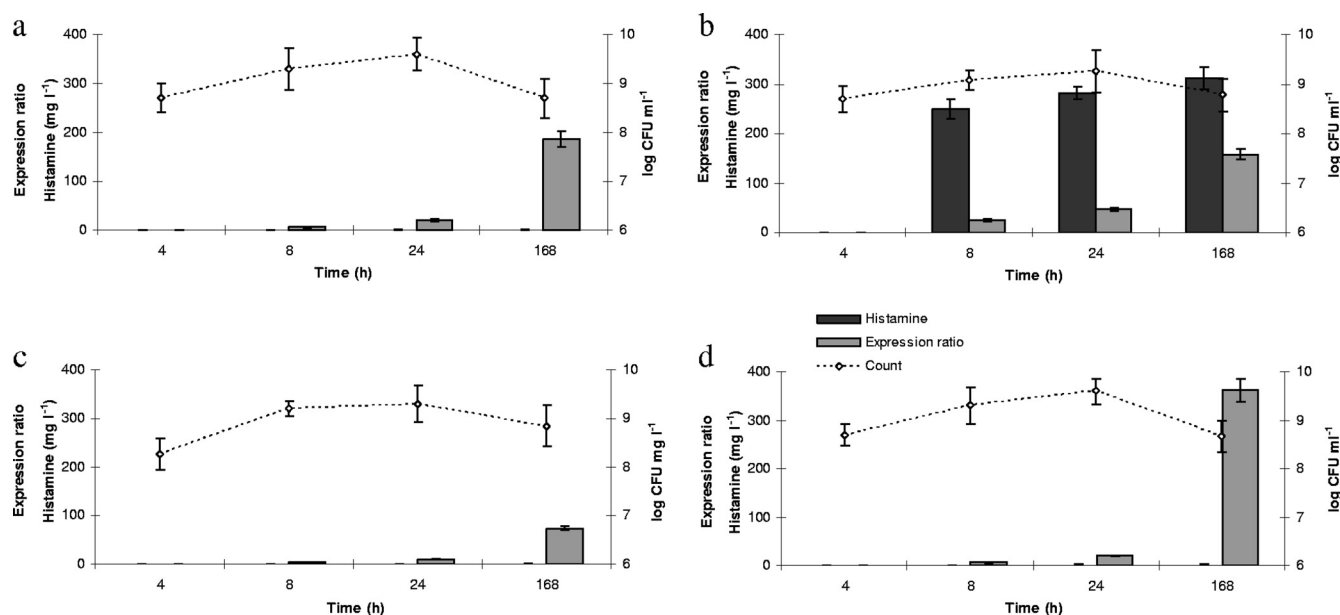


FIG. 2. Transcription ratio of the *hdcA* gene referred to the reference gene *rpoA* (gray bars), histamine formation (black bars), and counts (lines) of *S. thermophilus* PRI60 during growth under the following conditions: skim milk (a), skim milk plus 0.1% (wt/vol) histidine (added after 6 h) (b), thermized (67°C for 20 s) inoculum in skim milk (c), and skim milk plus 2% (wt/vol) NaCl (added after 24 h) (d).

***hdcA* transcriptional analysis and histamine production under conditions relevant for cheese making.** The expression level of the *hdcA* gene and the histamine accumulation by *S. thermophilus* PRI60 were quantified by RT-qPCR and HPLC, respectively, evaluating the effect of parameters relevant for cheese making, such as thermization, presence of 0.1% (wt/vol) histidine (a level possibly found in cheese), presence of 2% (wt/vol) NaCl, and ripening temperature of 20°C. *S. thermophilus* PRI60 was inoculated in skim milk at an initial cell number of 5×10^6 CFU ml⁻¹ and incubated at 37°C for 24 h. Later the samples were kept at 20°C for a further 6 days. Histidine and NaCl were added in some samples after 6 and 24 h, respectively. In other cultures an inoculum heated at 67°C for 20 s was used to study the effect of thermization.

The transcriptional level of the *hdcA* gene was referred to the constitutive gene *rpoA*, encoding the *S. thermophilus* RNA polymerase alpha subunit. This was chosen as a reference gene among those reported to be stably expressed in bacteria (23). The stability of expression of *rpoA* in *S. thermophilus* PRI60 was experimentally confirmed as described in a previous paper (13), with a maximum intertreatment variation of the transcript $2^{\Delta CT} = 2.0$. An RT-qPCR test was assessed for each target gene using the primer pairs STHDEC-F/STHDEC-R and RPOST-F/RPOST-R (Table 1), specific for *hdcA* and *rpoA*, respectively.

The qPCRs were carried out as described previously (13) using the Platinum SYBR green Supermix UDG with Rox (6-carboxy-X-rhodamine) dye (Invitrogen, Milan, Italy). Calibration curves were constructed by using as targets duplicate 10-fold serial dilutions of the *hdcA*- and *rpoA*-specific amplicons. The linearity ranges of both tests included points between 0.3 and 6.3 log copies of target in the reaction (see Fig. S2 in the supplemental material). The efficiencies [E =

$10^{(-1/\text{slope})} - 1$] of the *hdcA*- and *rpoA*-specific qPCR tests were 0.98 and 1.02, respectively.

RNA extraction from skim milk cultures and reverse transcription for the RT-qPCR tests were carried out as previously described (13). Duplicate RT-qPCR trials were carried out amplifying *hdcA* and *rpoA* in separate sets of reactions on the same cDNAs. The expression ratio of *hdcA* referred to *rpoA* was determined according to the method of Pfaffl (22), and the C_T value of each gene at 4 h of incubation was used as a control.

The growth of *S. thermophilus* PRI60 during the trials was very similar in all the cultures (Fig. 2). A pH decrease to 4.23, or to 4.28 in the cultures with added histidine, was observed after 24 h; then the values remained stable for the whole incubation period.

Based on the C_T values, which depend on the transcript copy number, the *hdcA* gene of *S. thermophilus* PRI60 was highly expressed since the early stages of incubation and it further increased in the stationary phase. This is in contrast with the results of Calles-Enríquez et al. (5), who found higher expression at the onset of the stationary phase. Indeed, only at 4 h was the average copy number of the *hdcA* transcript (6.1 log) lower than that of the *rpoA* transcript (6.9 log), while after 8 h from the inoculum the copy number of the *hdcA* transcript was found to be equal to or higher than that of the *rpoA* transcript. The increase of *hdcA* expression during incubation suggested a positive regulation by acidification and energy source depletion. This provided evidence that the activation of HdcA reported under such conditions for *O. oeni* and *S. capitis* (7, 10) occurs at the transcriptional level in *S. thermophilus*.

In Fig. 2, the normalized *hdcA* transcription levels during the trial are reported, together with the histamine production. Histamine was detected in relevant amounts only in the sam-

ples supplemented with histidine (Fig. 2b), while *hdcA* was expressed in all cases. After 8 h, the expression ratio of *hdcA* varied from 4.9 to 24.7 compared to the control (4 h), depending on the growth conditions. However, in the samples supplemented with histidine, the expression ratio of *hdcA* increased faster than in the cultures without histidine, indicating that the level of transcription was enhanced by the addition of the amino acid. At 24 h the *hdcA* expression ratio in the presence of histidine was about 2-fold higher than in cultures without histidine (Fig. 2a and b). It must be noticed that about 80% of the total histamine produced after 7 days was accumulated in the first 2 h after the addition of histidine, when the cultures were not yet in stationary phase. The observation that a large amount of histamine is rapidly formed in the presence of histidine indicated that *S. thermophilus* PRI60 has a high potential of efficiently accumulating this BA in dairy substrates.

Thermization of the inoculum reduced the expression level of *hdcA*, and it remained lower at all time intervals compared to that in the other trials (Fig. 2c). No explanation of this finding is available from the literature. Therefore, the regulation mechanisms involved need to be clarified in order to exploit heat treatment to reduce histamine formation by *S. thermophilus*.

On the other hand, the addition of salt led to an upregulation of *hdcA* (Fig. 2d) as observed at 7 days, when the expression ratio was 1.9-fold the corresponding value in skim milk. This result suggests a potential role of *hdcA* in osmoprotection mechanisms, though no literature data are available on this respect. Moreover, this finding can have implications for cheese making and can promote further research on the physiological role of histidine decarboxylation in bacteria.

Effects of histidine and cell integrity on histamine production. In order to evaluate if culturing in the presence of histidine could influence the overall decarboxylase activity of *S. thermophilus* PRI60 and to determine the activity of the HdcA enzyme in cell lysates, enzymatic assays were carried out with whole and disrupted cells. The strain was previously subcultured twice for 24 h in skim milk with or without the addition of 0.1% (wt/vol) histidine. Skim milk was chosen as the precultivation medium because of its inability to support histamine formation in the absence of added histidine (Fig. 2). Cultures of 100 ml were centrifuged at $4,000 \times g$ for 10 min in the presence of two volumes of EDTA 26.8 mM, pH 12, washed twice with physiological solution (0.9% wt/vol NaCl), and resuspended in 20 ml of the same solution. Cell suspensions were subdivided into two aliquots: one was used for testing the HdcA activity of whole cells, and the other was submitted to three passages at $18,000 \text{ lb/in}^2$ through a French pressure cell press (SLM Aminco; Spectronic Instruments, Rochester, NY) to disrupt the cells and test the activity in cell extracts. The treated cells were cooled in ice between the breaking passages. Finally, both whole-cell suspensions and cell extracts were added to phosphate buffer (pH 5.3) containing 0.1% (wt/vol) histidine in triplicate at a concentration corresponding to about $2.5 \times 10^8 \text{ CFU ml}^{-1}$, and incubated at 37°C. Samples were collected at different times (0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, and 24 h) for the determination of histamine content. After centrifugation at $10,000 \times g$ for 10 min at 8°C, the supernatants were used for HPLC analysis. The series of

values of histamine amounts formed by whole or broken cells were compared by Student's *t* test.

The trial showed that histamine increased over time in both whole and broken cells, but the accumulation rates were not significantly affected by the presence of histidine in the precultivation step (see Fig. S3 in the supplemental material). In addition, the histidine decarboxylase was particularly active in the cell extracts, where histamine accumulation was faster than in the whole cells. Indeed, the histamine content after 24 h of incubation was higher for the cell extracts (approximately $220 \text{ mg liter}^{-1}$ against the $160 \text{ mg liter}^{-1}$ obtained with the whole cells).

Though histidine was found to enhance the expression of the *hdcA* gene, the increase observed at the transcriptional level did not correspond to significantly different levels of histamine produced (see Fig. S3 in the supplemental material). An explanation could be that a probable high affinity of the HdcA for its substrate and consequent high speed of the enzymatic reaction may have masked the differences in the amounts of enzyme.

It is noteworthy that the *S. thermophilus* HdcA was more active in cell extract. The higher activity observed can be explained by the slowing down of the decarboxylase reaction by the histamine/histidine exchange across the membrane in whole cells. The high level HdcA activity after cell lysis is a relevant concern for cheese production, since in these products HdcA could remain active for a long time during ripening and determine high histamine contents.

On the whole, results indicated that the presence of *S. thermophilus* strains carrying HdcA activity is a risk unknown until recently, and further research efforts should be focused on its better characterization and prevention. Remedies that can be carried out to reduce the risk of histamine formation in cheeses by *hdcA* gene-positive *S. thermophilus* strains are (i) the introduction of a screening for decarboxylases, such as a PCR-based detection of the *hdcA* gene, among the procedures applied for the selection of starter cultures and (ii) the possibility of using autochthonous nonhistaminogenic *S. thermophilus* isolates to outcompete histamine producers in artisanal dairy products.

A safer exploitation of this bacterial species can be promoted by revising its QPS (qualified presumption of safety) or GRAS (generally recognized as safe) status by taking into account the occurrence of BA-producing strains.

Nucleotide sequence accession number. The EMBL/GenBank/DBJ databases accession number of the sequence determined in this study is FR693807.

This research was partly supported by a grant from the Italian Ministry of University and Research within the program PRIN 2006 no. 2006072328_004.

We acknowledge Carlo Rugolotto for technical assistance.

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