Lactobacillus reuteri 2'-Deoxyribosyltransferase, a Novel Biocatalyst for Tailoring of Nucleosides^{▽†‡}

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A novel type II nucleoside 2'-deoxyribosyltransferase from *Lactobacillus reuteri* (LrNDT) has been cloned and overexpressed in *Escherichia coli*. The recombinant LrNDT has been structural and functionally characterized. Sedimentation equilibrium analysis revealed a homohexameric molecule of 114 kDa. Circular dichroism studies have showed a secondary structure containing 55% α -helix, 10% β -strand, 16% β -sheet, and 19% random coil. LrNDT was thermostable with a melting temperature (T_m) of 64°C determined by fluorescence, circular dichroism, and differential scanning calorimetric studies. The enzyme showed high activity in a broad pH range (4.6 to 7.9) and was also very stable between pH 4 and 7.9. The optimal temperature for activity was 40°C. The recombinant LrNDT was able to synthesize natural and nonnatural nucleoside analogues, improving activities described in the literature, and remarkably, exhibited unexpected new arabinosyltransferase activity, which had not been described so far in this kind of enzyme. Furthermore, synthesis of new arabinonucleosides and 2'-fluorodeoxyribonucleosides was carried out.

Nucleoside 2'-deoxyribosyltransferases (NDTs) (EC 2.4.2.6) catalyze the exchange between the purine or pyrimidine base of 2'-deoxyribonucleosides and free pyrimidine or purine bases (10, 25). These enzymes are specific for 2'-deoxyribonucleosides, regioselective (N-1 glycosylation in pyrimidine and N-9 in purine), and stereoselective (β -anomers are exclusively formed) (26) (Fig. 1).

Deoxyribosyltransferases are classified into two classes depending on their substrate specificity: type I (NDT I), specific for purines (Pur $\leftrightarrow$ Pur), and type II (NDT II), which catalyzes the transfer between purines and/or pyrimidines (Pur $\leftrightarrow$ Pur, Pur $\leftrightarrow$ Pyr, Pyr $\leftrightarrow$ Pyr) (10, 25). These enzymes were initially described for lactobacilli (27, 28), and they are involved in the nucleoside salvage pathway for DNA synthesis (23), although this remains unclear in *Lactococcus lactis* subsp. *lactis* (36). NDTs have been also found in some species of *Streptococcus* (11), in parasitic unicellular eukaryotic organisms such as *Crithidia luciliae* (49, 50), in *Trypanosoma brucei* (6), and in *Borrelia burgdorferi* (33). NDTs from *Lactobacillus helveticus* and *Lactobacillus leichmannii* have been well studied (2, 25, 26, 28, 29), and their kinetic mechanisms as well as their catalytic and substrate binding sites have been characterized. The transferase reaction proceeds via a ping-pong bi-bi mechanism by formation of a covalent deoxyribosyl enzyme intermediate (3,

15, 16). Likewise, a glutamyl residue (Glu98) has been proven essential for activity (40, 41, 46).

Enzymatic natural and nonnatural nucleoside synthesis in a one-pot reaction by NDTs provides an interesting alternative to traditional multistep chemical methods (13, 34). Indeed, chemical glycosylation includes several protection-deprotection steps and the use of chemical reagents and organic solvents that are expensive and environmentally harmful. Whereas previously described NDTs accept different nucleosides fromazole derivatives (5, 39) to expanded-size purines (37, 45), they are highly specific for 2'-deoxyribose and do not accept ribonucleosides as donors, because the nucleophilic oxygen atom of the catalytic glutamic hydrogen bonds to the O-2' atom of ribonucleosides and is, thus, inactive (1).

Since several nonnatural nucleosides acting as antiviral or anticancer agents have modifications on their sugar moiety, research on new biocatalysts able to synthesize them as alternatives to chemical synthesis is still relevant.

Here we report the cloning and expression of a putative *ndt* gene encoding a putative nucleoside 2'-deoxyribosyltransferase from *Lactobacillus reuteri* (LrNDT), and we show that LrNDT is a type II NDT. Moreover, we have characterized the purified LrNDT structurally and functionally. Remarkably, LrNDT synthesizes natural and nonnatural nucleosides and bases with higher activities than those described in the literature. More interestingly, LrNDT is able to synthesize new nonnatural nucleosides: 2'-fluorodeoxyribonucleosides and arabinonucleosides. It is important to note that arabinosyltransferase activity has not been described in this kind of enzyme before, this being the first time that an NDT enzyme has shown arabinosyltransferase activity. These results are very interesting since LrNDTs, inactive for ribonucleosides, can recognize arabinonucleosides and 2'-fluorodeoxyribonucleosides as substrates.

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

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FIG. 1. 2'-Deoxyribosyltransferase reaction catalyzed by NDTs. E, enzyme; B₁ and B₂, purine or pyrimidine.

MATERIALS AND METHODS

Chemicals. Cell culture medium reagents were from Difco (St. Louis, MO). Enzyme substrates and chemical reagents were from Sigma-Aldrich. Arabinonucleosides were a gift from Bio Sint (Italy), and 2'-fluoro-2'-deoxyribonucleosides were supplied by Rasayan Inc. (California).

Microorganisms, culture conditions, plasmids, DNA manipulation, and sequencing. *Lactobacillus reuteri* CECT 925, a putative nucleoside 2'-deoxyribosyltransferase (NDT) producer, was used as a chromosomal DNA source. *Escherichia coli* DH5 α was used as a host for subcloning experiments, and *Escherichia coli* BL21(DE3) was used as a host for gene expression. *L. reuteri* cells were cultured at 30°C in MRS (De Man-Rogosa-Sharpe) medium (17). *E. coli* cells were cultured at 37°C in LB (Luria-Bertani) medium, and the transformations were carried out by standard procedures (43). pGEM-T Easy vector (Promega) was used for subcloning experiments, and pET28a(+) (Novagen) was used for gene expression in *Escherichia coli* BL21(DE3). Antibiotics were added at the indicated concentrations: ampicillin (100 μ g/ml) and kanamycin (50 μ g/ml). All DNA manipulations were carried out according to standard procedures for *E. coli* (43). DNA sequences were determined by the dideoxy chain termination method (44) with an automated sequencer, a DNA Analyzer 3730 (Applied Biosystems).

Construction of *E. coli* strain overexpressing the *ndt* gene. The *ndt* gene encoding putative NDT from *L. reuteri* was amplified by PCR using chromosomal DNA from *L. reuteri* CECT 925 as template. The PCR primers used were designed according to the DNA sequence of *ndt* from *L. reuteri*: LRNCO (5'-C ATGCCATGGATGATAAAATCAAAAAAGTAAGACAG-3') and LRBAM (5'-CGGGATCCTTAATATATACCTCCACCATCC-3'). The restriction sites NcoI and BamHI, respectively (underlined), were included in the primers to facilitate subcloning. PCR amplification was carried out under standard conditions in a Mastercycler gradient thermocycler (Eppendorf, Germany) using *Pfu* DNA polymerase (Promega). The amplified 0.5-kb product was purified with GeneClean (Bio 101) and inserted into pGEM-T Easy vector (Promega). The resulting recombinant plasmid was purified with the High Pure isolation kit (Roche, Switzerland) and sequenced to confirm the absence of mutations. The *ndt* gene was then rescued as an NcoI and BamHI fragment and cloned into the BamHI-NcoI site of pET28a(+), giving pT28*ndt*, which was purified with the High Pure isolation kit (Roche, Switzerland), sequenced, and used to transform competent *E. coli* BL21(DE3) cells, producing the recombinant *E. coli* CECT 7435.

Overproduction and purification of recombinant *Lr*NDT. *E. coli* CECT 7435 cells harboring pT28*ndt* were grown at 37°C in LB liquid medium containing kanamycin at 50 μ g/ml. When the culture reached an optical density at 600 nm (OD₆₀₀) of 0.8, it was induced with 0.4 mM IPTG (isopropyl- β -D-thiogalactopyranoside) for 2.5 h. Then, cells were harvested by centrifugation at 3,500 $\times$ g for 15 min, resuspended in 10 mM potassium phosphate buffer (pH 7; buffer A), and broken by ultrasonic treatment using a Branson digital sonifier. The cell extract was applied onto a 5-ml Econo-Pac High Q cartridge (Bio-Rad) equilibrated in the same buffer. The column was extensively washed with buffer A, and then the protein was eluted with a linear gradient of 0 to 0.5 M NaCl in buffer A. The protein fractions containing *Lr*NDT enzyme were pooled and concentrated with polyethylene glycol 35000 (Sigma) (reverse dialysis) and loaded onto a Superose 12 Fast Flow column (Amersham Biosciences, United Kingdom) equilibrated with 50 mM potassium phosphate buffer, pH 7.0 (buffer B). Protein concentration was determined according to the method of Bradford (7). Electrophoresis on 0.1% sodium dodecyl sulfate (SDS) was carried out on a polyacrylamide slab gel (12.5%) with 25 mM Tris-HCl buffer, pH 8.6, according to the method of Laemmli (30). To determine the N-terminal sequences of the *Lr*NDT, the protein bands were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Bio-Rad) (47), and sequenced by automatic Edman degradation.

2'-Deoxyribosyltransferase assays. The standard activity assay was performed with incubation of 5 μ l of cell extract or 0.34 μ g of pure enzyme with 10 mM 2'-deoxyuridine and 10 mM adenine in 50 mM MES (morpholineethanesulfonic acid) buffer, pH 6.5. The reaction mixtures were incubated at 40°C for 5 min in a final volume of 40 μ l. Then, reactions were stopped by addition of 40 μ l of cold

methanol in an ice bath and heated at 95°C for 5 min. After centrifugation of the samples at 9,000 $\times$ g for 2 min, 50 μ l of supernatant was diluted with 50 μ l of water and the production of nucleosides was analyzed by high-pressure liquid chromatography (HPLC) (Agilent 1100 series) with a Luna C₁₈ column, 5 μ m, 250 $\times$ 46 mm (Phenomenex). The elution conditions were as follows: 0 to 10 min, 100 to 90% trimethyl ammonium acetate and 0 to 10% acetonitrile, and 10 to 20 min, 90 to 100% trimethyl ammonium acetate and 10 to 0% acetonitrile. The flow rate was fixed at 1 ml/min, and the pressure was 18,000 kPa. Four wavelengths were used for detection: 254, 260, 240, and 230 nm. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μ mol of product per min under standard conditions: 50 mM MES buffer, pH 6.5, 40°C, 10 mM substrate concentration. Under these conditions the reaction was linear at least up to 15 min of reaction and up to 40 μ g of pure protein (for an example, see document S2 in the supplemental material).

Enzymatic synthesis of natural nucleosides was performed as described for the NDT standard assay using different 2'-deoxyribonucleosides and bases (10 mM). Enzymatic synthesis of nonnatural nucleosides was carried out from natural or nonnatural deoxyribonucleosides and bases at different conditions. The production of nucleosides was detected by HPLC as described above (for an example, see document S2 in the supplemental material). All activity determinations were performed in triplicate.

Retention times (*t_r*) for the reference compounds were as follows: (i) natural compounds, uracil (Ura), 5.41 min; 2'-deoxyuridine (dUrd), 9.16 min; adenine (Ade), 10.14 min; 2'-deoxyadenosine (dAdo), 15.50 min; hypoxanthine (Hyp), 7.34 min; 2'-deoxyinosine (dIno), 10.95 min; cytosine (Cyt), 9.14 min; 2'-deoxycytidine (dCyd), 8.22 min; thymine (Thy), 9.68 min; thymidine (dThd), 13.25 min; uric acid (UA), 3.50 min; (ii) nonnatural compounds, 5-fluorouracil (5-FUra), 5.94 min; 5-chlorouracil (5-ClUra), 8.71 min; 5-bromouracil (5-BrUra), 10.28 min; 5-iodouracil (5-IUra), 13.10 min; flucytosine (5-FCyt), 5.41 min; 5-fluoro-2-methoxy-4(1H)pyrimidinone (FMP), 8.47 min; 2,6-diaminopurine (DAP), 8.71 min; 6-mercaptopurine (6-M), 8.85 min; benzimidazole (B), 24.26 min; 5-fluoro-2'-deoxyuridine (5-FdU), 9.8 min; 5-chloro-2'-deoxyuridine (5-ClUrd), 13.3 min; 5-bromo-2'-deoxyuridine (5-BrUrd), 15.7 min; 5-iodo-2'-deoxyuridine (5-IdUrd), 17.73 min; 5-fluoro-2'-deoxycytidine (5-FdCyd), 9.16 min; 5-fluoro-2-methoxy-4(1H)pyrimidinone-2'-deoxyribose (5-FMP-dRib), 13.42 min; 2,6-diaminopurine-2'-deoxyribose (2,6-DAP-dRib), 14.29 min; 6-mercaptopurine-2'-deoxyribose (6-MdRib), 11.96 min; benzimidazole-2'-deoxyribose (BdRib), 28.95 min; *ara*-adenine (*ara*-Ade), 13.14 min; *ara*-uracil (*ara*-Ura), 8.68 min; *ara*-cytosine (*ara*-Cyt), 7.3 min; 2'-fluoro-2'-deoxyuridine (2'-FdUrd), 10.3 min; 2'-fluoro-2'-deoxycytidine (2'-FdCyd), 8.7 min; 2'-fluoro-2'-deoxyadenosine (2'-FdAdo), 16.07 min; 5-fluoro-2'-fluorodeoxyuridine (5-F,2'-FdUrd), 13.94 min; 5-fluoro-2'-fluorodeoxycytidine (5-F,2'-FdCyd), 13.1 min; 5-chloro-2'-fluorodeoxyuridine (5-Cl,2'-FdUrd), 16.2 min; 5-bromo-2'-fluorodeoxyuridine (5-Br,2'-FdUrd), 18.2 min; 5-iodo-2'-fluorodeoxyuridine (5-I,2'-FdUrd), 20.1 min; 5-fluoro-2-methoxy-4(1H)pyrimidinone-2'-fluorodeoxyribose (5-FMP,2'-FdRib), 16.4 min; 2,6-diaminopurine-2'-fluorodeoxyribose (2,6-DAP,2'-FdRib), 17.29 min; *ara*-5-fluorouracil (*ara*-FUra), 10.9 min; *ara*-5-chlorouracil (*ara*-ClUra), 13.2 min; *ara*-5-bromouracil (*ara*-BrUra), 15.7 min; *ara*-5-iodouracil, 17.9 min; *ara*-flucytosine (*ara*-FCyt), 10.12 min; *ara*-5-fluoro-2-methoxy-4(1H)pyrimidinone (*ara*-5-FMP), 13.8 min; *ara*-2,6-diaminopurine (*ara*-2,6-DAP), 15.1 min.

Analytical ultracentrifugation analysis. Sedimentation velocity and equilibrium experiments for *Lr*NDT were carried out in the Centro de Investigaciones Biológicas (CSIC; Spain). Experiments were performed in 50 mM potassium phosphate buffer (pH 7)-0.5 M NaCl at 20°C and 50,000 $\times$ g in an Optima XL-I analytical ultracentrifuge (Beckman-Coulter Inc.), equipped with absorbance optics, using an An-60Ti rotor and standard (12-mm optical path) double-sector center pieces of Epon-charcoal. Baseline offsets were measured afterwards at 200,000 $\times$ g. The apparent sedimentation coefficient of distribution, *c*(s), and sedimentation coefficient *s* were calculated from the sedimentation velocity data using the program SEDFIT (8). The whole-cell weight-average *bM_w* (buoyant molar mass) values were obtained by fitting the experimental data to the equation for the radial concentration distribution of an ideal solute at sedimentation

equilibrium, using the program EQASSOC supplied by Beckman-Coulter (32). The corresponding apparent weight-average molar masses (M_w) were determined from the buoyant masses, taking into account the partial specific volumes of the protein (0.738 ml/g) obtained from the amino acid composition using the program SEDNTERP (35).

Spectrometric determinations. The molar extinction coefficient of *Lr*NDT was determined by the method of Edelhoch (18) according to the following equation: $(A_{\text{nat}}/A_{\text{unf}}) = (\epsilon_{\text{nat}}/\epsilon_{\text{unf}})$, where A_{nat} and A_{unf} are the absorbance values at 280 nm for the soluble protein in 10 mM potassium phosphate buffer, 0.5 M NaCl, pH 7.0, and that for unfolded protein in the presence of 6 M guanidine hydrochloride, respectively. ϵ_{unf} , the molar extinction coefficient of unfolded protein, was calculated according to the amino acid sequence of the enzyme by using the ProtParam program (<http://us.expasy.org/cgi-bin/protparam>) (21).

Fluorescence emission spectra of pure recombinant *Lr*NDT were monitored at 25°C using an SLM-Aminco 8000C fluorescence spectrophotometer with thermostat-linked 0.4-cm- and 1-cm-path-length quartz cells of excitation and emission, respectively. The excitation and emission slit widths were 5 nm. The scan rates were 60 nm/min. Two excitation wavelengths were used, 280 nm and 295 nm. Protein concentration was 0.1 mg/ml in 50 mM potassium phosphate buffer, pH 7.0. Temperature dependence studies of the fluorescence emission spectra of pure recombinant *Lr*NDT were performed between 20 and 85°C.

Circular dichroism (CD) spectra of pure recombinant *Lr*NDT were recorded using a Jasco J-715 (Japan) spectropolarimeter with a thermostat-linked 0.1-cm-path-length quartz cell in the far-UV region. The protein concentration was 0.21 mg/ml in 50 mM potassium phosphate buffer, pH 7, at 25°C. The CD readings were expressed as the mean residue molar ellipticity ($\text{deg} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$), assuming a residue molecular mass of 110 kDa. Secondary structure information on *Lr*NDT has been obtained from CD spectra by using the CDSTRR, CONTILL, and SELCON3 programs of the CDPACK pack (46).

DSC studies. Differential scanning calorimetry (DSC) experiments were performed using a Microcalorimeter VP-DSC calorimeter (Microcal). Six hundred microliters of pure enzyme solution (0.21 mg/ml) in 50 mM potassium phosphate buffer, pH 7, was charged in the reference cell and exposed under several temperatures between 20 and 90°C.

Temperature and pH studies. Thermal stability was studied by incubating pure NDT solutions (0.0675 mg/ml) in 50 mM MES buffer, pH 6.5, at different temperatures (20 to 80°C). At regular intervals of time, 5- μ l aliquots were extracted from the incubation mixture and the residual activity was determined at 40°C using 2'-deoxyadenosine synthesis from 2'-deoxyuridine and adenine under the standard assay conditions. The experimental deactivation data were modeled by equation 1 or equation 2 using a nonlinear regression procedure based on the Levenberg-Marquardt method of iterative convergence (24) included in the TableCurve 2D v5.01 program:

$$a = a_0 \cdot \exp(k_1 t) \quad (1)$$

$$a = \frac{E + \alpha_1 \cdot E_1 + \alpha_2 \cdot E_2}{E_0} = \alpha_2 + \left[1 + \frac{\alpha_1 k_1}{k_2 - k_1} - \frac{\alpha_2 k_2}{k_2 - k_1} \right] \cdot [\exp(-k_1 \cdot t)] - (\alpha_1 - \alpha_2) \left[\frac{k_1}{k_2 - k_1} \right] \cdot [\exp(-k_2 \cdot t)] \quad (2)$$

where a is the residual activity; E , E_1 , and E_2 are the specific activities of native, intermediate, and denatured enzyme, respectively; α_1 and α_2 are the ratios of specific activities E_1/E and E_2/E , respectively; and k_1 and k_2 are first-order deactivation coefficients. The values of k_1 , k_2 , α_1 , and α_2 were constrained to be nonnegative, and the theoretical deactivation curves were obtained from equation 1 or 2 by substitution of the calculated convergence values. Agreement between the experimental and theoretical data was considered good when the convergence relation coefficient was higher than 0.98 for each deactivation experiment.

The optimal temperature for enzyme activity was also determined by measuring the activity between 20 and 80°C using 2'-deoxyadenosine synthesis as described above.

The *Lr*NDT stability at different pHs was studied by incubating 0.34 μ g of pure recombinant enzyme at pH 4.0 to 8.0 for 15 min at 4°C in 10 mM potassium citrate-phosphate buffer at a constant ionic strength (I) of 150 mM, and after that, enzyme samples were adjusted to pH 6.5 by the addition of 50 mM MES buffer and the *Lr*NDT activity was measured at 40°C using thymidine synthesis from 2'-deoxyuridine and thymine under the standard conditions. Optimal pH was determined by measuring the *Lr*NDT activity in 10 mM potassium citrate-phosphate buffer at different pHs (4.0 to 8.5). The ionic strength (I) at each pH was adjusted to 150 mM by addition of NaCl in amounts calculated using a Visual

FIG. 2. Purification of *Lr*NDT. SDS-PAGE of different purification steps. Lane 1, 20 μ g of total protein after Econo-Pac High Q cartridge chromatography. Lane 2, 10 μ g of protein after size-exclusion chromatography on Superose 12. Lane P, prestained standard proteins from Bio-Rad used as molecular mass markers. The N-terminal amino acid sequence of the *Lr*NDT subunit is shown in single-letter code.

Basic program developed in our laboratory, which allows analysis of buffer systems with up to four tetraprotic species.

All activity determinations were performed in triplicate.

RESULTS

Purification and structural characterization of type II nucleoside 2'-deoxyribosyltransferase from *Lactobacillus reuteri*. The putative gene *ndt*, encoding nucleoside 2'-deoxyribosyltransferase from *Lactobacillus reuteri* (*Lr*NDT), was amplified by PCR, cloned, and overexpressed in *Escherichia coli* BL21 (DE3) (Materials and Methods). The recombinant *Lr*NDT produced by *E. coli* CECT 7435 was purified by two chromatographic steps. SDS-PAGE analysis of purified enzyme shows only one protein band with an apparent molecular mass of 18 kDa (Fig. 2). The N-terminal sequence (12 amino acids long) of the recombinant NDT confirmed that the purified enzyme was *Lr*NDT. In order to determine the 2'-deoxyribosyltransferase activity shown by *Lr*NDT, different reactions with ribo- and 2'-deoxyribonucleosides (dUrd + dAdo, dAdo + Hyp, dUrd + Thy, Ino + Ade, and Urd + Ade) were performed. The pure enzyme did not interchange bases between ribonucleosides; in contrast, it was able to catalyze the transfer of 2'-deoxyribose between purine or pyrimidine and/or pyrimidine bases, showing that *Lr*NDT should be classified as a type II NDT and is free of nucleoside phosphorylase traces.

In addition, analytical ultracentrifugation studies have been carried out in order to determine the molecular mass and the oligomeric state of *Lr*NDT in solution as well as its shape. Figure 3A shows the apparent sedimentation coefficient distributions, $c(s)$, for *Lr*NDT calculated from sedimentation velocity experiments using the program SEDFIT (8). All of the distributions were symmetrical, and a single major molecular species is in solution, which allows the conversion to $c(M)$, resulting in a molar mass of 112.7 kDa, thus indicating a hexameric state.

Moreover, sedimentation equilibrium experiments were performed to obtain an accurate measure of mass. *Lr*NDT sedimented at equilibrium as a single species with a best-fit bM_w of

FIG. 3. Analytical ultracentrifugation analysis for *LrNDT*. Sedimentation experiments were carried out using a protein concentration of 0.675 mg/ml in 50 mM potassium phosphate buffer (pH 7)-0.5 M NaCl, at 20°C and $50,000 \times g$. (A) Sedimentation coefficient distribution $c(s)$. (B) Sedimentation equilibrium gradient. The solid lines show the corresponding best-fit gradient for a single sedimenting species at sedimentation equilibrium with a bM_w value of $28,901 \pm 149$ Da.

$28,901 \pm 149$ Da (Fig. 3B), which, after buoyancy correction, is compatible with a single-species homohexameric model too of 113 ± 3 kDa. This value is similar to the molecular mass calculated from the amino acid sequence (109 kDa). These results agree with previous reports for this kind of enzyme (1, 3, 14, 25, 28, 36, 49, 50).

In order to characterize *LrNDT*, several properties have been further investigated. Thus, the molar extinction coefficient in solution, $\epsilon_{280} = 34,996 \text{ M}^{-1} \text{ cm}^{-1}$, was determined as described above. On the other hand, the fluorescence spectra of *LrNDT* were recorded at two excitation wavelengths, 280 nm and 295 nm (see Fig. S1 in the supplemental material). Since a single maximum emission peak at 334 nm was observed

in all spectra, we were able to conclude that tryptophans are the main residues responsible for the emission and all of them must be located inside the *LrNDT* structure.

Moreover, the content of regular elements of secondary structure in *LrNDT* was determined by obtaining its far-UV CD spectrum (Fig. 4). The protein contains 55% α -helix, 16% β -sheet, 10% β -turns, and 19% random coil. These results agree with the secondary structure predicted from its amino acid sequence and are similar to those described for *Lactobacillus leichmannii* (2, 14).

Furthermore, CD thermal denaturation curves (inset in Fig. 4) as well as temperature-dependence decay studies of fluorescence and calorimetry experiments (see Fig. S2 in the sup-

FIG. 4. Spectroscopic characterization of purified recombinant NDT from *Lactobacillus reuteri*. Far-UV CD spectra of *LrNDT* (0.21 mg/ml) in 50 mM potassium buffer (pH 7.0) at 25°C. (Inset) Thermal unfolding of *LrNDT* monitored by CD variation at 220 nm between 25 and 85°C scanned at 20°C/hour.

plemental material) revealed that the *LrNDT* global structure was highly thermostable, showing in all cases similar T_m s of 64°C.

Biochemical characterization. Since the optimal conditions for NDT activity are not well established in the literature, we have determined them for the reactions catalyzed for *LrNDT*. Thus, temperature, pH, and ionic strength (I) dependence on the stability and the activity of the enzyme as well as the effect of several ions on NDT activity were studied.

An important criterion for enzyme activity determination is that the enzyme must be stable during the assay time, and thus, the effect of temperature on *LrNDT* was examined at different temperatures ranging from 20 to 80°C. Thus, thermal stability of the recombinant NDT was studied by preincubating the enzyme at different temperatures, after which aliquots were withdrawn at the indicated times and tested for activity (Fig. 5A). The activity was stable up to 50°C, whereas activity progressively decreased to become almost abolished when the enzyme was stored at temperatures ranging from 50°C to 80°C (Fig. 5A and B). In addition, the effect of temperature on *LrNDT* activity was examined by measuring its activity in the temperature range from 20 to 80°C using 2'-deoxyadenosine

FIG. 5. Temperature and pH dependence of *LrNDT* activity. (A) Thermal deactivation profile at 20°C, 30°C, 40°C, and 50°C (■); 60°C (●); 65°C (▲); 70°C (□); 75°C (○); and 80°C (△). Residual activity, a , of *LrNDT* was measured at 40°C under standard conditions. (B) Effect of temperature on the *LrNDT* activity (●) and stability (△). (C) *LrNDT* stability at different pHs. (D) Optimal pH for *LrNTD* activity. The ionic strength (I) at each pH was adjusted to 150 mM by addition of NaCl.

TABLE 1. Natural nucleoside synthesis catalyzed by *Lr*NDT^a

Acceptor	Sp act (U/mg protein) with donor:			
	dUrd	dThd	dAdo	dIno
Ura		48	51	21
Thy	64		41	22
Ade	103	36		34
Hyp	43	31	8	
Cyt	117	85	66	35

^a Reaction conditions were 0.34 μ g of enzyme in 40 μ l at 40°C for 5 min. Substrate concentrations were 10 mM in 50 mM MES buffer, pH 6.5.

synthesis from 2'-deoxyuridine and adenine under standard conditions. The highest activity was achieved between 30 and 50°C (Fig. 5B). On the other hand, the deactivation parameters (α_1 , α_2 , k_1 , and k_2) were calculated (see Table S1 in the supplemental material) by fitting the experimental data to equation 1 or 2. As shown, the deactivation at 60 and 65°C followed a first-order exponential decay model whereas the depicted deactivation curves between 70 and 80°C exhibited classically biphasic decays, with an initial period of quick deactivation followed by a period of slower deactivation. Moreover, it is remarkable that the most important change occurred between 65 and 70°C. These results agree with thermal denaturation curves observed by fluorescence, CD, and microcalorimetry, in which unfolding was observed up to 50°C.

With regard to pH studies, synthesis of thymidine (Tyd) from 2'-deoxyuridine (dUri) and thymine (T) was selected as the standard reaction due to pK_a values of thymine (9.7) and uracil (9.2), which guarantee the correct protonated form of nucleosides and bases in solution at the pH range assayed (4 to 8). The enzyme was completely stable in this pH interval and showed optimal activity in a wide pH range (5.0 to 7) (Fig. 5C and D). Since the enzyme shows optimal activity in a broad temperature and pH range, 40°C and pH 6.5 were selected as conditions for the standard synthesis reaction catalyzed by *Lr*NDT.

Likewise, the effect of cations on the base exchange activity of the recombinant *Lr*NDT was examined (see Table S2 in the supplemental material). In contrast to other reports (36), the enhanced activity effect exerted by NH_4^+ , K^+ , or Rb^+ was not observed, whereas divalent cations showed inhibitory effects as previously described (36).

Finally, when the ionic strength (I) effect on NDT activity was studied (data not shown), a slight activity decrease was observed when NaCl concentration was increased (70% activity remained at 1 M NaCl).

Nucleoside synthesis. Synthesis of natural and nonnatural nucleosides catalyzed by recombinant *Lr*NDT has been extensively explored (see Tables 1, 3, 4, and 5). With regard to natural nucleoside synthesis, *Lr*NDT catalyzes the 2-deoxyribose transfer reaction between purine or pyrimidine and/or pyrimidine bases (Table 1). The enzyme prefers pyrimidines to purines as substrates, dUrd being the best nucleoside donor, followed by dThd, dAdo, and dIno, whereas cytosine (Cyt) was the best base acceptor and hypoxanthine (Hyp) was the worst. These results agree with those previously described for other NDTs. It should be noted that *Lr*NDT shows higher specific activities than do other reported NDTs (28, 29, 36): (i) the

TABLE 2. Kinetic parameters of *Lr*NDT with dUrd as nucleoside donor and cytosine (Cyt) as base acceptor

Parameter	Value with variable substrate:	
	dUrd	Cyt
K_m (mM)	1.142 ± 0.221	10.79 ± 1.18
k_{cat} (s^{-1})	483.20 ± 13.046	$2,083.42 \pm 95.99$
k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)	4.24×10^5	1.93×10^5

specific activity with dThd and Ade ($36 \mu\text{mol min}^{-1} \text{mg}^{-1}$) was three times and two times higher than the values showed by *Lactobacillus leichmannii* NDT (*L*INDT) and *Lactobacillus fermentum* NDT (*L*fNDT) ($11.9 \mu\text{mol min}^{-1} \text{mg}^{-1}$ and $20.1 \mu\text{mol min}^{-1} \text{mg}^{-1}$, respectively), whereas specific activities with dThd and Cyt ($85 \mu\text{mol min}^{-1} \text{mg}^{-1}$) were 11 and 12 times higher than those of *Lactobacillus helveticus* NDT (*L*hNDT) ($7.75 \mu\text{mol min}^{-1} \text{mg}^{-1}$) and *Lactococcus lactis* subsp. *lactis* (*L*cNDT) ($6.67 \mu\text{mol min}^{-1} \text{mg}^{-1}$), respectively; (ii) the specific activity for 2'-deoxyadenosine (dAdo) synthesis from 2'-deoxyinosine (dIno) ($34 \mu\text{mol min}^{-1} \text{mg}^{-1}$) was 10 and 30 times higher than values from *L*INDT ($3.6 \mu\text{mol min}^{-1} \text{mg}^{-1}$) and *L*fNDT ($1.08 \mu\text{mol min}^{-1} \text{mg}^{-1}$), respectively; and (iii) the specific activity for 2'-deoxycytidine (dCyd) synthesis from 2'-deoxyuridine (dUrd) ($117 \mu\text{mol min}^{-1} \text{mg}^{-1}$) was three times higher than those described for both *L*INDT and *L*fNDT ($42.5 \mu\text{mol min}^{-1} \text{mg}^{-1}$ and $40.2 \mu\text{mol min}^{-1} \text{mg}^{-1}$), respectively. In addition, kinetic constants (K_m , k_{cat} , and k_{cat}/K_m) of *Lr*NDT with dUrd as nucleoside donor and cytosine (Cyt) as base acceptor have been determined (Table 2). *Lr*NDT shows lower K_m values for dUrd than for Cyt, according to the ping-pong mechanism described for other NDTs (15, 16). It is notable that the k_{cat} and k_{cat}/K_m values for both substrates are higher than those described for other NDTs (15, 16, 29, 41).

To determine whether *Lr*NDT would be able to synthesize nonnatural nucleosides (purines and pyrimidines) from natural nucleosides and nonnatural bases, the best donors, dUrd (as pyrimidine 2'-deoxyribonucleoside) and dAdo (as purine 2'-deoxyribonucleoside) were selected (Table 3). Likewise, the best acceptors, cytosine, uracil, and adenine, were chosen to

TABLE 3. Nonnatural nucleoside synthesis by *Lr*NDT from natural nucleosides and nonnatural bases^a

Acceptor	Sp act (U/mg protein) with donor:	
	dUrd	dAdo
Benzimidazole (B)	1.45	0.6
2,6-Diaminopurine (2,6-DMP)	1.7	0.9
6-Mercaptopurine (6-M)	1.1	0.5
5-Chlorouracil (5-ClUra)	0.9	0.4
Flucytosine (5-FCyt)	1.0	0.5
5-Fluoro-2-methoxy-4(1H)pyrimidinone (5-FMP)	0.4	0.1
5-Fluorouracil (5-FUra)	1.1	ND ^b
5-Bromouracil (5-BrUra)	1	0.6
5-Iodouracil (5-IUra)	1.1	0.1

^a Reaction conditions were 0.34 μ g of enzyme in 40 μ l at 40°C for 2 h. Substrate concentrations were 2 mM in 50 mM MES buffer, pH 6.5.

^b ND, not detectable.

TABLE 4. Nonnatural nucleoside synthesis by *LrNTD* from nonnatural nucleosides and natural bases^a

Donor	Sp act (10 ³ U/mg protein) with acceptor:		
	Ura	Ade	Cyt
<i>ara</i> -Uracil		0.5	2.2
<i>ara</i> -Adenine	1		1.7
2'-Fluoro-2'-deoxyuridine		0.3	4.1

^a Reaction conditions were 0.34 µg of enzyme in 40 µl at 40°C for 24 h. Substrate concentrations were 1 mM in 50 mM MES buffer, pH 6.5.

synthesize nonnatural nucleosides from nonnatural nucleosides and natural bases (Table 4). As shown, *LrNTD* is able to catalyze most of the transfer reactions assayed with good yields. Remarkably, synthesis of 5-fluoro-2'-deoxycytidine and 5-fluoro-2-methoxy-4(1H)pyrimidinone-2'-deoxyribose by *LrNTD* has been performed, something which had not been described so far for 2'-deoxyribosyltransferases.

Most important, synthesis of nonnatural nucleosides such as *ara*-adenine, *ara*-uracil, *ara*-cytosine, 2'-fluoro-2'-deoxyuridine, and 2'-fluoro-2'-deoxycytidine catalyzed by *LrNTD* was performed (Table 4), being the first time that arabinosyltransferase activity is described for an NDT enzyme. Although wild-type NDTs from *Lactobacillus fermentum* and *Lactobacillus leichmannii* can slightly recognize 2',3'-dideoxyribonucleosides (28), up to now no NDT enzyme described so far has recognized arabinonucleosides.

Furthermore, synthesis of nonnatural nucleosides from nonnatural nucleosides and nonnatural bases has been carried out for the first time (Table 5). All of these synthetic activities are very interesting for the aim of using *LrNTD* as a novel biocatalyst to obtain new arabinonucleosides and 2'-fluoro-2'-deoxyribonucleoside analogues, which could be used as possible therapeutic agents.

DISCUSSION

Modified nucleosides are extensively used as antiviral and antitumor agents (19, 20). These molecules have been synthesized by different chemical methods (27); however, the necessary tedious protection and deprotection steps often lead to low yields and increased costs. Indeed, chemical methods usually increase the difficulty of obtaining products with correct stereo- and regioselectivity, generating secondary products (13, 34, 56). As an alternative to the synthesis approach, enzymatic synthesis performed under very mild conditions in a stereo- and regiospecific manner (42, 55) is a valuable option. Nowadays, 2'-deoxyribosyltransferases are quite interesting since these enzymes can be used to carry out the synthesis of natural and nonnatural nucleosides with antiviral and antitumor activity in a one-pot reaction. Actually few NDTs are described, and all of them are specific for deoxyribose. Moreover, since several nonnatural nucleosides acting as antiviral or anticancer agents have modifications on their sugar moiety, it is interesting to explore the possibility of developing a novel and effective industrial biocatalyst to catalyze the enzymatic synthesis of natural and modified nucleosides.

In this sense, we have cloned and overexpressed in *Escherichia coli* BL21(DE3) the putative *ndt* gene from *Lactobacillus*

TABLE 5. Synthesis of nonnatural nucleosides from nonnatural nucleosides and nonnatural bases catalyzed by *LrNTD*^a

Acceptor	Sp act (10 ³ U/mg protein) with donor:	
	<i>ara</i> -Uracil	2'-FdUrd
Benzimidazole (B)	ND ^b	0.1
2,6-Diaminopurine (2,6-DAP)	0.15	0.15
6-Mercaptopurine (6-M)	ND	ND
5-Chlorouracil (5-ClUra)	1.1	1.5
Flucytosine (5-FCyt)	1.4	4.1
5-Fluoro-2-methoxy-4(1H)pyrimidinone (5-FMP)	0.65	0.5
5-Fluorouracil (5-FUra)	1.2	2.0
5-Bromouracil (5-BrUra)	1	1.65
5-Iodouracil (5-IUra)	0.1	1.15

^a Reaction conditions were 3.4 µg of enzyme in 40 µl at 40°C for 24 h. Substrate concentrations were 1 mM in 50 mM MES buffer, pH 6.5.

^b ND, not detectable.

reuteri CECT 925 by using the expression vector pET28a(+). We have determined that *ndt* encodes a type II 2'-deoxyribosyltransferase (*LrNTD*) which catalyzes the transfer between purines and/or pyrimidines and is more promising for use in the industrial synthesis of nucleosides.

The pure *LrNTD* has been structurally characterized by analytical ultracentrifugation, UV-visible light spectroscopy, CD, fluorescence, and calorimetry studies (DSC). The enzyme is a homohexamer of 113 kDa, in agreement with other described lactobacillus NDTs (1, 3, 5, 14, 25, 28, 36, 49). Fluorescence emission spectra suggested that the recombinant NDT was perfectly folded since the fluorescence emission maximum of the protein was blueshifted, indicating that all tryptophan residues are buried inside the hydrophobic protein structure. Likewise, CD spectra recorded in the far-UV spectra evidence a folded structure that perfectly agrees with the secondary structure predictions based on amino acid sequence and is similar to those described for other NDTs from lactobacilli (2, 14).

Interestingly, the high *T_m* value (64°C) determined by temperature-dependent decay studies of fluorescence, CD thermal denaturation curves, and microcalorimetry experiments allows us to classify this enzyme as a stable protein. Moreover, *LrNTD* also shows high stability in a broad range of pHs and temperatures (Fig. 5). Furthermore, enzyme activity was totally maintained at 40°C for 20 h, and 70% of its activity remained after 24 h of storage time at the same temperature (see Fig. S3 in the supplemental material). It could be pointed out that the highest activity was achieved in a pH range from 4.5 to 7.9 and in the temperature interval from 30 to 50°C. These data, taken together, allow us to conclude that *E. coli* is able to produce *LrNTD* in active form and showing a high stability which provides an additional advantage for consideration as a promising biocatalyst for industrial nucleoside synthesis.

Furthermore, natural and nonnatural nucleoside synthesis by *LrNTD* has been performed to determine substrate specificity. *LrNTD* has a strong preference for pyrimidines as substrates; thus, regarding nucleoside donors the enzyme prefers dUrd > dThd > dAdo > dIno, whereas it recognizes Cyt > Ade > Ura ≅ Thy > Hyp as base acceptors. Similar behavior has been observed in other NDTs, where dUrd and cytosine

FIG. 6. Hypothetical active site structure of *Lr*NTD. The active center of *Lr*NTD has been determined by alignment of different 2'-deoxyribosyltransferase amino acid sequences of *Lactobacillus* strains. The program used was CLUSTAL W of the Biology Work Bench Server (31). A, *ara*-uracil (*ara*U); B, 2'-fluoro 2'-deoxyuridine.

were the best donor and acceptor, respectively (3, 28, 36). Likewise, hypoxanthine was the worst base acceptor, which supports the idea that *Lr*NTD is a type II NDT like *Lh*NTD, in contrast to *Lh*PDT, a type I NDT, which prefers hypoxanthine as base acceptor due to its specific role in the metabolism of dIno (28). Interestingly, *Lr*NTD shows higher specific activities than do other described NDTs (27, 56): for instance, dAdo synthesis is 30-fold higher than the value reported for *Lf*NTD, actually showing this enzyme as a very suitable biocatalyst to be used in industrial nucleoside synthesis.

Finally, nonnatural nucleoside synthesis has been performed with quite interesting results. Thus, we have showed that *Lr*NTD recognizes an extensive group of nucleosides and synthesizes a wide range of nonnatural nucleosides. For instance, the enzyme is able to synthesize 5-fluoro-2'-deoxycytidine, 5-fluoro-2-methoxy-4(1*H*)-pyrimidinone-2'-deoxyribose, arabinonucleosides, and 2'-fluoro-2'-deoxyribonucleosides, this being the first time that an NDT enzyme has shown arabinosyltransferase activity.

These unexpected results are rather surprising because, whereas NDTs accept different bases (natural and modified), they are highly specific for 2'-deoxyribose due to interaction of 3'-OH sugar with catalytic Glu, which seems critical for adequate orientation of the sugar moiety and optimal catalysis. When the donor is a ribonucleoside, the nucleophilic oxygen atom of the catalytic Glu hydrogen bonds to the O-2' atom of ribose and the enzyme is inactive (1). However, according to our experimental results, this hydrogen bond could not be produced in arabinonucleosides and 2'-fluoro-2'-deoxyribonucleosides, and *Lr*NTD can recognize these substrates and catalyze the transfer of sugar at long reaction times. This sub-

strate specificity could be explained according to the catalytic active site structure (Fig. 6), where the presence of a 2'-OH or a 2'-fluor atom does not allow the interaction by hydrogen bonds to carboxylate the oxygen atom of the putative catalytic Glu101, whereas the 3'-OH of the sugar shows the normal hydrogen bond essential for catalysis (1, 29). In this sense it is interesting that *Lr*NTD shows a high sequence identity with other described NDTs from *Lactobacilli*, where the essential amino acids involved in catalysis are conserved. So the amino acids proposed in substrate binding in *L. leichmannii* NDT, Asp72, Gln46, Asp92, and Asn123, as well as the catalytic residue Glu98, are conserved in the *Lr*NTD sequence, a finding which supports an active site similar to those of other *Lactobacilli* (see Fig. S4 in the supplemental material).

It could be emphasized that enzymatic arabinonucleoside synthesis by *Lr*NTD is performed by one enzyme, in contrast to nucleoside phosphorylases that require two purine and/or pyrimidine nucleoside phosphorylases or whole cells to carry out the synthesis (4, 9, 12, 38, 51–54). In conclusion, our results are an important contribution toward obtaining a biocatalyst to be used in industrial natural and, more interestingly, nonnatural tailored nucleoside synthesis, such as arabino- and 2'-fluoro-2'-deoxyribonucleoside analogues, which could be used as potential therapeutic agents. Furthermore, from an industrial point of view, *Lr*NTD is an excellent candidate for obtaining an immobilized derivative to be used in large-scale bioreactors (unpublished data).

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