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Characterization of Aerobic Denitrification Mechanism in *Klebsiella pneumoniae* Strain F-5-2 That Can Utilize Ammonium and Nitrate Ions Simultaneously and Application of Nitrifying and Denitrifying Processes to Waste Water Treatment

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Research aims

As countermeasures against eutrophication in lakes, wetlands, rivers, and enclosed areas of a sea, the removal of nitrogen compounds (NH_4^+ and NO_3^-) from industrial and domestic waste waters has become an urgent and crucial subject. In the microbial removal of NH_4^+ and NO_3^-, two processes have been thought to participate: oxidation of NH_4^+ to NO_3^- by nitrifying autotrophic bacteria under aerobic conditions and denitrification of nitrate by denitrifying bacteria under anaerobic conditions. We isolated a heterotrophic bacterium, *Klebsiella pneumoniae* F-5-2, which can utilize both NH_4^+ and NO_3^- simultaneously. This strain removed 4 mg/ml (1,400 ppm as N) of NH_4NO_3 completely in 48 h of aerobic cultivation with controlled Fe^{2+} and MoO_4^{2-} concentrations. GC-MS analysis indicated that the N_2 gases were derived from the nitrogen atoms of both NH_4^+ and NO_3^-. For these reasons, we concluded that *K. pneumoniae* F-5-2 has pathways for heterotrophic nitrification and aerobic denitrification. Our aim is to reveal the mechanism of nitrification and denitrification in this strain under aerobic condition and to investigate the effects of factors on the removal of NH_4^+ and NO_3^- simultaneously for application.

Methods

1. Functional analysis of the genes involved in the nitrification and denitrification steps.

On the basis of conserved region of previous reported nitrate reductases, the primers were synthesized. The amplified DNA fragment was used as a colony hybridization probe. The gene library of strain F-5-2 was constructed and screened by colony hybridization using the labeled probe.

2. Effects of various factors on the removal of NH_4^+ and NO_3^-.

i) Growth condition: Strain F-5-2 was cultured in the basal medium containing 0.1% (w/v) NH_4NO_3.

ii) Analytical tests: NH_4^+, NO_2^-, and NO_3^- were measured by a modification of the indophenol method, the diazo-coupling reaction, and nitration of salicylic acid, respectively.

iii) Effect of phosphate concentration on the removal of NH_4^+ and NO_3^-: Media containing 0 to 128 mM sodium-potassium phosphate were prepared. The cultures were taken every 12 hours. Growth and concentrations of N-compounds were determined.

iv) Effect of carbon sources on the removal of NH_4^+ and NO_3^-: Test medium was a modified NH_4NO_3 medium containing either sugar (8 kinds), organic acid (4 kinds), amino acid (7 kinds), or nutrient broth (4 kinds). The cultures were taken every 12 hours. Growth and concentrations of N-compounds were determined.

Results

1. Functional analysis of the genes involved in the nitrification and denitrification steps.

Sequence analysis of the PCR product showed high identities to those of the previous reported nitrate reductase genes. In colony hybridization using the labeled PCR product, three positive clones (p68D7, p24A12, and p6E12) were selected in the gene library of strain F-5-2. The inserts of these plasmids p68D7, p24A12, and p6E12 covered 12 Kb and 6 ORFs were found in the determined sequence. Each ORF was high identity to previous reported enzymes involved in denitrification step in the deduced amino acid sequence (Table 1).

2. Effects of various factors on the removal of NH_4^+ and NO_3^-.

Strain F-5-2 could grow well and remove NH_4^+ and NO_3^- completely in 60 h of aerobic cultivation in a medium containing more than 64 mM of sodium potassium phosphate. Among the tested carbon sources, sucrose, lactose, D-mannose, maltose, D-fructose, and D-glucose promoted the growth and the removal of NH_4^+ and NO_3^- (Table 2). The removal of NH_4^+ and NO_3^- was inhibited in the medium containing L-arabinose, D-galactose, or sodium acetate. L-arabinose, D-galactose, or sodium acetate was added to the medium containing effective sugar (sucrose, lactose, D-

Table 1. Comparison of primary amino acid sequences deduced from ORF 1 to 6.

ORF	Predicted function	Homology			
		Amino acid identity (%)	Protein	Source	Accession No.
1	Nitrate reductase (small subunit)	66	NasC	*Klebsiella oxytoca*	L27431
2	Nitrite reductase	89	NasB	*Klebsiella oxytoca*	L06800
3	Nitrate reductase (large subunit)	79	NasA	*Klebsiella oxytoca*	L06800
4	Nitrate/Nitrite response regulator	93	NarL	*Salmonella typhimurium*	AE008778
5	Nitrate/Nitrite sensor protein	80	NarX	*Salmonella typhimurium*	AE008778
6	Nitrite extrusion protein	—[a]	NarK	*Salmonella typhimurium*	AE008778

[a] partial sequence

Table 2. Effects of carbon sources on the removal of NO_3^- and NH_4^+ from 0.2% (w/v) (25 mM) $NHNO_3$ medium under aerobic conditions.

Carbon source	Remaining (mM, after 60 h cultivation)			Growth (OD_{660})
	NO_3^-	NO_2^-	NH_4^+	
D-Glucose	0	0	0	13
Lactose	0	0	0	11
L-Arabinose	15.5	1.5	0.65	9.1
D-Mannose	0	0	0	13
Maltose	0.4	0	0	13
D-Galactose	16.8	1.0	4.25	8.7
D-Fructose	0	0	0	11
Sodium acetate	18	3.0	23	0.2
DL-Malic acid	7.5	1.4	0	3.1
Disodium succinate	20	1.5	3.0	4.3

mannose, maltose, D-fructose, or D-glucose). Strain F-5-2 was cultivated in these media. The removal of NH_4^+ and NO_3^- was inhibited in the medium containing sodium acetate.

Conclusion

A 12 Kb-region, which contained several genes involved in the nitrification and denitrification steps, was cloned from *K. pneumoniae* F-5-2. The region contained six ORFs. Some of the deduced amino acid sequences of these ORFs showed high identities with those of putative nitrate reductase or nitrite reductase. Further genetic studies are needed to clarify the denitrification and nitrification in strain F-5-2.

Effects of various factors on the removal of NH_4^+ and NO_3^- simultaneously were examined. With the growth of strain F-5-2, pH of the medium was changed to weak acidic condition. This condition affected the removal of NH_4^+ and NO_3^- and further growth. Among the tested carbon sources, the sugars which promoted the growth and the removal of NH_4^+ and NO_3^- are generally metabolized to intermediates of glycolytic route or TCA cycle via fructose 6-phospahte. Disaccharides, which contained D-glucose as a constituent promoted the removal of both N-compounds. Strain F-5-2 is a heterotrophic bacterium; the important thing to lead to more effective denitrification and nitrification is not only to control concentration of metal ions (Fe^{2+} and MoO_4^{2-}) but also to select these sugars.

Reference

Kim, Y-J., Yoshizawa, M., Takenaka, S., Murakami, S., and Aoki, K., Isolation and culture conditions of a *Klevsiella pneumoniae* strain that can utilize ammonium and nitrate ions simultaneously with controlled iron and molybdate ion concentrations. *Biosci. Biotechnol. Biochem.*, **66**, 996–1001 (2002).