NisC, the Cyclase of the Lantibiotic Nisin, Can Catalyze Cyclization of Designed Nonlantibiotic Peptides†

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ABSTRACT: Nisin is a pentacyclic peptide antibiotic active against Gram-positive bacteria. Its thioether rings are formed by two enzymatic steps: nisin dehydratase (NisB)-mediated dehydration of serines and threonines followed by nisin cyclase (NisC)-catalyzed enantioselective coupling of cysteines to the formed dehydroresidues. Here, we report the in vivo activity of NisC to cyclize a wide array of unrelated and designed peptides that were fused to the nisin leader peptide. To assess the role of NisC, leader peptide fusions, secreted by *Lactococcus lactis* cells containing NisBT with or without NisC were compared. In hexapeptides, a dehydroalanine could spontaneously react with a more C-terminally located cysteine. In contrast, peptides containing dehydrobutyrines require NisC for cyclization. In agreement with in silico predictions NisC could efficiently cyclize the hexapeptides ADhbVECK and IDhbPGCK, but ADhbVWCE was not cyclized. Interestingly, NisC could efficiently catalyze the synthesis of peptides with intertwined rings and of a designed polyhexapeptide containing four thioether rings. Taken together the data demonstrate that NisC can be widely applied for the cyclization and stabilization of nonlantibiotic peptides.

The in vivo efficacy of many therapeutic peptides is seriously hampered by instability and proteolytic degradation. Stabilization of peptide drugs and protection against degradation may enhance the therapeutic potential, might allow lower doses, less frequent administration, longer shelf life, and possibly oral delivery. Oral delivery of a disulfide-stabilized C-terminal fragment of human growth hormone (*1*) was very effective against obesity (*2*). Cyclization is indeed a powerful method to protect peptides against peptidase and protease activity (*3*). Thioether cyclization gave full stabilization of a Herpes simplex derived epitope when tested in serum and rat lysosomal preparations (*4*), while in parallel experiments the presence of a disulfide bridge or peptide bond only gave partial protection against proteolytic enzymes. Thioether ring introduction in several other peptides significantly enhanced the stability and efficacy (*5*−*10*). Another way of protection against proteolysis is the insertion of D-amino acids in peptides. In lantibiotics, the cyclase-induced (methyl)-lanthionines have the DL configuration. By the excellent study of Meyer et al. for the first time the catalyzation of cyclization by a lantibiotic enzyme, PepC, was demonstrated (*11*).

Lantibiotics are (methyl)lanthionine (Ala−S−Ala and Abu−S−Ala)-containing bacterial peptides, most of which have antibiotic activity. The thioether bond is intrinsically significantly more stable than a disulfide bond. (Methyl)-lanthionine rings in lantibiotics may contain 0−19 amino acids under the sulfur bridge (*12*). The best known lantibiotic is the pentacyclic nisin, which is applied for food conservation in more than 80 countries (*13*). Nisin is produced by *Lactococcus lactis* (*14*, *15*). Its serines and threonines are dehydrated by the dehydratase, NisB, resulting in dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively. The resulting dehydroresidues are stereo- and regiospecifically coupled to cysteines by the cyclase, NisC (*16*),[1] a zinc protein (*17*). Export of fully modified prenisin is mediated by the dedicated transporter, NisT, after which the leader peptide is cleaved off by the leader peptidase, NisP. For decades it was believed that lantibiotic enzymes would be rather specific. Indeed, NisP cleaves fully modified prenisin but not dehydrated or unmodified prenisin (*18*). NisT and NisB, on the other hand, are equipped with a broad substrate specificity (*12*, *18*, *19*) but may require the leader peptide for substrate recognition.

Recently by van der Donk and co-workers the determination of the structure, the elucidation of the mechanism, the in vitro reconstitution of NisC activity, and the identification of essential catalytic residues have been achieved successfully (*16*, *20*). We previously could demonstrate the

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[1] Abbreviations: NisC, the nisin cyclase; NisB, the nisin dehydratase; NisT, the nisin transporter; LanB, lantibiotic dehydratases; TFA, trifluoroacetic acid; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; CDAP, 1-cyano-4-dimethylaminopyridinium tetrafluoroborate; TCEP, triscarboxyethyl phosphine.

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Table 1: Bacterial Strains and Plasmids

	characteristics	ref
	Strains	
NZ9700	*nisABTCIPRKEFG*	*31*
NZ9000	*nisRK*$^+$	*32*
	Plasmids	
pIL253-derived		*33*
pIL3BTC	Cm-r	*12*
pIL3BT	Cm-r	this study
pNZ8048-derived		*34*
pNZE3	Pnis + nisin prepeptide sequence, Em-r	*18*
pTP-ATVECK		*12*
pTP-ASVECK		*12*
pTP-ITPGCK		*12*
pTP-ATVWCE		*12*
pTP-ASVWCE		*12*
pTP-ITRICK		*12*
pTP-rings	leader−ATVATCKGCK	this study
pTP-ring DE	leader−KTATCHCSK	this study
pTP-4hexa	leader−ITPGCK−ATVECK−ITPGCK−ATVECK	this study
pTP-nisinVE	P9V, G10E nisin mutant	this study

presence of thioether rings in both model peptides and variants of therapeutic peptides. However, these studies did not establish whether thioether ring formation in these nonlantibiotic peptides was induced by NisC, as spontaneous ring formation could not be ruled out. Here, we have assessed the role of NisC in *L. lactis* in the cyclization of designed peptides by comparing peptides exported via NisBT in the absence and presence of NisC. Our studies elucidate important aspects of the substrate specificity of NisC and reveal that it can catalyze the cyclization of a variety of de novo designer peptides.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The host *L. lactis* NZ9000 was used for expression of modification enzymes and peptides. *L. lactis* 108 (pORI280) was used as indicator strain for antimicrobial activity. Strains and plasmids are listed in Table 1.

Molecular Cloning. PCR was performed with Phusion DNA polymerase (Finnzymes, Finland). Ligation was carried out with T4 DNA ligase (Roche). Restriction enzymes used for cloning strategies were purchased from Biolabs (New England). The different peptide constructs were made by amplification of the plasmid pNZ-E3 (*18*), using antisense primers with a complementary part to the nisin leader and a 5′ overhang, encoding the peptide sequences, and one universal phosphorylated sense primer annealing downstream the NisA peptide-coding sequence. The nisin leader sequence is MSTKDFNLDLVSVSKKDSGASPR, the sequence of unmodified nisin is ITSISLCTPGCKTGALMGCNMK-TATCHCSIHVSK. The linear peptide-encoding plasmid was self-ligated. Construction of pIL3BT (Table 1) was carried out as follows. The deletion of the *nisC* gene from pIL3BTC was carried out by removing the *nisTC* genes by *Pml*I/*Sph*I digestion and replacing them by the *Pml*I/*Sph*I digested *nisT* gene from pNGnisBT (*18*). Electrotransformation of *L. lactis* was carried out as described previously (*21*) using a Biorad gene pulser (Biorad, Richmond, CA). Nucleotide sequence analysis was performed by BaseClear (Leiden, The Netherlands).

Growth conditions. *L. lactis* was grown in M17 broth (*22*) supplemented with 0.5% glucose (GM17) or minimal medium (*12*, *23*) with chloramphenicol (5 μg/mL) and erythromycin (5 μg/mL). Cultures were grown on minimal medium after induction with nisin prior to sample preparation for mass spectrometry.

Nonenzymatic Ring Closure. In vitro ring closure was induced by increasing the pH to 10 with 10 mM NaOH and incubating samples at 50 °C for 3 h.

Peptide Purification. Overnight cultures of *L. lactis* in GM17 were diluted 100 times in 500 mL of minimal medium buffered with 0.12 M MOPS (pH 7.0) and supplemented with 0.1 nM nisin for induction. The cultures were grown for 16 h at 30 °C in 500 mL sealed flasks in a rotary incubator at 200 rpm. After centrifugation to remove the cells, the supernatant was diluted with an equal volume of 100 mM lactic acid pH 4, and subsequently, purification proceeded by a single passage of diluted supernatant over a 5 mL FPLC HiTrap SP cation exchange column. Block elution was performed with 1 M NaCl in 50 mM lactic acid pH 4. The fraction containing the peptide was desalted on a PD10 column and freeze-dried. The leader was completely cleaved off in 1 h at 37 °C with 0.1 mg/mL trypsin in 100 mM Tris−HCl buffer pH 8 containing 10 mM $CaCl_2$. The digested peptides were separated on a semipreparative C18 column with reversed-phase HPLC with a 1%/min gradient of 5−35% acetonitrile in 0.1% TFA at a rate of 2 mL/min. Separate peaks were collected and dried in a speed-vac. The eluted peptides were identified with MALDI-TOF mass spectrometry.

Peptide Hydrolysis. Partial acid hydrolysis of peptides was performed in 6 N HCl in polypropylene screw cap tubes at 120 °C. Speed-vac-dried peptide was dissolved in 6 N HCl and placed in a heating block for 15 min. The reaction was terminated by evaporating the solution in a speed-vac apparatus. The dry material was dissolved in 50% acetonitrile and 0.1% TFA and directly used for MALDI-TOF analysis with α-cyano-4-hydroxy-cinnamic acid as matrix.

Mass Spectrometry. Peptides were purified from the medium fraction by ziptip purification (C18 ziptip, Millipore) or by subjecting medium volumes up to 1 mL to zipplate (Millipore) purification. The CDAP (1-cyano-4-dimethyl-aminopyridinium tetrafluoroborate) modification at 2 mg/mL was used to convert free cysteine residues to isothiocy-

Table 2: NisC-Mediated Cyclization of Designed Peptides[a]

	mass ($M + H^+$) w/o Met1 (Da)					
	export via NisBT		export via NisBTC		*theoretical value of dehydrated peptide without ring*	
peptide fused to the leader	−CDAP	+CDAP	−CDAP	+CDAP	−CDAP	+CDAP
ITPGCK	2934	2960	2933	2934	*2934*	*2959*
ITRICK	3049	3076	3049	3076	*3049*	*3074*

[a] The supernatant of producing *L. lactis* cultures was analyzed by MALDI-TOF. NisC-mediated cyclization was assessed by addition of CDAP to peptides exported via NisBT and absence of CDAP reaction with peptides exported via NisBTC. Observed masses are of peptides that contain the nisin leader without Met1. Peptide masses with dehydration are given in Da. Calculated values are displayed in italics.

anates in a 25 mM citrate buffer pH 3 (*19*, *24*). Formation of the isothiocyanate part will result in a mass increase of 25 Da, whereas the absence of a mass shift indicates that the cysteine is involved in ring formation. Neither thioethers (van der Donk, personal communication) nor other residues react with CDAP as long as large excess of CDAP is avoided (*25*). The samples were allowed to react with CDAP in the presence of 10−20% acetonitrile for 15 min at room temperature. The peptides CRYTDPKPHIRLRIK and leader−ITRICK were used as positive controls for the CDAP treatment, leading to an increase of 25 Da. The reaction with CDAP (*19*, *24*) was carried out with and without prior treatment with 0.5 mg/mL triscarboxyethyl phosphine (TCEP). TCEP removes the cysteine additions which are often observed in peptides having cysteines that do not participate in intramolecular cross-linking. Trypsin treatment was performed at 0.01 mg/mL. For MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, 1 μL of sample was spotted directly on target, dried, washed with 3 μL of MilliQ or 5% acetonitrile, dried, and overlaid with 1 μL of 5 mg/mL α-cyano-4-hydroxycinnamic acid in 50% acetonitrile in 0.1% (v/v) TFA. Mass spectra were recorded with a Voyager DE Pro MALDI-TOF mass spectrometer. Deviations of 1 Da were observed. In order to maintain high sensitivity, an external calibration was applied. MS/MS experiments were performed on an API3000 triple-quadrupole apparatus equipped with ion spray source (Applied Biosystems SCIEX). Product ion scans were performed using HPLC-purified peptides in the double-charged state with 30 V collision energy.

RESULTS

NisC-Mediated Cyclization of Designed Peptides. Here we tested whether thioether ring formation in a variety of peptides is mediated by NisC action or due to spontaneous ring formation. Herein, we compared the peptides secreted by *L. lactis* containing NisBTC with those secreted via NisBT. The peptides in the supernatants of the cultures were analyzed by mass spectrometry. Absence of thioether ring formation in the peptides was demonstrated by addition of CDAP that reacts with the nonmodified cysteines (*19*, *24*), i.e., those that did not participate in thioether ring formation. As previously (*12*) we observed in various experiments N-terminal truncations of the leader peptide. Peptides lacking one or more of the N-terminal leader peptide residues MSTKD and even further truncation were observed. Purified cyclized peptide occasionally contained at its N-terminus only the arginine, the C-terminal residue of the leader peptide. N-terminal truncation is not observed after incubation of leader peptide fusions in the medium without cells. This indicates that the truncation results from activity by peptidases of cellular origin.

The hexapeptide ATVECK was designed on the basis of a prediction model (*12*) as being an ideal substrate for both the dehydratase, NisB, and the cyclase, NisC. Mass spectrometric examination of the leader−ATVECK recovered from the supernatant of NisBT-containing cells revealed product with an N-terminal truncation of the leader peptide. The major peak of 2535 Da (Figure 1A, solid line) corresponds to fully dehydrated peptide (−18 Da) without the initial leader peptide residues MSTKD. Treatment of the dehydrated peptide with CDAP resulted in a mass upshift to 2561 Da (Figure 1A, dotted line), demonstrating a lack of cyclization. However, when the same peptide was expressed by NisBTC cells, a similar sized peak was observed by mass spectrometry but CDAP addition did not cause an increase in mass (Figure 1B). This strongly suggests that NisC catalyzes methyllanthionine formation in this peptide. Similar data were obtained for leader−ITPGCK (Table 2, 2934 Da corresponds to leader−ITPGCK without the initiating M). These data clearly demonstrate that NisC catalyzes the coupling of the Dhb to a cysteine in designed peptides.

Sequence-Dependent Interference with NisC-Mediated Thioether Ring Formation. We next investigated the modification of the peptide leader−ATVWCE. In silico analysis suggests that this peptide would be dehydrated, but not—or less readily—be cyclized (*12*). In lantibiotics, tryptophan is not frequently found as an N-flanking residue toward the lanthionine-forming cysteine, whereas glutamate does not occur at the C-flanking position. Indeed, the leader−ATVWCE exported via NisBT (Figure 1F) as well as via NisBTC (Figure 1G) were efficiently dehydrated as evidenced by an 18 Da lower mass (2593 Da corresponding to dehydrated leader−ATVWCE without the N-terminal MSTKD). CDAP addition efficiently modified the dehydrated peptide as evidenced by a mass increase to 2619 Da, irrespective the absence (Figure 1F) or presence (Figure 1G) of NisC. These data show that the cysteine in ADhbVWCE is not modified by ring formation despite the presence of the dehydrobutyrine. Similar observations were made for the leader−ITRICK (Table 2, 3049 Da corresponds to dehydrated leader−ITRICK without the initiating M). According to a previous in silico analysis of all known lantibiotic structures (*12*), ITRICK is predicted to be cyclized. Apparently, the substrate specificity of the individual cyclase used, NisC, differs from that of other cyclases.

We further investigated ring formation by examining sensitivity of the exported peptides to trypsin treatment. Modified prenisin is readily cleaved by trypsin after the

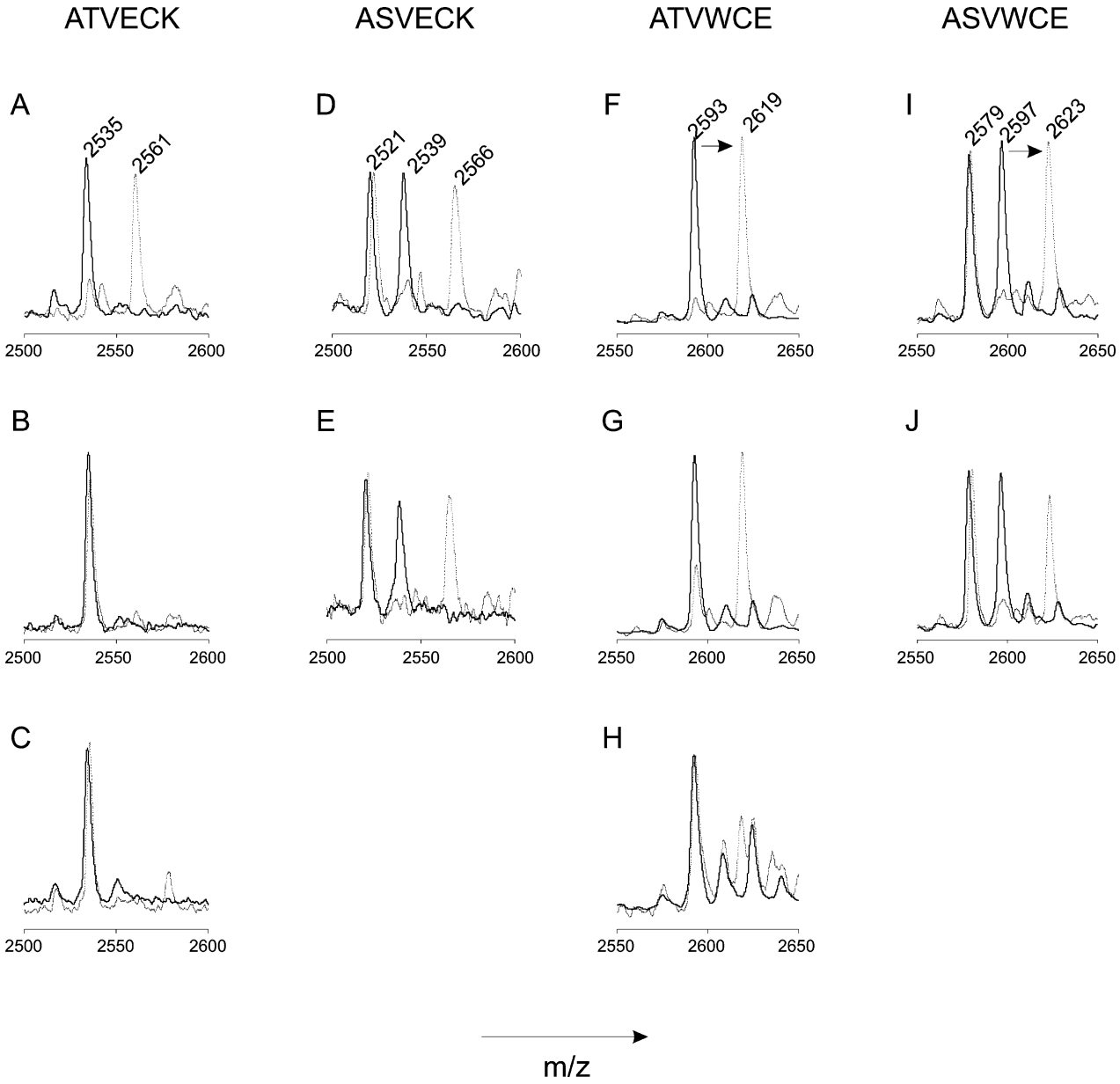

FIGURE 1: NisC catalyzes the cyclization of designed and unrelated peptides fused to the nisin leader peptides. *L. lactis* containing a leader fusion peptide-encoding plasmid together with either pIL3BT or pIL3BTC was induced, and peptides were analyzed by mass spectrometry. Samples were treated with (dotted lines) or without (continuous lines) the combination of TCEP and CDAP as described in Materials and Methods. (A) Leader−ATVECK via NisBT; (B) leader−ATVECK via NisBTC; (C) leader−ATVECK via NisBT treated at pH 10, 50 °C for 3 h; (D) leader−ASVECK via NisBT; (E) leader−ASVECK via NisBTC; (F) leader−ATVWCE via NisBT; (G) leader−ATVWCE via BTC; (H) leader−ATVWCE via NisBT treated at pH 10, 50 °C for 3 h; (I) leader−ASVWCE via NisBT; (J) leader−ASVWCE via NisBTC. The peaks of 2535 Da (A), 2521 Da (D), 2593 (F), and 2579 Da (J) correspond to dehydrated peptide (−18 Da) without the initial leader peptide residues MSTKD. CDAP addition (dotted lines) to uncyclized (A, F, and G) and undehydrated peptide (middle peak in D, E, I, and J) is indicated by the arrows.

C-terminal arginine of the leader peptide. We hypothesized that the presence of a ring behind the leader peptide at positions 2−5 might inhibit cleavage of the leader. Leader−ITPGCK exported either via NisBT (Figure 2A) or via NisBTC (Figure 2B) was incubated during 10 min in the presence of trypsin. In the case of export via NisBT virtually no IDhbPGCK peptide remained after trypsin treatment (Figure 2A). In contrast, a substantial amount NisBTC-produced leader−ITPGCK peptide remained after trypsin addition (Figure 2B), suggesting that the presence of a NisC-induced thioether ring in hexapeptide interferes with proteolytic digestion. Leader−ITRICK exported via NisBTC was cleaved by trypsin (not shown), consistent with an absence of ring formation. Taken together, these data demonstrate that NisC efficiently introduces a thioether ring into the leader−ITPGCK peptide which renders the modified peptide more resistant to trypsin cleavage.

Spontaneous Cyclization of Dehydroalanine-Containing Peptides. A higher extent of dehydration of threonines compared to serines was apparent by comparing the N-terminally truncated leader−ATVECK (Figure 1A−C) with leader−ASVECK (Figure 1, parts D and E) and the leader−ATVWCE (Figure 1F−H) with leader−ASVWCE (Figure 1, parts I and J). The threonine-containing peptides, ATVECK and ATVWCE, were fully dehydrated. By contrast, mass peaks of nondehydrated peptide were observed for the

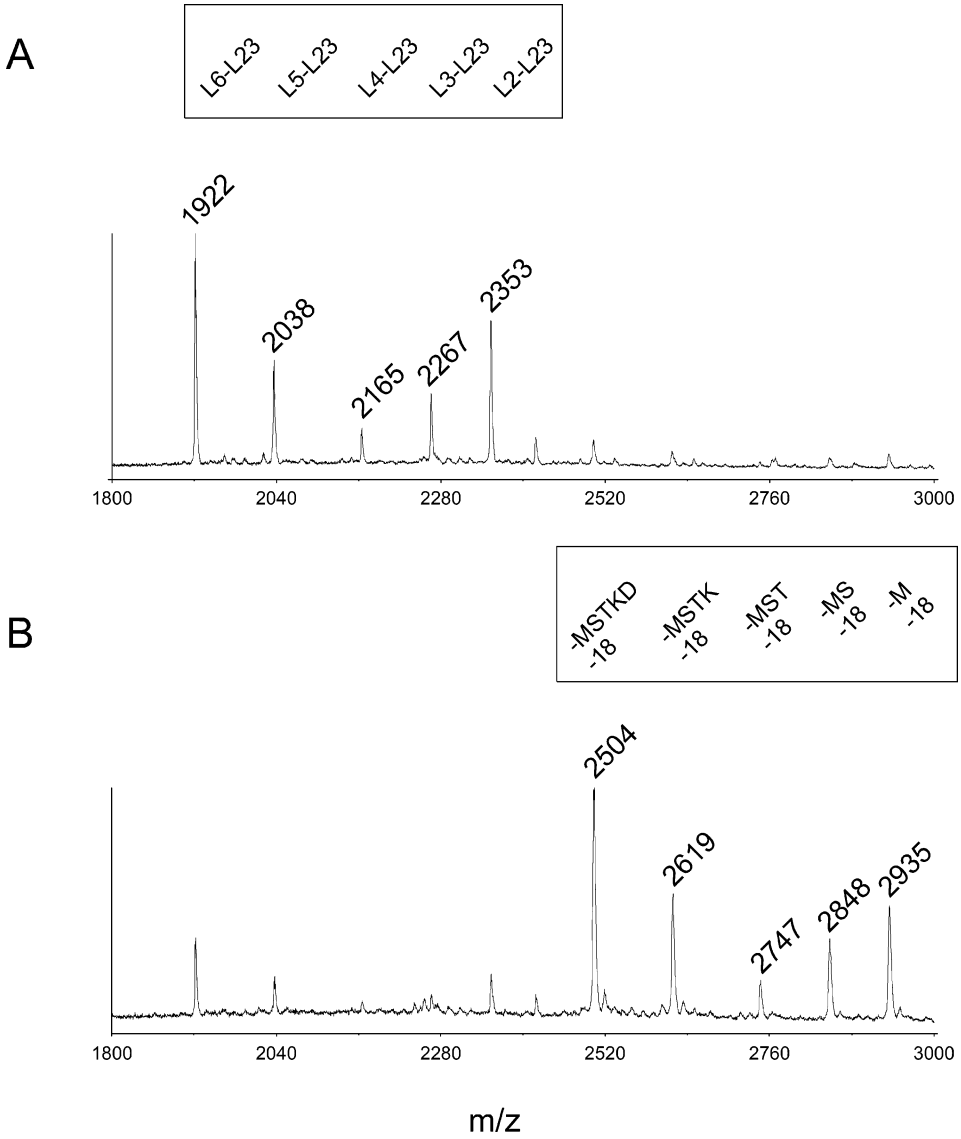

FIGURE 2: NisC catalyzes thioether ring formation in leader−IDhbPGCK. *L. lactis* containing pIL3BT (A) or pIL3BTC (B) and pTP−ITPGCK was induced, and its supernatant was analyzed by mass spectrometry. Samples were treated with trypsin during 10 min as described in the Materials and Methods section. Mass values ($M + H^+$) correspond to leader fragments (L1−L23) extended (B) or not extended (A) with I*Abu*PGAK having a thioether bridge between Abu and A.

leader−ASVECK (Figure 1, parts D and E, 2539 Da, corresponding to leader−ASVECK without MSTKD) and the leader−ASVWCE (Figure 1, parts I and J, 2597 Da, corresponding to leader−ASVWCE without MSTKD). The nondehydrated peptides still contained an unmodified cysteine as CDAP addition led to a mass upshift to 2566 Da (Figure 1, parts D and E) and 2623 Da (Figure 1, parts I and J), respectively. However, CDAP did not react with dehydroalanine-containing peptides produced via NisBT, ADhaVECK (Figure 1D, 2521 Da), and ADhaVWCE (Figure 1I, 2579 Da). Apparently, the dehydroalanines had spontaneously reacted with cysteines, preventing CDAP reaction. An attempt to isolate nonring closed leader−ADhaVECK and leader−ADhaVWCE by using medium with a low pH, to minimize spontaneous cyclization, was not successful. Hence for dehydroalanine-containing peptides, it could not be established if ring closure is due to NisC, spontaneous, or the result of both processes.

pH-Induced Cyclization. Ring formation in peptides that contain a dehydrobutyrine and a cysteine can be induced by incubation at pH 10. Indeed incubation at pH 10 of leader−ADhbVECK (Figure 1C) and leader−ADhbVWCE (Figure 1H) resulted in ring closure as evidenced by a lack of CDAP modification. Interestingly, also a peptide in which the order of cysteine and dehydrobutyrine was reversed, i.e., ICPG-DhbK, could be cyclized at alkaline pH. No ring closure was observed when leader−ICPGTK was exported via NisBTC (not shown). These data demonstrate that pH-induced cyclization can be used to ascertain whether or not cyclization is possible in peptides that are not modified by NisC.

NisC Cyclizes a Ring B Double Mutant of Nisin. In silico analysis suggested that ATVECK was an ideal substrate of lantibiotic enzymes (*12*), and here we experimentally confirmed its cyclization (Figure 1B). To further investigate the cyclization of this peptide inserted in a peptide with multiple rings we mutagenized nisin's ring B. To the best of our knowledge thus far no mutants of nisin's ring B have been reported. We decided to replace the ring B of nisin by the introduction of two mutations, i.e., P9V and G10E. Mutant

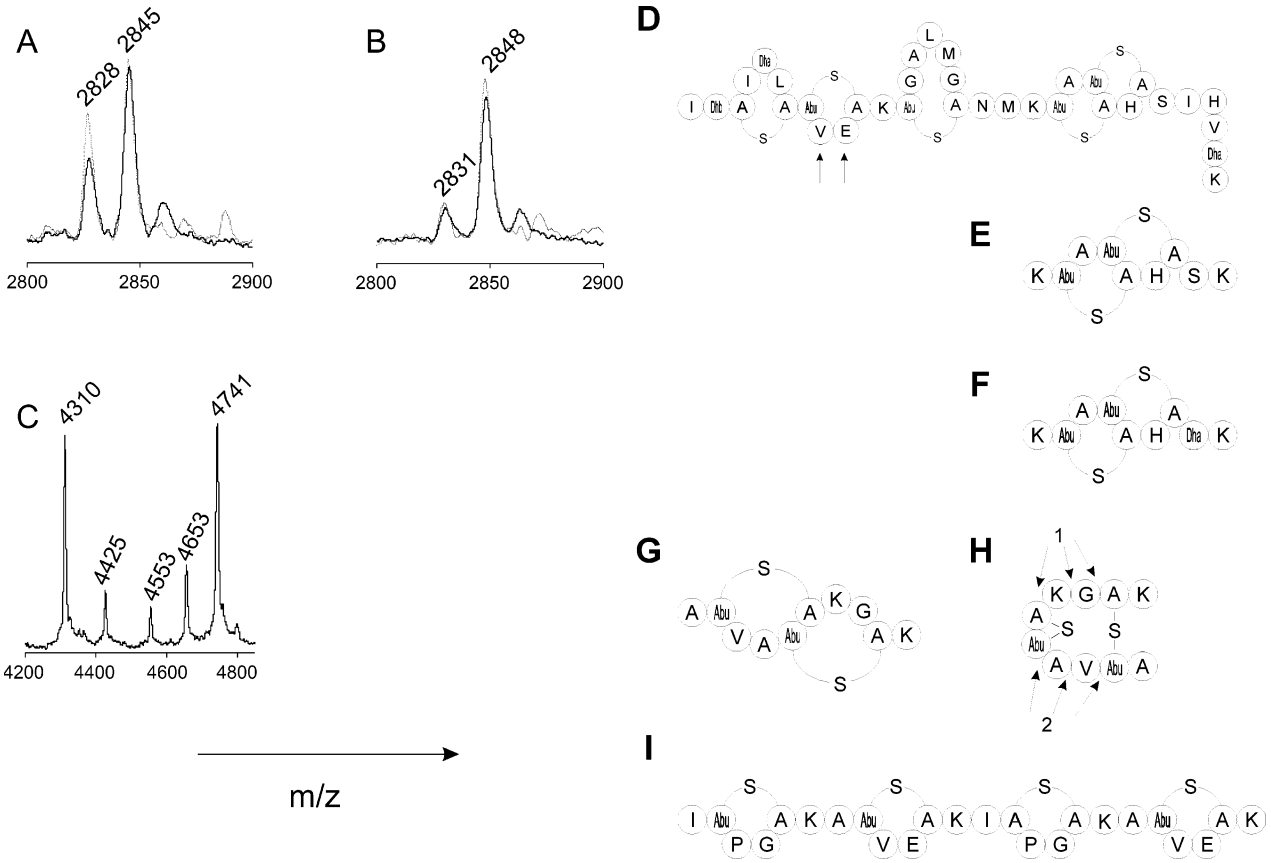

FIGURE 3: NisC catalyzes the formation of thioether rings in a nisin ring B mutant, of intertwined rings in designed peptides, and rings in designed polyhexapeptides. *L. lactis* containing a leader fusion peptide-encoding plasmid together with pIL3BTC was induced, and peptides from its supernatant were analyzed by mass spectrometry. Samples were treated with (dotted lines) or without (continuous lines) the combination of TCEP and CDAP as described in Materials and Methods. (A) Leader−KTATCHCSK; (B) leader−ATVATCKGCK; (C) leader−ITPGCK−ATVECK−ITPGCK−ATVECK. The mass peaks in (A) correspond to two (2845 Da) and three (2828 Da) times dehydrated peptide lacking the MSTKD residues of the leader peptide. The mass peak in (B) (2848 Da) corresponds to fully dehydrated ATVATCKGCK behind the leader without MSTKD. The masses in (C) correspond to fully modified leader−ITPGCKATVECKITPGCKATVECK without M (4741 Da), MS (4653 Da), MST (4553 Da), MSTK (4425 Da), or without MSTKD (4310 Da). Identical mass peaks were observed after CDAP treatment (not shown). (D) Proposed structure of P9V,G10E nisin. (E and F) Proposed structures of NisBTC-exported and modified KTATCHCSK. (G) Proposed structure of NisBTC-exported and modified ATVATCKGCK. (H) Purely theoretical alternative structure for structure G. Arrows in (H) indicate that by combined hydrolysis at one of the sites indicated by arrows 1 with one of the sites indicated by arrows 2 the molecule would be split in two parts, which would have been conclusive for the existence of the structure of (H). (I) Proposed structure for NisBTC-exported and modified ITPGCKATVECKITPGCKATVECK.

nisin was successfully produced, but all rings had closed as evidenced by absence of CDAP addition (not shown). Apparently, the P9V, G10E mutations in nisin still allow NisC-catalyzed formation of ring B. The resulting sequence approaches the fully modified ATVECK sequence, with the difference that the Ala at position 7 is part of a lanthionine. The obtained nisin mutant had lost both its antimicrobial and autoinducing capacity (data not shown). Apparently, the nisin activity requires specific structural requirements of ring B (Figure 3D).

NisC-Catalyzed Formation of Intertwined Rings. We subsequently investigated whether NisC is capable of catalyzing the formation of intertwined thioether rings in nonlantibiotic peptides. In a first approach, the peptide region corresponding to rings DE of nisin was extended with a lysine and fused to the leader peptide, i.e., leader−KTATCHCSK. The peptide was expressed and exported via NisBTC. Mass spectral analysis revealed a product lacking the MSTKD residues of the leader peptide with two (Figure 3A, 2845 Da) or three (Figure 3A, 2828 Da) dehydrated residues. With either peptide, no reactivity with CDAP was observed, neither in the presence nor absence of TCEP (Figure 3A, dotted line). This suggests NisC-mediated formation of two thioether bridges. The peptide of which the leader was cleaved off by trypsin was purified by HPLC. Two separate peaks were observed (16.2′ and 18.8′ elution time) which corresponded to the 2-fold and 3-fold dehydrated peptide with monoisotopic masses $[M + H]^+$ of 942.40 Da (calculated mass 942.43 Da) and 924.39 Da (calculated mass 924.42 Da), respectively, as determined by MALDI-TOF. Analyses of the HPLC-purified peptides by MS/MS (Figure 4, parts A and B) revealed dipeptide fragments composed of serine and lysine (y2 ion 234.2 Da, Figure 4A) for the 2-fold dehydrated peptide and a fragment of dehydroalanine and lysine (y2 ion 216.1 Da, Figure 4, parts A and B) for the 3-fold dehydrated peptide. Furthermore, a clear a7−17 ion could be detected in a single (664.4 Da) and double-charged (332.6 Da) state for both the 2-fold (Figure 4A) and 3-fold (Figure 4B) dehydrated peptides, which further proves that the dehydroalanine is not involved in ring formation. The structures of these peptides derived from rings DE of nisin are depicted in Figure 3E (2-fold dehydrated) and Figure 3F (3-fold dehydrated). Partial acid hydrolysis of the purified peptide showed the presence of one and two water

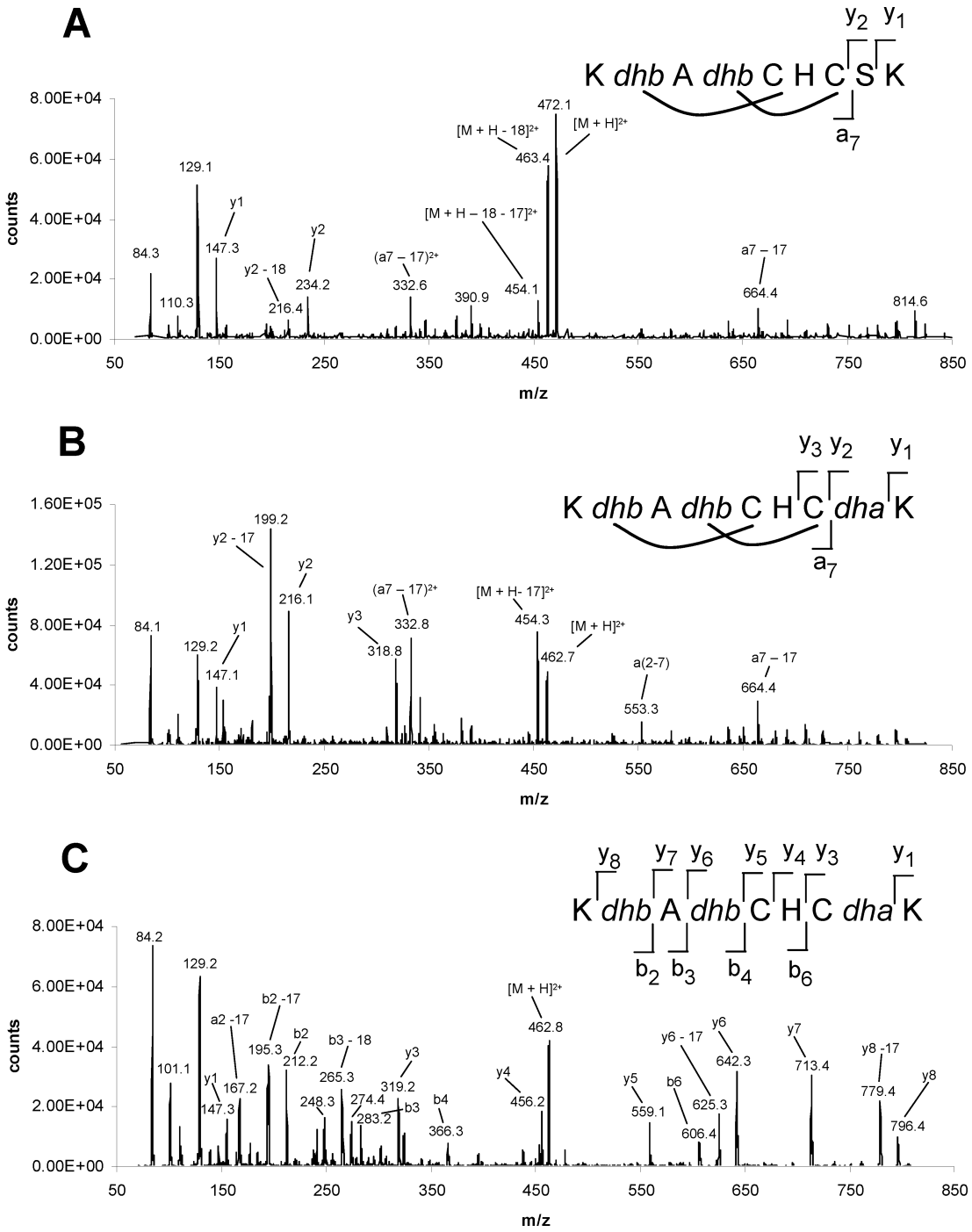

FIGURE 4: MS/MS spectra of posttranslationally modified KTATCHCSK peptides produced by a NisBTC- and NisBT-containing strain. All peptides were purified by HPLC prior to analysis. All spectra were recorded by using the double-charged parent ion and 30 V collision energy. Curved lines next to the inserted sequence in (A) and (B) indicate cyclizations as in rings D and E of nisin. (A) Product ion scan of cyclized KDhbADhbCHCSK peptide produced by NisBTC strain (16.2′ fraction of HPLC). The double-charged parent ion minus water and minus water and ammonia could also be observed. The serine has undergone partial water loss under the used conditions. (B) Product ion scan of cyclized KDhbADhbCHCDhaK peptide produced by NisBTC strain (18.8′ fraction of HPLC). Only loss of ammonia could be observed for the parent ion. (C) Product ion scan of KDhbADhbCHCDhaK peptide produced by NisBT strain (19.2′ fraction of HPLC; the 17.9′ fraction gave similar spectrum (not shown)). Dhb is dehydrobutyrine, Dha is dehydroalanine.

additions, meaning that the peptide could be hydrolyzed twice without breaking up, further demonstrating that both thioether rings are present.

When leader−KTATCHCSK was exported via NisBT, mass spectrometric analysis of the supernatant revealed that the peptide was fully dehydrated. After digestion with trypsin, two peaks were purified by HPLC; both peptides had a mass of 924.41 Da, corresponding to the 3-fold dehydrated peptide. MS/MS analysis showed clearly that the peptides were linear as was demonstrated by the presence of b and y ions (Figure 4C). The y2 ion which was clearly present in the 3-fold dehydrated peptide produced by a BTC strain (Figure 4B) was not detectable. Most likely the reactive dehydroalanine had spontaneously formed a lanthionine with the neighboring free cysteine residue. Two peaks of masses 248.3 and 274.4 Da were observed that were not explained by fragment ions of a linear peptide. They could be fitted by a y2 and x2 ion with an additional mass of 32 (y2 + 32 = 248.2 Da; x2 +

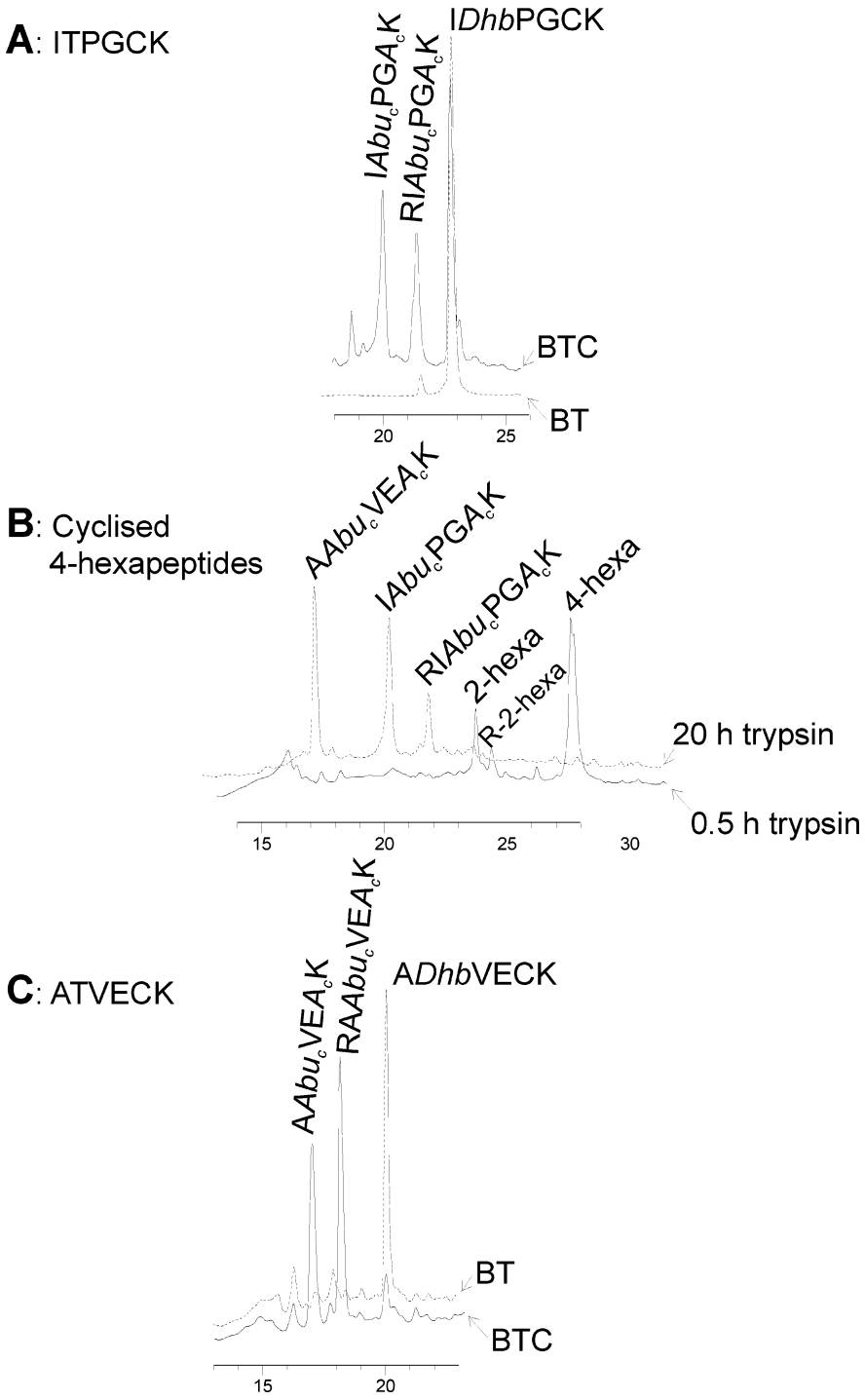

FIGURE 5: Regioselectivity of NisC-catalyzed thioether bridge formation in polyhexapeptides analyzed by trypsin and HPLC. (A) Trypsin digest of fully dehydrated Lp-ITPGCK peptide isolated from *L. lactis* containing NisBT strain (dotted line) and NisBTC (solid line). I*Abu*$_c$-PG*A*$_c$K is dehydrated and cyclized ITPGCK; I*Dhb*PGCK is dehydrated, noncyclized ITPGCK. (B) The 0.5 h (solid line) and 20 h (dotted line) trypsin hydrolysis of Lp-4hexa peptide. A*Abu*$_c$VE*A*$_c$K is dehydrated and cyclized ATVECK, 2 hexa is I*Abu*$_c$PG*A*$_c$K A*Abu*$_c$VE*A*$_c$K; 4-hexa is I*Abu*$_c$PG*A*$_c$KA*Abu*$_c$VE*A*$_c$KI*Abu*$_c$PG*A*$_c$KA*Abu*$_c$VE*A*$_c$K. (C) Trypsin digest of fully dehydrated Lp-ATVECK peptide isolated from *L. lactis* containing NisBT strain (dotted line) and NisBTC (solid line). N-terminal arginine (R) corresponds to the ultimate residue of the leader peptide.

32 = 274.1 Da). Perhaps the lanthionine could be opened by MS/MS transferring the sulfur atom to the position of the dehydroalanine generating these fragments. Taking the data on NisBT- and NisBTC-exported peptide together they demonstrate that the dehydroalanine, although very reactive in nature, is not part of the NisC-mediated ring structures.

Next, a designed peptide was used related to the ATVECK sequence that could be efficiently dehydrated and cyclized. The leader−ATVATCKGCK allows for two intertwined rings each harboring three residues under the thioether bridge. After export via NisBT and CDAP treatment two CDAP additions were observed (not shown) indicating absence of thioether rings in the absence of NisC. By contrast, after production via NisBTC, the fully dehydrated peptide (Figure 3B, 2848 Da, corresponding to leader−ATVATCKGCK without MSTKD) present in the supernatant did not react

with CDAP (Figure 3B, dotted line). Hence, the data suggest that also in this peptide, NisC had catalyzed the formation of two thioether rings. In principle two different structures are possible for the NisBC-modified peptide ATVATCK-GCK, which contains two thioether rings, i.e., one structure with (Figure 3G) and one without (Figure 3H) intertwined rings. The structure of Figure 3H needs only two hydrolysis sites to release conclusive fragments. Hydrolysis at one of the sites indicated by arrows 1 in Figure 3H combined with hydrolysis at one of the sites indicated by arrows 2 should release fragments specific for the structure depicted in Figure 3H. To discriminate between the two structures of Figure 3G and Figure 3H, the purified peptide was treated with 6 N HCl at 120 °C for 15 min resulting in partial hydrolysis. Subsequently MALDI-TOF analyses were performed (Table S1, Figure S1). If there were no intertwined rings (Figure 3H), conclusive fragments (lower part of Table S1) should have been detectable whose release should have resulted from hydrolysis at two sites. The existence of not one of these fragments could be demonstrated by mass analysis. The peaks above 400 Da could be assigned to peptides resulting from two or more hydrolysis events and—in some cases—lanthionine ring opening with transfer of the sulfur atom to the dehydrobutyrine. The data are therefore consistent with the structure with intertwined rings (Figure 3G).

NisC-Mediated Ring Closure in Polyhexapeptides. Since both the leader−ITPGCK and leader−ATVECK were cyclized by NisC, we investigated the export and modification via NisBTC of a fusion sequence leader−ITPGCKATVEC-KITPGCKATVECK. Fully dehydrated peptides corresponding to variants with N-terminal truncations lacking the M, MS, MST, MSTK, or MSTKD of the leader peptide were detected in the culture medium (Figure 3C). Ethanethiol treatment confirmed the full dehydration. No CDAP addition was observed indicating that all cysteines are present in the thioether ring form (not shown). In order to investigate the regioselectivity of the NisC-mediated cyclizations we treated the purified peptide with trypsin. After 30 min of incubation peptide corresponding to two cyclized hexapeptides could be isolated by HPLC (Figure 5B, solid line, two hexa). After prolonged incubation during 24 h, only peaks corresponding to the cyclized individual hexapeptides were present (Figure 5B, dotted line). These data indicate that the thioether bridges are formed as depicted in Figure 3I. Control HPLC runs were performed with NisBT- and NisBTC-modified ITPGCK (Figure 5A) and ATVECK (Figure 5C). Peptides exported via NisBTC sometimes had the C-terminal arginine from the leader peptide at their N-terminus. The data in Figure 5B show for the first time that a completely designed polyhexapeptide with multiple thioether rings can be produced (Figure 3I).

DISCUSSION

NisC catalyzes the enantioselective coupling of cysteines to the formed dehydroresidues in nisin. However, little is known about its specificity and ability to introduce thioether rings in unrelated peptides. We have investigated the versatility of the NisC activity by comparing peptides produced by *L. lactis* cells harboring either NisBT or NisBTC. Our results for the first time demonstrate that the cyclase NisC can catalyze the regiospecific formation of thioether rings in peptides unrelated to nisin or any other

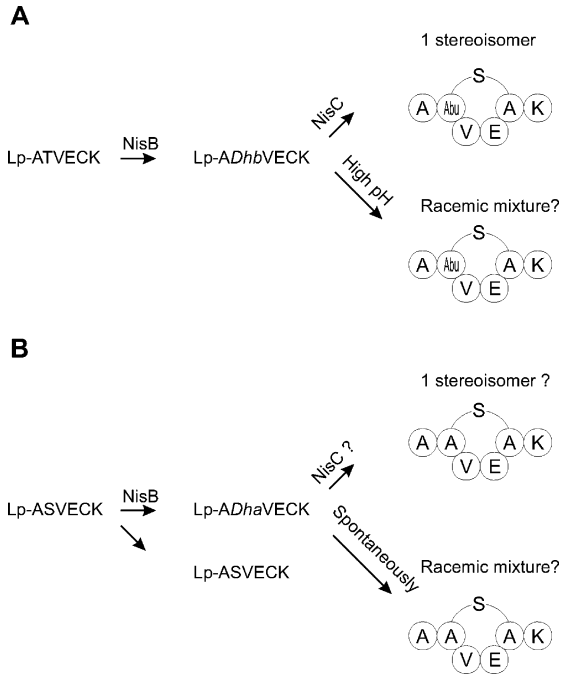

FIGURE 6: Thioether ring formation in ATVECK and ASVECK. Leader peptide constructs, Lp-ATVECK (A) and Lp-ASVECK (B), produced by *L. lactis* via NisBT or via NisBTC. ATVECK is fully dehydrated by NisB, whereas ASVECK partly escapes dehydration. NisC causes ring closure in ADhbVECK. ADhaVECK has undergone ring closure both when exported via NisBT or NisBTC. The differences between ATVECK and ASVECK are caused by the fact that threonines are more readily dehydrated, whereas dehydroalanines are more reactive than dehydrobutyrines.

naturally occurring lantibiotic (Figure 3, parts G and I, Figure 4A−C, and Figure 5A−C). In dehydrated prenisin, NisC regio- and stereospecifically catalyzes the cyclization of five rings. Rings A, B, and C have different sizes, whereas the intertwined rings D and E have a similar size as ring B. Furthermore, the distance between each ring and the leader peptide, which is necessary for cyclization (*16*) probably because of a targeting and/or activation function, is different. In our study, we used peptides that were fused immediately behind the leader peptide and the generated peptides were unrelated to the ring A or AB of nisin.

We previously compared all known lantibiotic structures to reveal possible rules on the substrate specificity of NisC and other cyclases with respect to the flanking amino acids of cysteine that becomes modified. This in silico analysis suggested some preference and exclusion for particular amino acids at the N- and C-flanking positions of the cysteine. For instance, N-flanking glutamate or C-flanking lysine seems to be favorable for ring formation, whereas N-flanking tryptophan appears unfavorable. We previously observed thioether rings in leader−ATVECK, and leader−ITPGCK, which were exported via NisBTC (*12*). Here we demonstrate that these rings are not spontaneously formed but introduced by NisC.

In line with the higher reactivity of dehydroalanines compared to dehydrobutyrines (*26*), we found that peptides with a dehydroalanine spaced by two amino acids from a cysteine, e.g., ADhaVECK, spontaneously formed rings under the experimental conditions employed (Figure 6B) while such spontaneous ring formation was not observed with peptides containing dehydrobutyrines, e.g., ADhbVECK

(Figure 6A). In the case of three amino acids distance between the dehydroalanine and the cysteine, in ring A of dehydrated prenisin, ring formation does not or only partially occurs spontaneously (*16*). When threonines in peptides that can be cyclized by NisC are replaced by serines, also these serines can be subject to NisC-catalyzed ring formation provided that they are dehydrated. Generally, serine residues are less readily dehydrated by NisB, but the formed dehydroalanines often easily cyclize spontaneously by reacting with cysteines. Threonine residues on the other hand are more efficiently dehydrated by NisB, but spontaneous cyclization involving the less reactive dehydrobutyrine occurs less readily.

Peptides with spontaneously formed or pH-induced thioether bridges may in many cases consist of a mixture of stereoisomers. With the use of a model peptide a three to one stereo preference was shown for ring A of subtilin (*27*). Depending on the amino acid composition stereospecific ring closure can occur nonenzymatically such as for rings B and E of nisin (*27*, *28*). NisC-catalyzed cyclization is—at least for dehydrated prenisin—stereospecific. As a consequence, the lanthionine and methyllanthionines in nisin are in the DL configuration. Van der Donk and co-workers discussed the possible mechanistic role of some residues of NisC, consistent with stereospecific cyclization (*16*) and subsequently demonstrated that the three zinc ligands Cys284, Cys330 and His331 as well as His212 and Asp141 were essential for cyclization, whereas Arg280 and Tyr285 were not (*20*). It will be of interest to experimentally investigate whether also in the case of designed nonlantibiotic peptides NisC-catalyzed cyclization is stereospecific and whether this might be an intrinsic and general property of this cyclase-mediated catalysis.

NisC-induced ring formation is regiospecific in nisin but also in the intertwined thioether rings of different sizes and in the thioether-rings-containing polyhexapeptide generated in this study. Such intertwined and multiple rings may protect the peptides against proteolytic degradation even to a larger extent than single thioether rings, but they also provide a specific constrained conformation to the peptide important for bioactivity. Regioselectivity of lacticin 481 synthetase has recently been demonstrated by Zhang et al. (*29*). In some lantibiotics, lanthionine rings are observed in two different orientations, an N-terminal dehydroresidue linked to a C-terminal cysteine or an N-terminal cysteine linked to a C-terminal dehydroresidue (*12*). Our data on peptide KDhbADhbCHCDhaK exported via NisBT indicate a spontaneously formed thioether bridge with inverse orientation between the Dha and its N-sided cysteine. Interestingly, in nisin cyclization occurs only between dehydroresidues and more distally located cysteines and not to more proximally located cysteines. Also in this study, in the case of the leader−ICPGDhbK, we did not observe NisC-mediated cyclization of a methyllanthionine ring with an inversed orientation. This implies that some other cyclases are even more promiscuous than NisC. NisC seems to act as a processive enzyme as it can efficiently introduce thioether linkages in hexapeptides that are placed blockwise behind each other.

The regio- and chemoselectivity of NisC-induced cyclization, which is—at least for nisin—also stereospecific, allows the exclusive synthesis of complex molecules. Recently, detailed mechanistic insight in the enzymatic mechanism of NisC has been obtained by van der Donk and co-workers, who reported the NisC crystal structure, in vitro reconstituted NisC-catalyzed synthesis of nisin and identified essential catalytic residues in NisC (*16*, *20*, *30*). Here we report that this enzyme is rather versatile and capable to catalyze the cyclization of many unrelated peptides thereby yielding complex structures including intertwined rings.

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SUPPORTING INFORMATION AVAILABLE

MALDI-TOF mass spectrometry analyses of hydrolysis fragments of the NisC-cyclized peptide RADhbVADhbCKGCK (Table S1, Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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