Characterization of the ModABC Molybdate Transport System of Pseudomonas putida in Nicotine Degradation

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Pseudomonas putida J5 is an efficient nicotine-degrading bacterial strain that catabolizes nicotine through the pyrrolidine pathway. In our previous study, we used Tn5 transposon mutagenesis to investigate nicotine metabolism-associated genes, and 18 nicotine degradation-deficient mutants were isolated from 16,324 Tn5-transformants. Three of the mutants were Tn5 inserts into the modABC gene cluster that encoded an ABC-type, high-affinity, molybdate transporter. In-frame deletion of the modABC genes abolished the nicotine-degrading ability of strain J5, and complementation with modABC either from P. putida or Arthrobacter oxidans restored the degrading activity of the mutant to wild-type level. Nicotine degradation of J5 was inhibited markedly by addition of tungstate, a specific antagonist of molybdate. Molybdate at a non-physiologically high concentration (100 µM) fully restored nicotine-degrading activity and recovered growth of the modABC mutant in a nicotine minimal medium. Transcriptional analysis revealed that the expression of modABC was up-regulated at low molybdate concentrations and down-regulated at high molybdate concentrations, which indicated that at least one other system was able to transport molybdate, but with lower affinity. These results suggested that the molybdate transport system was essential to nicotine metabolism in P. putida J5.

Keywords: biodegradation, nicotine, Pseudomonas putida, molybdate transporter, ModABC

INTRODUCTION

Molybdenum (Mo) plays essential roles in bacteria, because it serves as a cofactor for a number of enzymes that catalyze a variety of oxidation/reduction reactions, and it is involved in microbial metabolism of carbon, nitrogen, and sulfur (Hille, 1996; Kisker et al., 1997). For the synthesis of molybdoenzymes, bacteria need to transport molybdate, activate it to an appropriate form, and incorporate it into the organic part of the molybdenum cofactor (Zhang et al., 2011). In nature,
the predominant form of Mo is molybdate oxyanion, which is transported by an ABC-type transport system (Self et al., 2001). In *Escherichia coli*, the first identified Mo transporter was the high affinity ModABC transport system, in which ModA was responsible for molybdate binding, ModB was the transmembrane component of the permease, and ModC provided the energizer function on the cytoplasmic side of the membrane (Maupin-Furlow et al., 1995). Based on genome sequences, *mod* homologs can be identified in ≥50 bacteria (Leimkuhler and Iobbi-Nival, 2015). Among these bacteria, *mod* genes have been functionally characterized in only a few species, which include *Rhodobacter capsulatus* (Wang et al., 1993), *Azotobacter vinelandii* (Luque et al., 1993), *Staphylococcus carnosus* (Neubauer et al., 1999), and *Bradyrhizobium japonicum* (Delgado et al., 2006). In *B. japonicum* USDA110, a nitrogen-fixing, root-nodule symbiont of soybean, *modA* and *modB* mutant strains were unable to grow with nitrate as a nitrogen source or as a respiratory substrate, and they lacked nitrate reductase activity (Delgado et al., 2006). Partially purified quinoline dehydrogenase from *P. putida* Chin IK indicated the presence of favin and molybdenum-binding pterin, and the enzyme activity was wholly dependent on the availability of molybdate in the growth medium (Blaschke et al., 1991).

Mo uptake occurred during nicotine biodegradation by gram-positive bacteria (Freudenberg et al., 1988; Menendez et al., 1997). The nicotine utilization of *Arthrobacter oxidans* P-34 (DSM419) required a supplementation with molybdate to the growth medium, and nicotine dehydrogenase (NDH) was identified as a molybdo-iron-sulfur-flavoprotein (Freudenberg et al., 1988). The nicotine-degrading plasmid pAO1 of *A. nicotinovorans* was sequenced and showed a remarkable gene cluster for biosynthesis of the molybdenum cytosine dinucleotide (MCD) cofactor and molybdate transporter system that were involved in nicotine degradation (Ganas et al., 2008). Despite the importance of Mo in nicotine metabolism by gram-positive bacteria, there has been very little work on the mechanisms involved in uptake of molybdate in nicotine metabolism by gram-negative bacteria. A recent report showed that conversion of 3-succinoylpyridine to 6-hydroxy-3-succinoylpyridine during the degradation of nicotine by *P. putida* S16 was catalyzed by a multi-enzyme reaction that consisted of a molybdopterin binding oxidase (SpmA), molybdopterin dehydrogenase (SpmB), and a (2Fe-2S)-binding ferredoxin (SpmC) with molybdenum molybdopterin cytosine dinucleotide as a cofactor (Tang et al., 2013). This result led us to presume that Mo uptake was also involved in nicotine metabolism by gram-negative bacteria.

*Pseudomonas putida* J5 is an efficient nicotine-degrading strain isolated from the tobacco rhizosphere which could catabolize 3 g/l nicotine in 24 h (Wei et al., 2008). In our most recent study on nicotine-degrading associated genes in *P. putida* J5, we generated a Tn5 transposon mutant library with 16,324 transformants and screened 18 nicotinedegrading deficient mutants in which three mutants were mutated directly by Tn5 in the *modABC* transport gene cluster (Xia et al., 2015). In this paper, we cloned the entire gene cluster that encodes the molybdate transport system from strain J5, in-frame deleted the *modABC* genes, and characterized the function of the ModABC system in nicotine metabolism.

**MATERIALS AND METHODS**

**Strains, Plasmids, and Growth Conditions**

Characteristics of strains and plasmids are listed in Table 1. *Pseudomonas putida* was grown in Luria-Bertani (LB) medium or NI medium at 28°C (Wei et al., 2009; Xia et al., 2015), and *Escherichia coli* was grown at 37°C in LB medium. For plasmid propagation and selection of transformants, media were supplemented with antibiotics at appropriate concentrations as follows: 100 µg/ml ampicillin, 50 µg/ml kanamycin, and 20 µg/ml tetracycline.

**DNA Manipulations and Sequencing**

Chromosomal and plasmid DNA isolations, restriction enzyme digestions, agarose gel electrophoresis, ligations, and *E. coli* transformations were performed according to standard protocols (Sambrook and Russell, 1998). Nucleotide sequencing was performed by Invitrogen Co., Ltd., China.

**Southern Hybridization and Cloning of the Tn5 Insertion Sites**

To determine the copy number of the Tn5 transposon in mutant strains, total DNA was digested with EcoRI, *Pst*I, and *BamHI* separated by electrophoresis on 0.8% (w/v) agarose gel and transferred onto nylon membranes (Hybond-N+; Amersham, GE Healthcare, Piscataway, NJ, United States). A digoxigenin-labeled, kanamycin-resistant, gene probe (Xia et al., 2015) was used to do hybridization and detection according to the protocol for the DIG High Primer DNA Labeling and Detection Starter Kit I (Roche).

Shotgun cloning was performed to determine the transposon insertion site. Chromosomal DNA samples were restricted with *PstI* and *EcoRI* and ligated into pBluescript II SK. *E. coli* DH5α transformants were selected on LB medium that contained kanamycin. Positive clones were sequenced with Tn5-39 and Tn5-1571 (Xia et al., 2015) to allow determination of the precise location of a transposon insertion. Sequences were then compared to the protein sequence database (GenBank) using the BlastX algorithm (Altschul et al., 1990). For each mutant, the joins between the transposon sequences were identified.

**Construction of a modABC in-Frame Deletion Mutant**

To create a *modABC* gene deletion allele, two fragments that flank *modABC* genes were generated by endonuclease digestion. A 2.4 kb fragment was created by digestion with *KpnI* and *SalI* from pBS-M728, and another 1.6 kb
TABLE 1 | Bacterial strains and plasmids used in this study.

| Strain or plasmid       | Genotype or relative phenotype                      | Source                 |
|-------------------------|-----------------------------------------------------|------------------------|
| Escherichia coli        |                                                     |                        |
| DH5α                    | F- recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1Δ (argF-lacZYA)169Φ80lacZ ΔM15 | Sambrook and Russell, 1998 |
| Pseudomonas putida      |                                                     |                        |
| J5                      | Apr; wild type                                      | Wei et al., 2009       |
| M430                    | Nicotine-degrading mutant inserted with Tn5         | This study             |
| M728                    | Nicotine-degrading mutant inserted with Tn5         | This study             |
| M9502                   | Nicotine-degrading mutant inserted with Tn5         | This study             |
| ΔmodABC                 | modABC deletion mutant                              | This study             |
| Arthrobacter oxidans    |                                                     |                        |
| J4                      | Nicotine-degrading bacterium                        | Lab collection         |
| Plasmids                |                                                     |                        |
| pBluescript II SK+      | ColE1 origin; Ap<sup>+</sup>                        | Stratagene             |
| pHSG299                 | ColE1 origin; Km<sup>+</sup>                        | Takara                 |
| pRK2013                 | ColE1 replicon with RK2 transfer region, helper plasmid; Km<sup>+</sup> | Figurski and Helsinki, 1979 |
| pRG970Km                | Cloning vector containing promoterless lacZYA for construction of transcriptional fusions; Km<sup>+</sup> | Yan et al., 2009 |
| pME6032                 | Shuttle vector; Tc<sup>+</sup>                      | Heeb et al., 2000      |
| pBBR1MCS-2              | Shuttle vector; Km<sup>+</sup>                      | Kovach et al., 1995    |
| pHSG299Δmod             | pHSG299ΔmodABC                                      | This study             |
| p6032-modABC-J5        | pME6032 with full length of modABC genes from J5   | This study             |
| p6032-modABC-J4        | pME6032 with full length of modABC genes from J4   | This study             |
| pMCS2-modABC-J5        | pBBR1MCS-2 with full length of modABC genes from J5 | This study             |
| pMCS2-modABC-J4        | pBBR1MCS-2 with full length of modABC genes from J4 | This study             |
| p970-PmodABC           | pRG970Km with a 580 bp fragment containing the promoter of modABC genes | This study             |

fragment was generated by digestion with XhoI and EcoRI from pBS-M9502. After being digested with relevant restriction enzymes, the two fragments were inserted into pBluescript to create pBSΔmod. An approximately 4.0 kb long KpnI-EcoRI fragment, which included the modABC genes with a 1.8 kb deletion, was lifted and ligated into pHSG299. The last suicide plasmid pHSG299Δmod was introduced into P. putida J5 by electroporation, and this was used in a two-step strategy to introduce the shortened modABC locus into the chromosome. Primers P3210 (5′-CACAGTGCGCGCGGTTTGGTGGGAAG-3′) and P5864 (5′-AGTTGAGATCCCGGGAATTCTGCTTG-3′) were used to confirm a double crossover event.

**Genetic Complementation of the modABC Mutant**

To complement the mod mutant, a 2.6 kb fragment that contained the putative upstream promoter and coding region of the modABC genes was amplified from P. putida J5 with primers P3210 and P5864. Primers M4J-F (5′-CTCTCTCTACTCGTCCCGC-3′) and M4J-R (5′-CTCTCTGACGGTGTCCCGC-3′) were also used to amplify the modABC gene from Arthrobacter oxidans J4 to complement the mod mutant. The amplified intact modABC genes were inserted into the shuttle vector pME6032 or pBRR1MCS2, and the resulting plasmids were introduced into strain J5Δmod by triparental mating (Wei and Zhang, 2006).

**Transcription Analysis of modABC Genes**

For determination of promoter activity, the promoter region that included the upstream fragment of modABC was amplified by PCR using primers P2925 (5′-GGTGTGGGTACCAATACGGA-3′) and P3505 (5′-CCATTCTTGGATCCCGGCA-3′), and a 580 bp BamHI fragment was cloned ahead of a promoterless lacZ in p970Km (Yan et al., 2009), which is a derivative plasmid of pRG970 (Van den Eede et al., 1992). The resulting plasmid, p970-PmodABC, was introduced into strain J5 and used to examine the activity of the modABC promoter. ß-galactosidase measurement was performed at 6 hpi according to the standard protocol.

A further RT-qPCR assay was conducted to check the expression of modABC. Fresh midexponential cells from a single colony of strain J5 were harvested using 12,000 rpm centrifugation. Total RNA was extracted from the pellets using RNAprep pure cell/bacteria kit (Tiangen), treated with DNase, and reverse transcribed to cDNA using random hexamer primers and SuperScript III reverse transcriptase (Invitrogen). Transcriptional expression of modA was determined using the CFX Connect™ Real-Time System (Bio-Rad) with Luna®Universal qPCR Master Mix (New England Biolabs). Transcript levels for modA were calculated relative to the level for the housekeeping gene gyrA.

**Detection of Nicotine Concentration**

P. putida J5 and its derivatives were cultured to stationary phase in LB medium, and 1 ml of the cell suspension was spun down, inoculated into 100 ml of NI media that contained 1.0/l nicotine, and incubated at 30°C. One mM sodium tungstate and 1, 10, 100 µM, and 1 mM sodium molybdate were added as needed. To determine cell density, the absorbance (600 nm) of 3 ml of culture was determined with a spectrophotometer at 2 h intervals. The cell suspensions were then centrifuged, and the nicotine concentration of the supernatant was determined by high-pressure liquid chromatography (HPLC) (Wei et al., 2009).
RESULTS

Isolation and Characterization of Nicotine-Degrading, Deficient Mutants M430, M728, and M9502

Previous analysis of the mutant strains M430, M728, and M9502 revealed that Tn5 was inserted into the homologs of modB and modC genes (Xia et al., 2015). Because the Tn5 insertion sites of M430 and M728 were very close to the modB gene, we only determined the degradation efficiency of mutants M728 and M9502, conducted Southern hybridization analysis, and subsequently cloned a larger fragment that covered the Tn5 transposon.

Under optimal conditions, wild type P. putida strain J5 thoroughly degraded 1.0 g/l nicotine in 12 h (Wei et al., 2009), but the Tn5 mutants, M728 and M9502, completely failed to degrade nicotine (Figure 1). The hybridization patterns of the two mutants were different (Figure 2A), which indicated that the mutants were not identical. Genomic sequences that flanked the insertion sites of M728 and M9502 were cloned by the shot-gun strategy with PstI and EcoRI, based on the results of Southern blotting. The resulting plasmids, pBSM728-P and pBSM9502-E, contained about 6.3 and 3.6 kb foreign fragments, respectively, which included 1.6 kb of the mini-Tn5 sequence. After assembling the two fragments and discarding the Tn5 sequence, a 7.0 kb fragment was obtained (Figure 2B).

modABC Genes

In the nucleotide sequence of the 7.0 kb fragment that was "rescued" from M728 and M9502, three open reading frames (ORFs) were found on one strand (Figure 2B). The genomic organization of the three ORFs suggested a transcriptional coupling. Potential Shine-Dalgarno sequences were identified at appropriate distances in front of the predicted start codons. These ORFs encoded 252 (26.7 kDa), 229 (24.2 kDa), and 363 (39.4 kDa) amino acid residues, which exhibited 88, 93, and 86% identities with ModA, ModB, and ModC in P. putida S16, respectively, which is a nicotine-degrading bacterium that has had the entire genome sequenced (Yu et al., 2011; Tang et al., 2013). Sequence analysis revealed accurately that the Tn5 cassettes of M728 and M9502 were inserted at the sites of 219 bp of modB and 36 bp of modC. The deduced N-terminus of ModA showed the typical features of lipoproteins (Sutcliffe and Russell, 1995). It resembled the molybdate-specific, periplasmic binding proteins. ModB of strain J5 was similar to the integral-membrane, channel-forming proteins of molybdate-specific, ABC transporters. ModC was predicted to be a cytosolic protein with typical consensus sequences for nucleotide binding. Additional studies focused on the function of ModABC in Mo uptake and nicotine biodegradation.

Effect of modABC Mutation on Nicotine-Degrading Activity

To assess the function of the modABC genes with respect to the nicotine-degrading activity of P. putida J5, the modABC genes were also mutated by in-frame deletions in addition to
the mod::Tn5 mutation (Figure 2B). The deletion-mutant was confirmed by PCR with primers P3210 and P5864. A 2.6 kb fragment was amplified from wild type strain J5, but only a 0.8 kb fragment was amplified from strain J5Δmod, which indicated that a sequence of the modABC gene that contained approximately 1.8 kp had been deleted from strain J5 as designed.

In contrast to wild type P. putida J5, cells of the modABC mutant strain were unable to grow in a minimal medium with nicotine as the sole source of carbon and nitrogen (Figure 3B) but had a similar growth rate to the wild in the medium with glucose (Supplementary Figure S1). After incubating the cells under these conditions, almost no nicotine-degrading activity in cells of the modABC mutant was detected compared with those in cells of strain P. putida J5 (Figure 3A). Complementation of P. putida J5Δmod with pME6032 that contained wild-type modABC genes of strain J5 restored both nicotine-degrading activity and the ability of the cells to grow in minimal medium with nicotine (Figures 3A,B). Similar results were obtained from complementation of Δmod with modABC genes of A. oxidans J4 (Figures 3A,B), which is an efficient nicotine-degrading, gram-positive bacterium that was isolated from tobacco rhizospheres. These results indicated that the molybdate transport system was involved in nicotine metabolism in P. putida J5.

**Effect of Molybdate on Nicotine Metabolism by P. putida J5 and the modABC Mutant**

We tested to determine whether the nicotine- and molybdate-degrading activity of wild type P. putida J5 was dependent on molybdate. Therefore, we employed tungstate, the specific antagonist of molybdate (Higgins et al., 1956; Nagel and Andreesen, 1989), to remove the molybdenum trace from the medium. We firstly determined if tungstate had impact on bacterial growth and nicotine metabolism. As expected, no difference was found on growth and metabolism when supplying 1 mM tungstate in LB liquid media with 1 g/l nicotine (Supplementary Figure S2). If the nicotine minimal growth medium was supplemented with tungstate (1 mM), no growth or nicotine consumption was found. These results seemed to be specific for nicotine, because no inhibition of growth and nicotine-degrading ability on succinate as a carbon source was detected after adding the same concentration of tungstate (Figure 4). Taken together, the results indicated that molybdate was a key element required by P. putida to degrade nicotine.

Although we already proved ModABC constituted a molybdate transport system and played an important role in nicotine biodegradation in P. putida J5, we were not sure if increased concentrations of molybdate complemented the mutation of modABC mutants. Therefore, P. putida J5Δmod was grown in minimal medium supplemented with nicotine and with different concentrations of molybdate. In the presence of molybdate, J5Δmod revealed divergent kinetics of nicotine reduction and growth rate (Figure 5). The nicotine-degrading activity of the P. putida J5Δmod was not influenced by supplementation with a low concentration of molybdate (1 µM).

However, as the molybdate supplementation was increased, P. putida J5Δmod restored nicotine-degrading activity and recovered growth in the nicotine medium. At a concentration of 100 µM, molybdate had the highest efficiency in supporting the growth and nicotine-degrading activity of the modABC mutant, while a higher concentration of molybdate up to 1 mM repressed uptake of Mo and resulted in a decreased rate of growth and nicotine-degrading activity. The results clearly indicated that at least one other system was able to transport molybdate, but with lower affinity.

**Transcription of modABC Is Molybdate-Dependent**

To investigate whether the expression of modABC genes was associated with molybdate, the effect of molybdate on the expression of modABC was first measured using a PmodABC::lacZ transcriptional fusion in the plasmid p970Km. Before doing this assay, we firstly tested the
growth of the wild type strain J5 supplied with selected titers of molybdate. No significant differences of the growth rates were found between the treatments (data not shown), which dismissed the possibility of growth affecting gene expression. The results revealed that modABC transcription under low-molybdate conditions was significantly enhanced compared with that under high-molybdate conditions, and 10 µM of molybdate induced the highest expression of modABC. After the molybdate concentration was increased to 100 µM, expression of the modABC genes were completely repressed (Figure 6A). We also employed RT-qPCR to double-check the expression of modA at different molybdate concentrations. The results were consistent with those in the promoter assay. The only exception was that modABC was induced moderately at higher molybdate concentrations (Figure 6B), which suggested that qPCR was more sensitive than transcriptional fusion. The expression of modABC was induced by the depletion of molybdate in P. putida J5, and the modABC molybdate transport system was a high affinity system.

DISCUSSION

Pseudomonas putida J5 is an efficient nicotine-degrading bacterium, which has been used to degrade employed in nicotine-contained tobacco waste (Wei et al., 2008). The molecular mechanisms of nicotine degradation by P. putida J5 was determined extensively by a genome-wide Tn5 mutagenesis strategy (Xia et al., 2015). In this study, we report on the physiological and genetic analysis of the three Tn5 mutants defective in modABC that encoded the molybdenum transport system. Both the random and site-directed mutants failed to degrade nicotine in minimal medium with nicotine as the sole source of carbon and nitrogen. Complementation of the mutants with native modABC genes or the homologs from well-studied A. oxidans fully restored nicotine-degrading activity. The effect of molybdate was observed for growth and utilization of the substrate of nicotine, as evidenced by the strong inhibition of tungstate, which was not observed for succinate, a control substrate reported before. The same phenomenon was also reported for degradation of quinolone and 2-furoic acid by Pseudomonas strains (Koenig and Andreesen, 1989; Blaschke et al., 1991). Overall, it seems likely that degradation by N-heterocyclic compounds generally involves a molybdenum-dependent reaction.

The trace element molybdenum is an important catalytic component of many enzymes involved in microbial metabolism of nitrogen, sulfur, and carbon. Enzymes that contain Mo at their active sites catalyze oxo-transfer reactions (Zhang et al., 2011). A variety of molybdoenzymes, such as xanthine oxidase,
catalyze oxidative hydroxylation of a wide range of aldehydes and aromatic heterocycles (Stephan et al., 1996; Parschat et al., 2003; Wagener et al., 2009; Yokoyama and Leimkuhler, 2015). In the best-elicited metabolic pathway of nicotine degradation by A. nicotinovorans, the heterotrimeric molybdenum enzyme NDH catalyzed the first nicotine-degrading reaction, which was hydroxylation of the pyridine ring to 6-hydroxynicotine (Brandsch, 2006; Fitzpatrick, 2018). But unfortunately, the nicotine oxidase NdaA from P. putida J5 and its homologs Nox from Pseudomonas sp. HZN6 and NicA2 from P. putida S16 have not shown such characters to known molybdoenzymes (Qiu et al., 2013; Tang et al., 2013). However, the key step in nicotine metabolism in the conversion of 3-succinylpyridine to 6-hydroxy-3-succinylpyridine by P. putida S16 was catalyzed by a multi-enzyme reaction that consisted of a molybdopeterin-binding oxidase (SpmA), molybdopterin dehydrogenase (SpmB), and a (2Fe-2S)-binding ferredoxin (spmC) with molybdenum molybdopterin cytosine dinucleotide as a cofactor (Tang et al., 2013). SmABC proteins showed significant sequence identities to the subunits of the xanthine dehydrogenase family, such as NDH, a molybdoenzyme of A. nicotinovorans (Brandsch, 2006; Tang et al., 2013). The SmABC homologs were also found from the draft genome of strain J5, which locates downstream of NadD, a homolog of Sapd in strain S16 (Tang et al., 2013). These reports support our conclusion of molybdenum-dependent nicotine degradation by Pseudomonas strains, although the SmABC products await further purification and characterization.

It is very interesting to find that the ModABC transport system was repressed by a high concentration of molybdate, but 100 µM molybdate restored the growth and nicotine-degrading activity of the modABC mutant. In Staphylococcus carnosus, the mutant of modABC reduced nitrates very slowly in the absence of molybdate and efficiently in the presence of added molybdate (100 µM) (Neubauer et al., 1999). In E. coli, the molybdate transport system was repressed by increasing the molybdate concentration in the medium, and the modABC mutant utilized other transport systems with a lower affinity for molybdate (Rosentel et al., 1995). Molybdate can be taken up by the sulfate-transport system in the absence of a functional, high-affinity, molybdate-transport system in E. coli and B. japonicum (Rosentel et al., 1995; Delgado et al., 2006).

Our results, together with the similarity to previous reports, clearly indicated an involvement of the ModABC of P. putida J5 in molybdate transport. This is the first research to report that molybdenum and the ModABC transport system were involved in nicotine degradation by P. putida, but further research needs to be done to elucidate the other nicotine-degrading related ModABC transport systems.

AUTHOR CONTRIBUTIONS

H-LW and ZX designed the research. ZX, LL, and H-YZ performed the research. H-YZ and H-LW analyzed the data. H-LW wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.03030/full#supplementary-material
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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