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Permeabilization induced by lipid II-targeting lantibiotic nisin and its effect on the bioconversion of vitamin D₃ to 25-hydroxyvitamin D₃ by *Rhodococcus erythropolis*

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Abbreviations:

P450, cytochrome P450 monooxygenase; Vdh, vitamin D₃ hydroxylase; VD₃, vitamin D₃; 25(OH)VD₃, 25-hydroxyvitamin D₃; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PMCD, partially methylated-β-cyclodextrin; NADH, β-nicotinamide adenine dinucleotide, reduced form; HPLC, high-performance liquid chromatography; GCCD, green chemiluminescent cyclodextrin.

Abstract

Vitamin D₃ (VD₃) is a fat-soluble prohormone in mammals. VD₃ is inert and must be activated by hydroxylation at the C-25 and C-1 α positions to exert its biological activity. We recently accomplished the bioconversion of VD₃ to 25(OH)VD₃ with a recombinant strain of *Rhodococcus erythropolis* and found that the permeability of VD₃ into the cytoplasm may be the rate-limiting step of 25(OH)VD₃ production (Sallam et al., 2010). When the cells were treated with the lipid II-targeting lantibiotic nisin, the permeability of green chemiluminescent cyclodextrin (GCCD), which is used as a model substrate instead of VD₃-partially methylated- β -cyclodextrin (PMCD) complex, was drastically induced. Nisin also induced VD₃ hydroxylation, and the rate was correlated with the expression levels of Vdh and its redox partner proteins. In the bioconversion reaction, the stability of the redox partner proteins and the additional NADH-regenerating system are crucial for VD₃ hydroxylation. The degradation rate of the [2Fe-2S] cluster of ferredoxin ThcC from *R. erythropolis* NI86/21 is faster than that of AciB from *Acinetobacter* sp. OC4. Therefore, the nisin-treated *R. erythropolis* cells coexpressing Vdh and AciBC (1176.5 μ g) exhibited much greater 25(OH)VD₃ production than the cells coexpressing Vdh and ThcCD (431.7 μ g) after 4 consecutive 16-h reactions. These results suggest that nisin forms nisin-lipid II pore complexes in the *Rhodococcus* membrane that increase the accessibility of VD₃-PMCD complexes to the inside of the cells. Furthermore, nisin-treated *Rhodococcus* cells can be utilized for the bioconversion of other fat-soluble chemicals.

Keywords *Rhodococcus erythropolis*, Vitamin D₃, Nisin, Bioconversion, Ferredoxin

Introduction

Vitamin D₃ (VD₃) is a fat-soluble prohormone in mammals that plays a crucial role in bone metabolism, immunity, and the control of cell proliferation and differentiation. VD₃ is obtained from food or is produced in skin when 7-dehydrocholesterol reacts with ultraviolet-B light from the sun or artificial sources [1, 2]. In mammals, VD₃ is hydroxylated by cytochrome P450 in the liver at the C-25 position forming 25-hydroxyvitamin D₃ (25(OH)VD₃); it is subsequently hydroxylated by another P450 in the kidneys at the C-1 α position forming 1 α ,25-dihydroxyvitamin D₃ (1 α ,25(OH)₂VD₃). The most active form, 1 α ,25(OH)₂VD₃, is used to treat osteoporosis, hyperparathyroidism, psoriasis, and VD₃ metabolic abnormality. The industrial production of 1 α ,25(OH)₂VD₃ is performed chemically or microbiologically. The processes for the microbiological production of the active form of VD₃ are simpler than those for chemical synthesis.

Recently, we identified a cytochrome P450 named Vdh (Vitamin D₃ hydroxylase) from the actinomycete *Pseudonocardia autotrophica*, which is capable of the bioconversion of VD₃ into its physiologically active forms of VD₃: 25(OH)VD₃ and 1 α ,25(OH)₂VD₃ [3]. Based on the structural analysis of a highly active Vdh mutant, Vdh-K1, the substrate-bound structure of Vdh-K1 accommodates both VD₃ and 25(OH)VD₃ but in an anti-parallel orientation. The occurrence of the 2 secosteroid-binding modes accounts for the regioselective sequential VD₃ hydroxylation activities [4]. The bioconversion of VD₃ to 25(OH)VD₃ is accomplished with a recombinant strain of the actinomycete *Rhodococcus erythropolis*, coexpressing Vdh and redox partner proteins, ThcC and ThcD from *R. erythropolis* NI86/21. By using a vector system for randomly integrating multiple copies of the Vdh expression cassette into the chromosomal DNA of *R. erythropolis*, the bioconversion rate of VD₃ to 25(OH)VD₃ is not accompanied by Vdh expression [5].

Cyclodextrins are cyclic oligomers of 1,4- α -D-linked glucose units. Cyclodextrin with its lipophilic inner cavities and hydrophilic outer surfaces is capable of interacting with a large

variety of guest molecules, including poorly water-soluble compounds, to form noncovalent inclusion complexes [6]. The complexation of progesterone with hydroxypropyl- β -cyclodextrin enhances androstadienedione production during bioconversion [7]. PMCD is a partially methylated- β -cyclodextrin that immobilizes VD_3 and its hydroxylated forms in its inner hydrophobic cavity. Consequently, the solubility of VD_3 in water is drastically increased (640 $\mu\text{g } VD_3/\text{ml}$, 1.5% PMCD); this improved solubility facilitates the hydroxylase activity of Vdh [8]. The conversion rate of VD_3 to its hydroxylated forms in a cell-free assay is much higher than that in *in vivo* experiments [3, 8]; this suggests that the low transport rates of VD_3 and its hydroxylated forms to and from cells are the rate-limiting steps in its bioconversion processes.

Nisin is a small antimicrobial peptide composed of 34 amino acids that are produced by *Lactococcus lactis* subsp. *lactis*. Nisin is highly effective against a range of gram-positive bacteria, including food-borne pathogens such as *Listeria monocytogenes* and *Clostridium botulinum* [9]. Its status is “generally recognized as safe”, and is used as a food biopreservative in more than 50 countries [10]. It exerts its biocidal activity via both pore formation in the membrane and the disruption of cell wall biosynthesis by binding to lipid II, which is an essential intermediate in peptidoglycan biogenesis. The pore structure is made up of 4 lipid II and 8 nisin molecules [11]. Previous studies demonstrate that nisin inhibits the growth of the actinomycetes *Mycobacterium smegmatis* and *M. tuberculosis*, by forming pores, which leads to proton motive force dissipation and the efflux of many vital intracellular compounds [12, 13].

In this study, we tested whether the nisin pore complex improves the accessibility of substrate-cyclodextrin complexes to the inside of *Rhodococcus* cells and enhances 25(OH) VD_3 production from VD_3 via biotransformation. Here, we report a new innovative procedure for enhancing the bioconversion of VD_3 to 25(OH) VD_3 with a recombinant strain of *R. erythropolis*.

Materials and methods

Strains, plasmids, oligonucleotides, and standard genetic manipulations

Wild-type *Rhodococcus erythropolis* JCM3201 was obtained from the Japan Collection of Microorganisms (JCM; RIKEN Bio Resource Center, Wako, Saitama, Japan). *Rhodococcus*, *Escherichia coli* XL1-Blue, and *E. coli* BL21-CodonPlus(DE3)-RIL cells were routinely cultured in Luria-Bertani (LB) broth (1% bacto tryptone, 0.5% bacto yeast extract, and 1% NaCl) in the presence or absence of appropriate antibiotics. Antibiotics were used at the following concentrations: chloramphenicol (17 µg/ml for *Rhodococcus*; 34 µg/ml for *E.coli*), kanamycin (20 µg/ml for *E.coli*), and ampicillin (100 µg/ml for *E. coli*), respectively. Cultures and agar plates were incubated at 28 °C for *Rhodococcus* species and at 37 °C for *E. coli*. We used *E. coli* XL1-Blue for vector maintenance and amplification. Competent *Rhodococcus* cells were prepared according to the procedure outlined by Shao et al. [14]. The transformation of *Rhodococcus* strains was performed using a previously described method [15].

Plasmid DNA isolation, restriction enzyme digestion, ligation, and transformation were performed according to the standard protocols for cloning [16]. Plasmid DNA from *E. coli* and genomic DNAs from *Rhodococcus* species were isolated using a previously described method [5]. Oligonucleotides were obtained from Hokkaido System Science Co., Ltd. (Hokkaido, Japan). PCRs were performed with *Pfu* Turbo DNA polymerase (Stratagene, CA, USA) or KOD plus polymerase (Toyobo Co., Osaka, Japan). Restriction endonucleases were purchased from New England BioLabs Inc. (MA, USA). T4 DNA ligase was purchased from Toyobo Co. Ltd. A Wizard SV Gel and PCR Clean-Up System (Promega Co., WI, USA) were used to purify the PCR products and isolate the DNA fragments from the gels before their subsequent cloning or sequencing.

DNA was sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit

(Applied Biosystems, CA, USA) on an ABI Prism 3100 automated sequencer (Applied Biosystems Inc., CT, USA) according to the manufacturer's instructions.

Construction of expression plasmids

For the construction of expression vectors, pTipQC-vdh, pTipQC-vdh-thcCD, and pTipQC-vdh-aciBC, each gene was amplified by PCR, digested with appropriate restriction enzymes, and introduced into the pTipQC2 vector [17]. Vdh expression levels were determined by reduced-CO difference spectral analysis. The detailed plasmid construction procedure is described in Supplementary Methods.

Structural stability of ThcC and AciB

To express recombinant ThcC and AciB proteins, their corresponding genes were cloned into the *NdeI* and *XhoI* sites of pET26b (Novagen, WI, USA). The expression, isolation, and the [2Fe-2S] cluster stability assay of recombinant ThcC and AciB are described in Supplementary Methods.

Determination of nisin sensitivity using the colony count method

To determine the effects of nisin on actively growing *R. erythropolis* JCM3201 cells, cells cultured overnight were diluted in LB broth to an OD₆₀₀ of 5.0 and were divided into 15-ml aliquots [12]. The cells were exposed to different concentration of nisin (0, 0.25, 0.5, 1.0, or 2.0 mg/ml; Sigma-Aldrich Co., MO, USA) and were incubated for 1–10 h at 28 °C under constant agitation. Cultured cells (100 µl) were plated onto LB agar without antibiotics, incubated for 3 days at 28 °C, and enumerated.

Permeability assay of cyclodextrin to cytosol in nisin-treated cells

To determine the effects of nisin on the permeability of cyclodextrin to the

cytoplasm of *R. erythropolis* cells, green chemiluminescent cyclodextrin (GCCD) (Wako Pure Chemical Industries Ltd., Osaka, Japan) [18] was used as a model substrate; GCCD in the cytoplasm was detected using superoxide-induced chemiluminescence intensity as described in Supplementary Methods.

Expression and isolation of recombinant GlcDH-IV

To express recombinant glucose dehydrogenase from *Bacillus megaterium* IAM1030 (GlcDH-IV), its corresponding gene was cloned into the *Nde*I and *Xho*I sites of pET28a (Novagen) [19]. The expression and isolation of recombinant GlcDH-IV is described in the Supplementary Methods.

Repeated bioconversion of vitamin D₃ using nisin-treated Rhodococcus erythropolis

Two cell strains, JCM3201/pTipQC-vdh-thcCD and JCM3201/pTipQC-vdh-aciBC, were prepared and independently cultured; their VD₃ bioconversion activities were measured in the presence or absence of nisin. The reaction solution containing 1 mM VD₃ was refreshed every 16 h, and 4 consecutive reactions were performed. Vitamin D₃ metabolites were determined on the basis of the bioconversion rate of inactive vitamin D₃ into its hydroxylated active form as described previously [3, 5]. The reconstitution system of NADH (2% glucose, 2 U/ml GlcDH-IV, and 4 mM NADH) was used for the bioconversion of VD₃ to 25(OH)VD₃ in nisin-treated cells. The detailed experimental procedure is described in Supplementary Methods.

Results and discussion

Permeability of cyclodextrin to cytosol in nisin-treated *Rhodococcus erythropolis* cells

We first determined the effects of a small antimicrobial protein, nisin, on the growth of *R. erythropolis* JCM3201. The nisin-resistant cells decreased in a dose-dependent manner: 1% residual *R. erythropolis* cells with 0.25 mg/ml nisin (Fig. 1A). The sensitivity of *R. erythropolis* to nisin is slightly lower than that of *M. smegmatis* [12]. No apparent cell lysis was observed after 5 h incubation with 0.4 mg/ml nisin, and no proteins were detected in the extracellular media by SDS-PAGE (data not shown). It is reported that nisin forms pores with the cell wall precursor lipid II that have diameters of 2 to 2.5 nm [20]. It may be possible that nisin pores function as open channels for the passage of VD₃–PMCD complexes. Then, we investigated whether cyclodextrin can be transported into the cytoplasmic space after cells are treated with nisin. For this purpose, GCCD was used as a model substrate and was detected by the superoxide-induced chemiluminescence intensity as described in Materials and methods. The GCCD luminescence intensity in the cytoplasmic space increased in the presence of nisin in a dose-dependent manner; the maximum luminescence intensity ranged between 0.25 and 1.0 mg/ml nisin. Meanwhile, almost no GCCD was detected in the cell extracts without nisin treatment (Fig. 1B), suggesting that VD₃ is released from PMCD and is transported to the cytoplasmic space by diffusion in *in vivo* VD₃ biotransformation experiments. Since the accumulation of GCCD in the cytoplasmic space increased in a time-dependent manner and required over 3 h to reach equilibrium, nisin continuously formed cell membrane pore complexes (Fig. 1C). GCCD is a γ -CD-modified probe; the dimensions of γ -CD (0.78 nm high \times 1.69 nm outer diameter) are smaller than those of nisin pores [6]. Taken together, these results demonstrate that nisin inhibits *R. erythropolis* growth and increases the permeability of GCCD into the cytoplasmic space, possibly by forming

nisin-lipid II pore complexes in the membrane.

NADH regeneration system enhances 25(OH)VD₃ production in nisin-treated cells

To clarify whether nisin enhances the bioconversion of VD₃ to 25(OH)VD₃, the cell strain (JCM3201/pTipQC-vdh-thcCD) coexpressing Vdh and redox partner proteins, ThcC and ThcD from *R. erythropolis* NI86/21, was prepared and incubated with VD₃-PMCD complexes in the presence or absence of nisin (Fig. 2A). Protein expression increased in the presence of thiostrepton in a dose-dependent manner, but 25(OH)VD₃ production was not enhanced in any thiostrepton-treated cells when nisin was absent. However, in the presence of nisin, the cells exhibited an increased (up to 3.9-fold) conversion rate of VD₃ to 25(OH)VD₃ that was correlated with the expression levels of the proteins. These data suggest that nisin enhances the transport of VD₃-PMCD complexes to the cytoplasm and increases the concentration of VD₃ in the intracellular space. For nisin-treated cells, an electron donor, NADH, and its regeneration system, which is composed of glucose and glucose dehydrogenase from *Bacillus megaterium* IAM1030 (GlcDH-IV) strongly supported Vdh activity. ThcD is an NADH-dependent ferredoxin reductase (Fig. 2B). When JCM3201/pTipQC-vdh-thcCD cells were treated with different concentrations of nisin (0–1.0 mg/ml), a time-dependent increase in 25(OH)VD₃ production was observed in the presence of the NADH-regenerating system. Approximately 50% of VD₃ was hydroxylated after a 16-h reaction with 0.75–1.0 mg/ml nisin, while only 13.8% was hydroxylated in the absence of nisin (Fig. 2C). The highest conversion rate of VD₃ was achieved in the cells treated with 1 mg/ml nisin (52.5% after 24-h reaction). These results indicate that the NADH-regeneration system is essential for enhancing the bioconversion of VD₃ to 25(OH)VD₃. Increases in the NADH pool may facilitate electron transfer from ferredoxin (ThcC) to Vdh.

Co-expression of Vdh and redox partner proteins

P450s generally require the sequential delivery of 2 electrons passed from redox partner proteins to facilitate its monooxygenase activity. In the case of Vdh, it is reported that in addition to ThcCD from *R. erythropolis* NI86/21 another exogenous redox partner, AciBC from *Acinetobacter* sp. OC4, provides efficient electron transfer to the heme iron of Vdh [3, 21]. Both AciB and ThcC are [2Fe-2S] cluster containing ferredoxins (2Fe-2S ferredoxin); AciB exhibits significant amino acid sequence similarity to ThcC (38.7% identity). ThcD and AciC are ferredoxin reductases; they also show significant amino acid sequence similarity to each other (41.8% identity). To clarify which redox partner protein is a suitable electron transfer system for Vdh, the *R. erythropolis* strain coexpressing Vdh and AciBC (JCM3201/pTipQC-vdh-aciBC) was prepared, and the biotransformation activity of VD₃ to 25(OH)VD₃ was measured (Fig. 3).

In the absence of nisin, JCM3201/pTipQC-vdh cells expressing Vdh alone showed a certain level of bioconversion of VD₃ to 25(OH)VD₃; this level is almost the same as those of the other 2 strains JCM3201/pTipQC-vdh-thcCD and JCM3201/pTipQC-vdh-aciBC. The expression levels of Vdh in each strain were somewhat similar: 3.29, 2.67, and 2.51 nmol/mg protein in the JCM3201/pTipQC-vdh, JCM3201/pTipQC-vdh-thcCD, and JCM3201/pTipQC-vdh-aciBC strains, respectively (Fig. 3B). This result suggests that endogenous redox proteins drive Vdh and that the overexpression of redox partner proteins is not essential for enhancing VD₃ hydroxylation in living cells (Fig. 3A). However, once cells were exposed to nisin, overexpressed redox partner proteins were essential, and expressed proteins drastically enhanced the production of 25(OH)VD₃ (Fig. 3A); in fact, VD₃ hydroxylation activity was abolished from JCM3201/pTipQC-vdh cells expressing Vdh alone (Fig. 3A). JCM3201 cells overexpressing endogenous [2Fe-2S] ferredoxin and ferredoxin reductase, which was revealed by the whole genome sequence of *R. erythropolis* JCM3201, showed lower enhancement of VD₃ hydroxylation than the cells overexpressing ThcCD or AciBC. Since only endogenous [2Fe-2S] ferredoxin could not be expressed using *E. coli* as

an expression platform, their structural stability might not be as high as those of ThcC and AciB (data not shown).

SDS-PAGE analysis revealed that the expression levels of ThcC in JCM3201/pTipQC-vdh-thcCD and AciB in JCM3201/pTipQC-vdh-aciBC were different; ThcC was observed as an intense band, while AciB was barely visible (Fig. 3B). When the degradation of the [2Fe-2S] cluster of recombinant ThcC and AciB was monitored, its degradation in holo-ThcC was much faster than that of holo-AciB; 77% of ThcC is inactivated after a 24-h incubation (Fig. 3C). Protein synthesis in nisin-treated cells may be disrupted by the efflux of vital intracellular compounds through nisin pores. The structural and functional stability of ferredoxin is therefore critical for the P450-mediated biotransformation process using nisin-treated cells.

Repeated bioconversion of VD₃ to 25-hydroxylated using nisin-treated cells

After the reaction conditions were optimized, the VD₃ hydroxylation activity of nisin-treated cells was investigated. Two strains, JCM3201/pTipQC-vdh-thcCD and JCM3201/pTipQC-vdh-aciBC, were independently cultured and their VD₃ bioconversion activity was measured as described in Materials and methods. The reaction solution was refreshed every 16 h, and 4 consecutive reactions (about 64 h in total) were performed (Fig. 4). The expression levels of Vdh and redox partner proteins are shown in Fig. 3B. In the absence of nisin, both strains showed almost the same bioconversion rate (10–17%) after 16 h during whole reaction. The total amount of 25(OH)VD₃ produced was not different between strains: 199.5 μg in JCM3201/pTipQC-vdh-thcCD versus 192.9 μg in JCM3201/pTipQC-vdh-aciBC. In the presence of nisin, the bioconversion rate in JCM3201/pTipQC-vdh-thcCD decreased regardless of reaction time; furthermore, the amount of 25(OH)VD₃ produced in the fourth reaction was less than that produced by the cells without nisin (Fig. 4A). Since 65% of holo-ThcC is inactivated after a 16-h incubation

(Fig. 3C), the synthesis of ThcC may have stopped; thus, the [2Fe-2S] cluster would not be assembled and transferred to apo-ThcC. In JCM3201/pTipQC-vdh-aciBC cells, VD₃ hydroxylation activity increased after the second reaction (Fig. 4C). This may be caused by the increased number of nisin pores in the membrane within the first 16 h; the maximum transportation rate of VD₃-PMCD complexes to the cytoplasm may be attained after at least a 3-h exposure to nisin (Fig. 1C). The total amount of 25(OH)VD₃ produced in each strain was markedly different: 431.7 versus 1176.5 μg in JCM3201/pTipQC-vdh-thcCD and JCM3201/pTipQC-vdh-aciBC, respectively (Fig. 4B and D). Based on the *in vitro* reconstitution experiment, Vdh and ferredoxin reductases, ThcD and AciC, were structurally and functionally stable (data not shown). Nisin pores are quite stable once formed as reported previously [11]. Taken together, the stable electron transfer from AciB to Vdh was crucial for the bioconversion of VD₃ to 25(OH)VD₃ in this experiment.

Further protein engineering studies of Vdh and redox partner proteins, and cell biological studies using nisin are required to elucidate the molecular mechanisms underlying the transport of VD₃ to and from cells as well as the hydroxylation of VD₃ in connection with PMCD. This experimental design is applicable for the biotransformation of other fat-soluble and toxic molecules for living cells.

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Figure legends

Fig. 1 Effect of nisin on actively growing cells

Rhodococcus erythropolis JCM3201 cells were inoculated in LB medium containing different concentrations of nisin: 0(◆), 0.25(■), 0.5(□), 1.0(▲), 2.0(◇), and 5.0(●) mg/ml (A). *R. erythropolis* cells were exposed to different concentrations of nisin (0, 0.025, 0.05, 0.10, 0.25, 0.50, or 1.0 mg/ml) for 1 h; GCCD was then added to the cell suspension (final concentration, 10 μ M) for 3 h. The transport of GCCD to the cytoplasm was measured (B). *R. erythropolis* cells were treated with nisin (0.5 mg/ml) for 0–3 h, followed by the addition of GCCD to the cell suspension (final concentration, 10 μ M) for 3 h. Time-course accumulation of GCCD in the cytoplasm of nisin-treated cells (C). GCCD was detected by superoxide-induced chemiluminescence intensity as described in Materials and methods. Values in (B) and (C) are means \pm SD ($n \geq 3$ independent cultures).

Fig. 2 Effect of nisin on VD₃ biotransformation activity in JCM3201/pTipQC-vdh-thcCD cells

(A) Bioconversion rate of VD₃ to 25(OH)VD₃ correlated with the expression level of Vdh in nisin-treated cells. The co-expression of Vdh and ThcCD was induced by various concentrations of thiostrepton for 16 h (0.1 μ g/ml (open bar), 0.2 μ g/ml (gray bar), and 0.5 μ g/ml (closed bar)), followed by VD₃ biotransformation activity was measured in the presence or absence of nisin (0.5 mg/ml) for 16 h. Inset shows expression level of Vdh and ThcD. Vdh expression level was determined by reduced-CO difference spectral analysis as described in Materials and methods.

(B) The NADH-regenerating system enhances 25(OH)VD₃ production. The expressions of Vdh and ThcCD were induced by thiostrepton (0.5 μ g/ml) when JCM3201/pTipQC-vdh-thcCD cell growth reached the mid-log phase. After a 16-h induction, VD₃ biotransformation activity was tested in the presence or absence of nisin (0.5 mg/ml) and NADH-regeneration system

(NR). The reaction was terminated after a 16-h reaction at 28 °C with agitation.

(C) Time-course of VD₃ hydroxylase activity. The thiostrepton-induced JCM3201/pTipQC-vdh-thcCD cells described in (B) were tested for their VD₃ biotransformation activity in the presence of different concentrations of nisin (0(◆), 0.25(■), 0.5(□), 0.75(▲), or 1.0(◇) mg/ml) and NADH-regeneration system for 24 h at 28 °C with agitation.

The amounts of 25(OH)VD₃ were measured by HPLC in each reaction as described in Materials and methods. Values are the means of more than 3 independent cultures (bars indicate standard deviations).

Fig. 3 Effect of redox partner proteins on VD₃ hydroxylation activity

(A) Biotransformation of VD₃ to 25(OH)VD₃ in JCM3201/pTipQC-vdh (Vdh), JCM3201/pTipQC-vdh-thcCD (Vdh-ThcCD), and JCM3201/pTipQC-vdh-aciBC (Vdh-AciBC) cells. The cells coexpressing Vdh and redox partners were prepared, and VD₃ hydroxylation activity was measured in the presence (closed bar) or absence (open bar) of nisin (0.5 mg/ml) for 16 h at 28 °C with agitation. The amounts of 25(OH)VD₃ were measured by HPLC in a 16-h reaction. Values are means ± SD (n ≥ 3 independent cultures).

(B) Expression profiles of Vdh and redox partner proteins in *Rhodococcus erythropolis*. Thiostrepton-induced crude proteins (15 μg) were loaded onto a 12.5% SDS-PAGE: wild-type JCM3201 (lane 1), JCM3201/pTipQC-vdh (lane 2), JCM3201/pTipQC-vdh-thcCD (lane 3), and JCM3201/pTipQC-vdh-aciBC cells (lane 4). Vdh and AciC showed the same migration in SDS-PAGE (indicated as asterisks) (lane 4).

(C) [2Fe-2S] cluster degradation of holo-ThcC and holo-AciB. Holo-ThcC (●) and holo-AciB (○) were incubated in 50 mM potassium phosphate buffer (pH 7.4) at 28 °C. The percentage of the [2Fe-2S] cluster preserved in ferredoxin solutions was calculated as the ratio of A₄₁₂ nm measured at a given time to A₄₁₂ nm measured at the beginning of the experiment. Values are

the means of 3 independent experiments.

Fig. 4 Repeated production of 25(OH)VD₃ using nisin-treated *Rhodococcus erythropolis* cells. The reaction solution was refreshed every 16 h, and 4 consecutive reactions (about 64 h in total) in the presence (■) or absence (○) of nisin (0.5 mg/ml) were performed using JCM3201/pTipQC-vdh-thcCD (A) or JCM3201/pTipQC-vdh-aciBC cells (C). The total amount of 25(OH)VD₃ produced in the presence (closed bar) or absence (open bar) of nisin (0.5 mg/ml) in JCM3201/pTipQC-vdh-thcCD (B) or JCM3201/pTipQC-vdh-aciBC cells (D). The amount of 25(OH)VD₃ was measured by HPLC in each reaction. Values are the means of more than 3 independent cultures (bars indicate standard deviations).

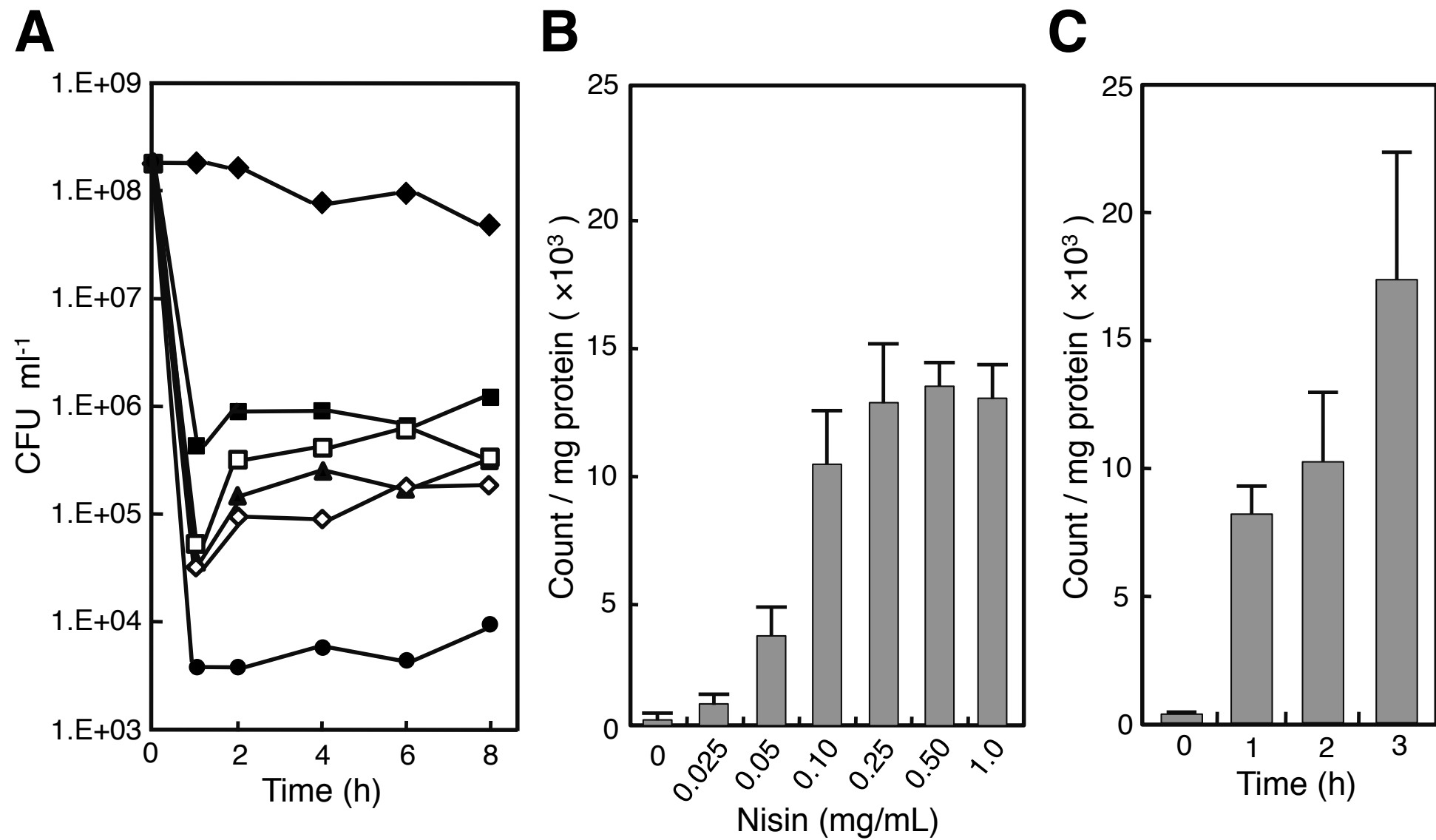


Fig. 1

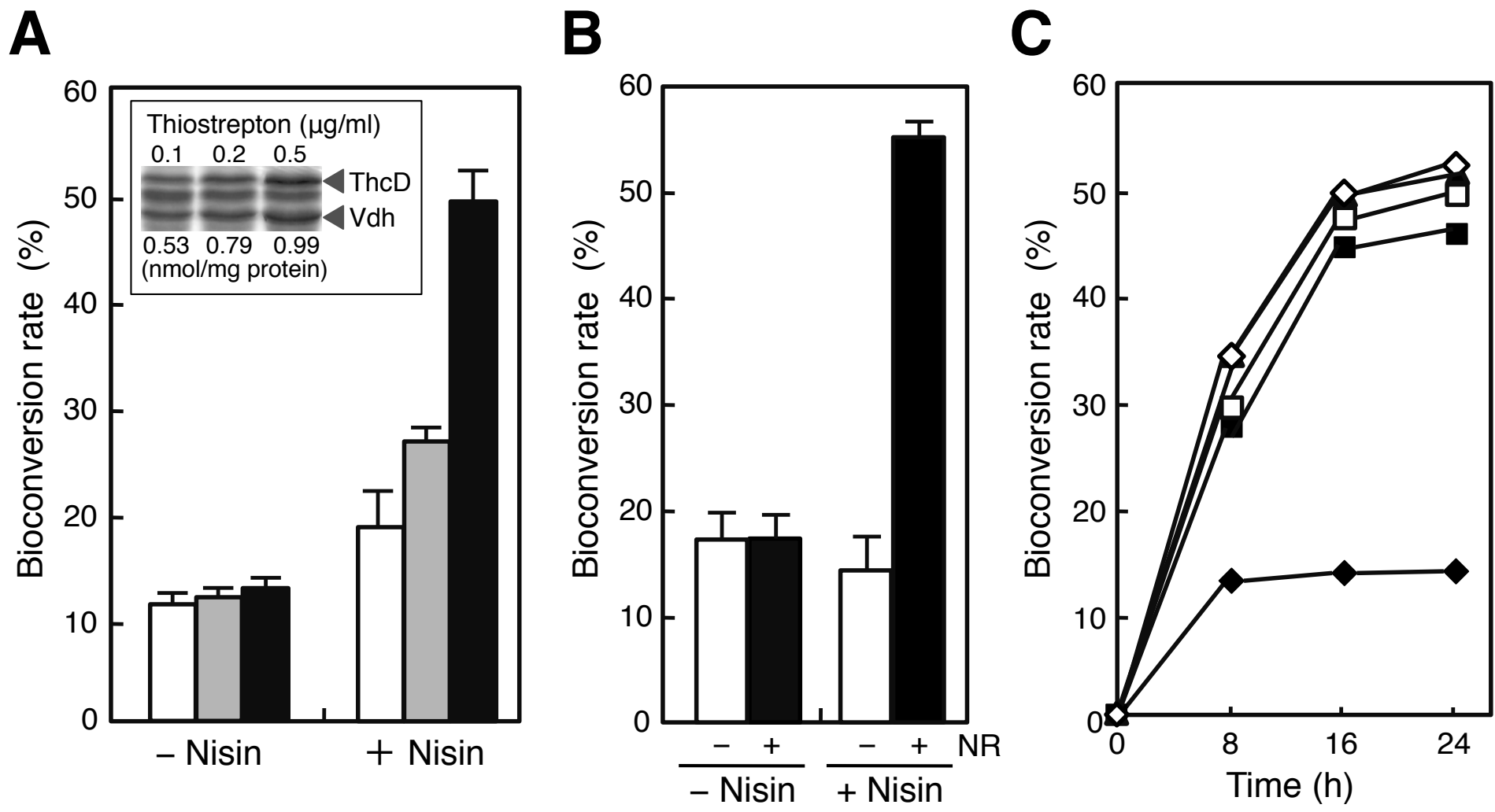


Fig. 2

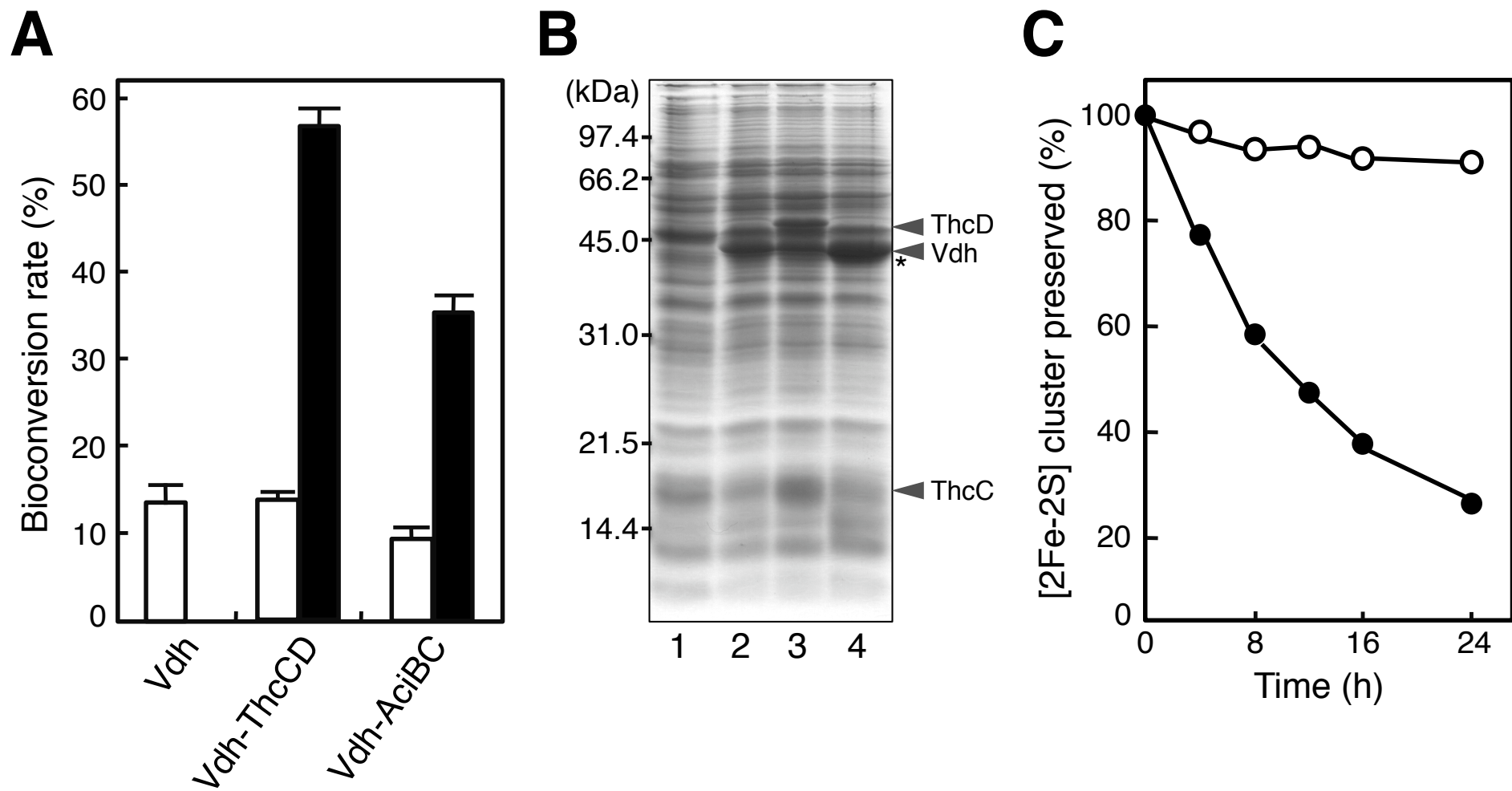


Fig. 3

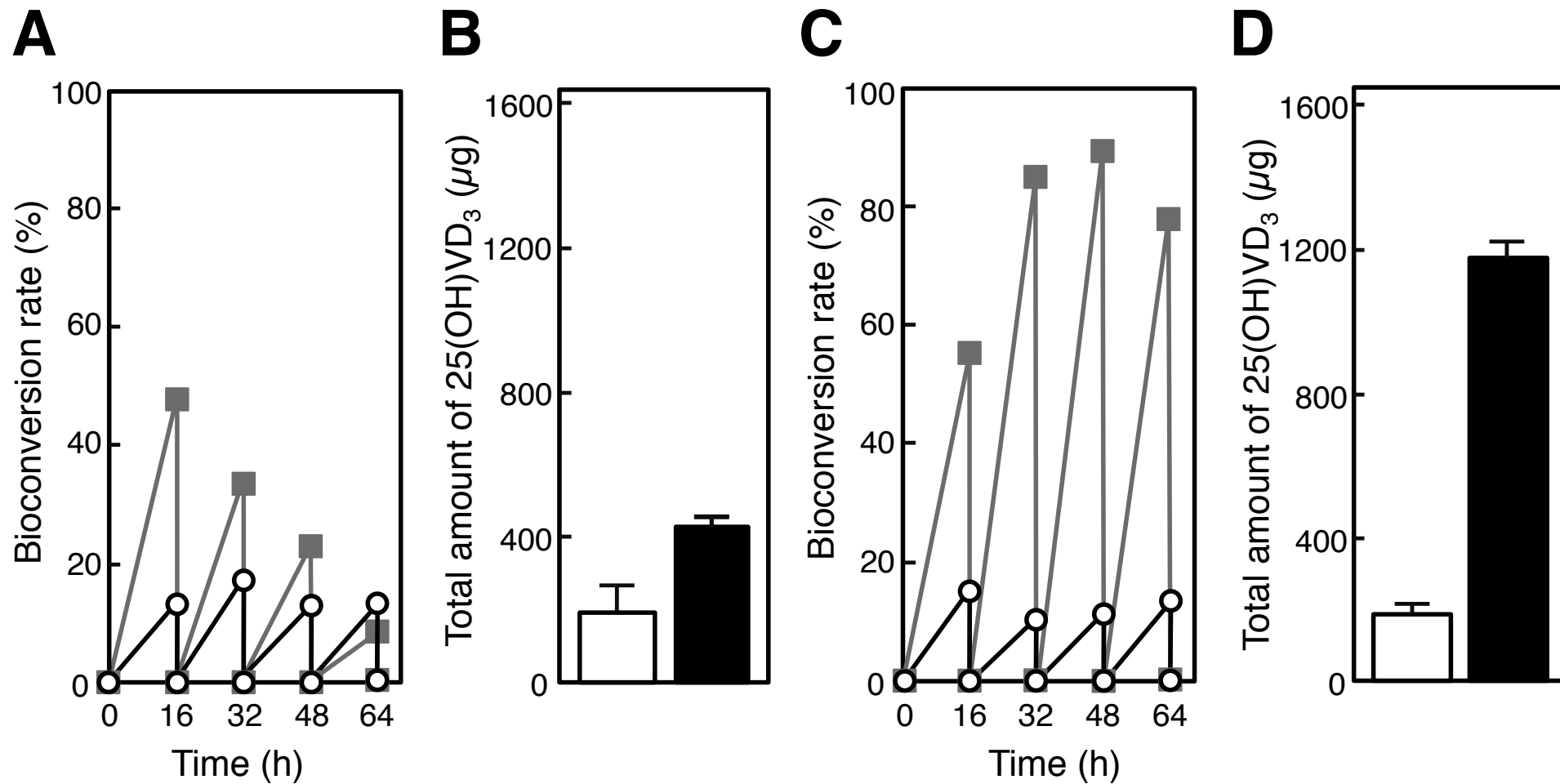


Fig. 4

Permeabilization induced by lipid II-targeting lantibiotic nisin and its effect on bioconversion of VD₃ to 25-hydroxyvitamin D₃ by *Rhodococcus erythropolis*

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Supplementary Methods

Expression vector construction

To construct the expression vectors pTipQC-vdh, pTipQC-vdh-thcCD, and pTipQC-vdh-aciBC, PCRs were performed using KOD plus DNA polymerase (Toyobo Co., Osaka, Japan). The *vdh* gene was amplified by PCR using the following primers with pTipQT2-vdh-thcCD as the template [1]: vdh-F, 5'-GGAATTCCCATATGGCGCTGACCACCACCGGCACC-3' (*Nde*I site is underlined) and vdh-R, 5'-GGAATTCTCAGGCGCTGCGCGGCCCCATC-3' (*Eco*RI site is underlined). The PCR fragment was isolated, digested with *Nde*I and *Eco*RI, and inserted into the corresponding sites of pTipQC2 [2], yielding pTipQC-vdh.

The *thcCD* gene was amplified by PCR using the following primers with pTipQT2-vdh-thcCD as the template: thcC-F, 5'-GGGAATTCCCATATGCCTACCGTCACCTACGTTAC-3' (*Nde*I site is underlined) and thcD-R, 5'-GACTAGTTCATGACGCCACCACCTGACGCTC-3' (*Spe*I site is underlined). The PCR fragment was isolated, digested with *Nde*I and *Spe*I, and inserted into the corresponding sites of pTipQC2, yielding pTipQC2-thcCD. The ribosome-binding region derived from pTipQC2 and the *thcCD* gene was amplified by PCR using the following primers with pTipQC2-thcCD as the template: LG10RBS_E-F, 5'-GGAATTCCTCTAGAAATAATTTTGTTTAAAC-3' (*Eco*RI site is underlined) and thcD-R, 5'-GACTAGTTCATGACGCCACCACCTGACGCTC-3' (*Spe*I site is underlined). Amplified

DNA was isolated, digested with *EcoRI* and *SpeI*, and cloned into the corresponding sites of pTipQC2-vdh, yielding pTipQC-vdh-thcCD.

The *aciB* gene was amplified using the following primers: *aciB*-F, 5'-GGAATTCCCATATGGGCCAAATTACATTTATTG-3' (*NdeI* site is underlined) and *aciB*-R, 5'-CCCAAGCTTTTACATCTGAAACTCAGGC-3' (*HindIII* site is underlined). The expression vector pNami2vdh (kindly provided by Dr. A. Arisawa, Mercian Corporation) was used as the template. The PCR fragment was isolated, digested with *NdeI* and *HindIII*, and cloned into the corresponding sites of pTipQC2, yielding pTipQC2-*aciB*. The ribosome-binding region derived from pTipQC2 and the *aciB* gene was amplified by PCR using the following primers with pTipQC2-*aciB* as the template: LG10RBS_E-F, 5'-GGAATTCCTCTAGAAATAATTTTGTTTAAC-3' (*EcoRI* site is underlined) and *aciB*-R, 5'-CCCAAGCTTTTACATCTGAAACTCAGGC-3' (*HindIII* site is underlined). Amplified DNA was isolated, digested with *EcoRI* and *HindIII*, and cloned into the corresponding sites of pTipQC2-vdh, yielding pTipQC2-vdh-*aciB*.

The *aciC* gene was amplified by PCR using the following primers with pNami2vdh as the template: *aciC*-F, 5'-GGAATTCCATATGCAAACAATCGTCATCATTG-3' (*NdeI* site is underlined) and *aciC*-R, 5'-GACTAGTCTACCCCATCAACGCCTGTAC-3' (*SpeI* site is underlined). The PCR fragment was isolated, digested with *NdeI* and *SpeI*, and inserted into the corresponding sites of pTipQC2, yielding pTipQC2-*aciC*. The ribosome-binding region derived from pTipQC2 and *aciC* gene was amplified by PCR using the following primers with pTipQC2-*aciB* as the template: LG10RBS_H-F, 5'-CCCAAGCTTCTCTAGAAATAATTTTGTTTAAC-3' (*HindIII* site is underlined) and *aciC*-R, 5'-GACTAGTCTACCCCATCAACGCCTGTAC-3' (*SpeI* site is underlined). Amplified DNA was isolated, digested with *HindIII* and *SpeI*, and cloned into the corresponding sites of pTipQC2-vdh-*aciB*, yielding pTipQC-vdh-*aciBC*.

Expression and purification of the recombinant ferredoxins

To express the ThcC and AciB proteins, their corresponding genes were cloned into the *Nde*I and *Xho*I sites of pET26b (Novagen, WI, USA).

The gene encoding *thcC* (gene bank accession number U17130) was amplified by PCR using *Pfu* Turbo DNA polymerase (Stratagene, CA, USA) with the following primers: *thcC*-F, 5'-GGGAATTCCCATATGCCTACCGTCACCTACGTTCAC-3' (*Nde*I site is underlined) and *thcC*-R, 5'-CGCTCGAGTCATACTTGCTCCTCCGGAAGCCGG-3' (*Xho*I site is underlined). The *R. erythropolis* NI86/21 genome DNA was used as the template.

The gene encoding *aciB* from *Acinetobacter* sp. OC4 (gene bank accession number AB221118) was amplified by PCR using the following primers: *aciB*-F, 5'-GGAATTCCCATATGGGCCAAATTACATTTATTG-3' (*Nde*I site is underlined) and *aciB*-R, 5'-CGCTCGAGTTACATCTGAAACTCAGGCAGA-3' (*Xho*I site is underlined). The expression vector pNami2vdh was used as the template.

Transformed *E. coli* BL21-CodonPlus(DE3)-RIL cells were grown at 37 °C in 100 ml LB medium supplemented with 20 µg/ml kanamycin and 34 µg/ml chloramphenicol.

This culture was then inoculated at a ratio of 100 ml overnight cultures into 900 ml LB medium. Expression was induced by the addition of 0.1 mM IPTG (isopropyl-β-D-thiogalactoside), and the culture was incubated overnight at 25 °C.

The cells were harvested from the culture by centrifugation at 3,000 × *g* for 10 min. The cell pellet was resuspended in Buffer A (50 mM Tris-HCl, 100 mM NaCl, and 1 mM DTT; pH 7.5) containing 1 mg/ml lysozyme (Sigma-Aldrich Co., MO, USA) and about 50 U/ml Benzonase (Merck & Co. Inc., NJ, USA). This cell suspension was incubated on ice for 30 min and sonicated for a few minutes. After centrifugation at 27,200 × *g* for 20min, the supernatant was collected and precipitated with polyethylene glycol 6,000 (Sigma-Aldrich Co.) in a final concentration of 5%. After excluding the insoluble proteins by centrifugation at 27,200 × *g* for 20 min, the supernatant was loaded onto a DEAE Sephacel Fast Flow Column

(GE Healthcare UK Ltd., UK) equilibrated with Buffer A. Bound proteins were eluted using a linear gradient of 0.1–0.6 M NaCl in Buffer B (50 mM Tris-HCl, 100 mM NaCl, and 10% glycerol; pH 7.5). The brown-colored fractions were collected and concentrated on a 10-kDa Amicon Ultra-15 (Millipore, MA, USA) at 4 °C.

The concentrated fractions were loaded onto a Sephacel S-100 High Resolution column (2.5 × 90 cm; GE Healthcare UK Ltd.) equilibrated with Buffer B. The brown-colored fractions were pooled and loaded onto a Q sepharose Fast Flow column (GE Healthcare UK Ltd.) equilibrated with Buffer B. Bound proteins were eluted using a linear gradient of 0.1–0.6 M NaCl in Buffer B. The purified fractions were pooled and dialyzed against a 25 mM Tris-HCl and 20% glycerol (pH 7.5) and stored at -20 °C.

Ferredoxin concentrations were estimated as described previously with minor modifications [3]. The sample in 100 mM Tris-HCl (pH 8.5) and 2% SDS was incubated for 15 min at 60 °C for denaturation, and further incubated with 0.2 mM BPS (bathophenanthroline disulfonate), and 8 mM dithionite (Sigma-Aldrich Co.) for 30 min at 30 °C. The iron content of ferredoxin was determined by the Fe^{2+} -(BPS) complex, which has a specific absorbance peak at 535 nm with $\epsilon_{\text{BPS}} = 25,100$.

The [2Fe-2S] cluster stability assays of 10 μM ThcC and AciB were performed by monitoring decreases in absorption at 412 nm using a JASCO V-630 biospectrophotometer with 1-cm path length quartz cells.

The samples were incubated at 28 °C in 50 mM potassium phosphate (pH 7.4). The [2Fe-2S] cluster preservation was calculated as the ratio of absorption at 412 nm measured at a given time to that measured at the beginning of experiment. The drop in absorption at 412 nm indicates the dissociation of [2Fe-2S] cluster.

Transport of cyclodextrin to the cytoplasm of nisin-treated cells

To determine the influence of nisin on the permeability of cyclodextrin (CD) to the

cytoplasm of *R. erythropolis* cells, cells were harvested from overnight cultures, washed twice with sterilized water, and resuspended in 50 mM potassium phosphate buffer (pH 7.4) to an OD₆₀₀ of 5.0. Resuspended cells (25 mL) were incubated with different concentrations of nisin (0, 0.025, 0.05, 0.1, 0.25, 0.5, or 1.0 mg/ml; Sigma-Aldrich Co., MO, USA), at 28 °C for 1 h under constant agitation. The mixture was then centrifuged at 1,870 × *g* for 10 min at 4 °C, and the cell pellet was resuspended in 900 μl of 50 mM potassium phosphate buffer (pH 7.4). Green chemiluminescence CD (GCCD) (Wako Pure Chemical Industries Ltd., Osaka, Japan)[4] dissolved in solution (50% methanol and 0.05% trifluoroacetic acid (TFA)) was added into the cell suspension at a final concentration of 1 × 10⁻⁵ mol/l. The reaction mixture was incubated at 28 °C for 3 h under constant agitation at 120 rpm. The mixture was then centrifuged at 6,800 × *g* for 15 min at 4 °C, and the pellet was washed with sterilized water, resuspended in 900 μl of 50 mM potassium phosphate buffer (pH 7.4), and disrupted for 20 min with a Multi Beads Shocker equipped with a cooling circulator set at 4 °C (Yasui Kikai Co., Osaka, Japan). The resultant lysate was centrifuged at 20,800 × *g* for 20 min at 4°C. The GCCD levels in 200 μl crude extract was measured by a luminometer (ATTO AB2350EX Luminescencer Phelios, ATTO Co., Tokyo, Japan) after adding 10 μl of 0.37 U/ml hypoxanthine and 10 μl of 0.3 mM xanthine oxidase. The luminescence intensity (expressed in count) was measured for 10 s with a 10 s delay after the addition of hypoxanthine.

Vdh expression and Vdh redox partner co-expression in Rhodococcus erythropolis cells

Rhodococcus erythropolis JCM3201 transformed with pTipQC-vdh, pTipQC-vdh-thcCD, or pTipQC-vdh-aciBC was grown at 28 °C in LB broth supplemented with 17 μg/ml chloramphenicol. The expressions of Vdh and its redox partner proteins were induced by the addition of 0.5 μg/ml thiostrepton when the cell growth reached the mid-log phase. At 24 h after induction, SDS-PAGE was used to determine the expression levels of

Vdh and its redox partners. The cell pellets were washed twice with Buffer C (50 mM sodium phosphate and 300 mM NaCl; pH 8.0), resuspended in Buffer C to 2× concentrated cell suspension and disrupted for 20 min using a Multi Beads Shocker equipped with a cooling circulator set at 4 °C. The resultant lysates were centrifuged at 20,800 × *g* for 20 min at 4 °C. Fifteen micrograms of the protein from each clone was loaded onto a 12.5% SDS-PAGE gel, followed by staining with Coomassie brilliant blue G-250. The concentration of Vdh was determined by reduced-CO difference spectral analysis [5].

Expression and purification of the recombinant GlcDH-IV

To express recombinant GlcDH-IV with N-terminal His tags, the corresponding gene (gene bank accession number D10626) was cloned into the *Nde*I and *Xho*I sites of pET28a (Novagen) [6].

Bacillus megaterium IAM1030 has 4GlcDH isozymes (I–IV). The nucleotide sequences of *gdhIII* and *gdhIV* are almost identical. Thus, the upstream and downstream regions of *gdhIV* were initially amplified by PCR using *Pfu* Turbo DNA polymerase with the following primers: *gdhIV*-UP, 5'-GAAAAGAAAACAAAGCGTCAGCTTATTTTC-3' and *gdhIV*-D, 5'-TGGCATTTCGCTTAGGTATAGAAAAAAG-3'. The *B. megaterium* IAM1030 genome DNA was used as the template. Finally, *gdhIV* was amplified by PCR using *Pfu* Turbo DNA polymerase with the following primers: glcDH-IV-F, 5'-GGAATTCCATATGTATACAGATTTAAAAGATAAAGTAG-3' (*Nde*I site is underlined) and glcDH-IV-R, 5'-CGCTCGAGTTAGCCTCTTCCTGCTTGGAAAGAA-3' (*Xho*I site is underlined). The amplified fragment described earlier was used as the template. Transformed *E.coli* BL21-CodonPlus(DE3)-RIL cells were grown at 37 °C in 100 ml LB medium supplemented with 20 µg/ml kanamycin and 34 µg/ml chloramphenicol. This culture was then inoculated at a ratio of 100 ml overnight cultures to 900 ml LB medium. The expression was induced by the addition of 0.4 mM IPTG, and the culture was incubated overnight at 30 °C.

The cells were harvested from the culture by centrifugation at $3,000 \times g$ for 10 min. The cell pellet was resuspended in Buffer C containing 1 mg/ml lysozyme and about 50 U/ml benzonase. The cell suspension was incubated on ice for 30 min and sonicated for a few minutes. After centrifugation at $27,200 \times g$ for 20 min, the supernatant was loaded onto a Ni-affinity column (HIS-SELECT resin; Sigma-Aldrich Co.) equilibrated with Buffer C. Bound proteins were washed with Buffer D (50 mM sodium phosphate, 300 mM NaCl, and 10% glycerol; pH 6.0) and eluted using a linear gradient of 0–0.4 M imidazole in Buffer D. Enzymatic activity was assayed as described previously [7].

The purified fractions were pooled and dialyzed against a 25 mM Tris-HCl and 20% glycerol (pH 7.5), and stored at $-20\text{ }^{\circ}\text{C}$.

HPLC analysis of vitamin D₃ metabolites produced in recombinant Rhodococcus erythropolis cells

The vitamin D₃ metabolites were determined on the basis of the bioconversion rate of inactive vitamin D₃ into its hydroxylated active form as described previously [1,8]. In brief, 24 h after the addition of thiostrepton, the cell pellet was washed twice with 50 mM potassium phosphate buffer (pH 7.4), resuspended in 50 mM potassium phosphate buffer (pH 7.4) containing 0.5 mM vitamin D₃ (Sigma-Aldrich Co.) as a substrate, 0.5% methyl- β -cyclodextrin (PMCD; Junsei Chemical Co. Ltd., Tokyo, Japan), 10 % glycerol, 1 mM thiamine, nisin (0, 0.25, 0.5, 0.75, or 1.0 mg/ml), and the reconstitution system of NADH (2% glucose, 2 U/ml GlcDH-IV, and 4 mM NADH)(OD₆₀₀ = 5.0). The bioconversion was performed by incubating the reaction mixture at 28 °C for 8, 16, or 24 h. The mixture was then centrifuged at $20,800 \times g$ for 10 min at 4 °C, and 100 μl from the supernatant was mixed with 900 μl methanol and centrifuged again for 10 min at the same speed and temperature. The methanol solution was analyzed by HPLC equipped with a J'sphere ODS-H80 (75 mm \times 4.6 mm internal diameter; YMC Co., Ltd., Kyoto, Japan) controlled at a column temperature of

40 °C. Samples were resolved on the column using a linear gradient of 70% to 85% methanol for 10.1 min, 85% to 100% methanol for 0.3 min, 100% methanol for 7.2 min, 100% to 70% methanol for 0.8 min, and 70% methanol for 1.6 min at a flow rate of 1.0 ml/min. The metabolites were detected by UV-monitoring at 265 nm. 25(OH)VD₃ and VD₃ had retention times of 13.5 min and 17.8 min, respectively.

Repeated VD₃ bioconversion using recombinant Rhodococcus erythropolis cells

Two cell strains (JCM3201/pTipQC-vdh-thcCD and JCM3201/pTipQC-vdh-aciBC) co-expressing Vdh and its redox partner proteins, ThcC and ThcD or AciB and AciC, were constructed. To determine of the effects of the reuse of nisin-treated cells for vitamin D₃ conversion, 24 h after the addition of thiostrepton, the cell pellet was washed twice with 50 mM potassium phosphate buffer (pH 7.4) and was resuspended in 50 mM potassium phosphate buffer (pH 7.4) containing 2% glucose, 10% glycerol, 0.5% methyl-β-cyclodextrin, 1 mM thiamine, 1.0 mM vitamin D₃ as a substrate, 0 or 0.5 mg/ml nisin, 2 U/ml GlcDH-IV, and 4 mM NADH to obtain an OD₆₀₀ of 5.0. The bioconversion was performed by incubating the reaction solution at 28 °C for 16 h with agitation. The reaction solution (without nisin) was refreshed every 16 h, and 4 consecutive reactions (about 64 h in total) were performed. The mixture was then centrifuged at 20,800 × *g* for 10 min at 4 °C, and 100 μl from the supernatant was mixed with 900 μl methanol and centrifuged again for 10 min at the same speed and temperature. These respective methanol solutions were analyzed by HPLC.

Assay for determination of protein concentration

Protein concentrations of crude extract and recombinant GlcDH-IV were determined using the Bradford assay with bovine serum albumin (BSA) as the standard [9].

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