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Nitrous oxide production in soil isolates of nitrate-ammonifying bacteria

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Summary

Here we provide the first demonstration of the potential for N_2O production by soil-isolated nitrate-ammonifying bacteria under different C and N availabilities, building on characterizations informed from model strains. The potential for soil-isolated *Bacillus* sp. and *Citrobacter* sp. to reduce NO_3^-, and produce NH_4^+, NO_2^- and N_2O was examined in batch and continuous (chemostat) cultures under different C-to-NO_3^- ratios, NO_3^--limiting (5 mM) and NO_3^--sufficient (22 mM) conditions. C-to-NO_3^- ratio had a major influence on the products of nitrate ammonification, with NO_2^-, rather than NH_4^+, being the major product at low C-to-NO_3^- ratios in batch cultures. N_2O production was maximum and accompanied by high NO_2^- production under C-limitation/NO_3-sufficiency conditions in chemostat cultures. In media with lower C-to-NO_3-N ratios (5- and 10-to-1) up to 2.7% or 5.0% of NO_3^- was reduced to N_2O by *Bacillus* sp. and *Citrobacter* sp., respectively, but these reduction efficiencies were only 0.1% or 0.7% at higher C-to-NO_3^- ratios (25- and 50-to-1). As the highest N_2O production did not occur under the same C-to-NO_3^- conditions as highest NH_4^+ production we suggest that a re-evaluation may be necessary of the environmental conditions under which nitrate ammonification contributes to N_2O emission from soil.

Introduction

Nitrate ammonification, or dissimilatory nitrate reduction to ammonium (DNRA), is one of the least well-characterized pathways of the soil N cycle in environmental terms even though it has been well characterized biochemically in the model laboratory organism *Escherichia coli* and the crystal structures of the key enzymes involved are known (Bamford *et al*., 2002; Jormakka *et al*., 2004). During nitrate ammonification NO_3^- is reduced to NO_2^- and NH_4^+ (Mohan *et al*., 2004), with N_2O, a potent greenhouse gas (IPCC, 2007), thought to be produced at the NO_2^- reduction stage (Costa *et al*., 1990; Kelso *et al*., 1997). However, N_2O production during nitrate ammonification has yet to be directly verified *in situ*, and so is often excluded from soil N budgets, and we know little about the response of this production to changing environmental conditions.

During nitrate ammonification bacteria respire NO_3^- in microoxic or anoxic environments, reducing it via NO_2^- to NH_4^+ (Cole, 1996; Simon, 2002). *Escherichia coli* is a prototype ammonifying bacterium. It has two periplasmic enzymes that can together reduce NO_3^- to NH_4^+, a periplasmic nitrate reductase NapA that reduces NO_3^- to NO_2^- (Potter and Cole, 1999) and a periplasmic nitrite reductase NrfA that reduces NO_2^- to NH_4^+ (Hussain *et al*., 1994). The structures of the two enzymes involved in this process have been determined (Bamford *et al*., 2002; Jepson *et al*., 2007). *Escherichia coli* also contains nitrate and nitrite reductases with active sites in the cytoplasm that can reduce NO_3^- through to NH_4^+, two integral membrane nitrate reductases, the structurally defined NarG (Jormakka *et al*., 2004) and the NarZ isozyme (Iobbi-Nivol *et al*., 1990), and the cytoplasmic nitrite reductase NirB (Macdonald and Cole, 1985). Alongside *E. coli*, emerging genome sequences show nitrate ammonification to be widespread among *Proteobacteria* (Simon, 2002). There are also reports demonstrating the ability of *Bacillus subtilis* to survive in the anoxic environments by utilizing dissimilatory nitrate reduction to ammonia and fermentation processes (Cruz Ramos *et al*., 2000). *Bacillus subtilis* utilizes the respiratory nitrate reductase system (NarGHIJ) and nitrite reductase NasDEF (with both assimilatory and dissimilatory roles) to complete reduction of nitrate to ammonia (Nakano *et al*., 1998).

There have been a few reports in the literature that *E. coli* produces N_2O during anaerobic nitrate metabolism (Bleakley and Tiedje, 1982; Smith, 1983). For example, in complex medium nutrient-sufficient batch culture experiments the rate of N_2O production during nitrate ammonification was around 5% of NO_3^- reduction

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(Bleakley and Tiedje, 1982). It has been suggested that enteric nitrate-ammonifying bacteria could be a significant source of N_2O in soil (Bleakley and Tiedje, 1982), although this remains to be directly verified in part due to problems in distinguishing nitrate ammonification from denitrification *in situ* as both processes may occur simultaneously in anaerobic microsites in soils (Fazzolari *et al*., 1990) depending on C and N availability and Redox status (Smith and Zimmerman, 1981; Stevens *et al*., 1998; Pett-Ridge *et al*., 2006; Wan *et al*., 2009; Schmidt *et al*., 2011). It has even been proposed that under high C-to-NO_3^- conditions nitrate ammonification may be faster and produce greater quantities of N_2O than denitrification, depending on enzyme regulation (Yin *et al*., 2002), although this has yet to be verified.

The increasing concern about emissions of N_2O from soil has focused interest on the microbial sources of this greenhouse gas and the conditions under which it is released by bacteria. However, there is debate on the relevance of regulation of N_2O production in model laboratory strains of ammonifying bacteria to the regulation in soil isolates. In response to this we have undertaken experiments in which we have isolated nitrate-ammonifying bacteria from soils and quantified N_2O production in batch and chemostat cultures at different C-to-NO_3^- ratios. We have established that N_2O production by these soil-isolated strains is most prevalent at low C-to-NO_3^- ratios and consider this in the context of N_2O production by laboratory *E. coli* wild-type strains and *Bacillus subtilis* 1A01, and the potential for N_2O production during nitrate ammonification in soil.

Results and discussion

Isolation of soil bacteria and verifying their ability to reduce nitrate to ammonium

Nitrate-reducing soil isolates were enriched from a sandy soil that was selected for its high NO_3^- concentration. Of 80 isolates randomly cultured from a nitrate broth selection medium in the absence of oxygen only five were capable of growth on glucose minimal P medium with NO_3^- as the only N source. During anaerobic growth on NO_3^- and glucose (on P medium) all five isolates reduced NO_3^-, but NH_4^+-N was present (extracellularly) in significant concentrations only in cultures of isolates 4, 15 and 45 (Table 1a). When grown on modified P medium (with glycerol as a C source; less susceptible to fermentation) NO_2^- and NH_4^+ were the products of nitrate reduction for isolates 4, 15 and 45 (Table 1b). These three isolates were thus considered capable of nitrate ammonification. Two isolates (4 and 45) were chosen for further analysis and were identified by analysis of 16S rDNA with primers 27F and 1492R (Lane, 1991). Isolated plasmids were sequenced at the University of Dundee, UK, compared with 16S rDNA sequences available in the NCBI database using BLAST, and identified (98% of similarity to database sequences) as *Bacillus* sp. (isolate 4) and *Citrobacter* sp. (isolate 45). Fragments of a gene encoding for the periplasmic nitrite reductase (nrf; Mohan *et al*., 2004) were amplified only from DNA isolated from *Citrobacter* sp. (isolate 45).

Table 1. Mineral N concentrations (NO_3-N, NO_2^--N and NH_4^+-N) at 120 h and optical density in batch cultures of soil isolates capable of growth on (a) minimal glucose P medium with KNO_3 as the only nitrogen source (Fazzolari *et al*., 1990) and (b) modified P medium with 20 mM glycerol, 10 mM KNO_3 and 2 mg NH_4^+-N l^{-1}.

Soil isolate No.	NO_3^--N (mg l^{-1})	NO_2^--N (mg l^{-1})	NH_4^+-N (mg l^{-1})	OD (600 nm)
(a)				
4	113 (1.2)	4 (0.01)	24 (2.0)	0.12
15	63 (38)	57 (28)	25 (12)	0.053
45	100 (3.9)	11 (0.02)	2 (0.42)	0.29
(b)				
4	25 (4.6)	94 (17)	31 (3.9)	0.063
15	18 (5.1)	128 (2.0)	20 (0.75)	0.053
45	9 (3.9)	96 (24)	21 (0.12)	0.30

Initial NO_3^--N concentration in both media was 140 mg NO_3^--N l^{-1}. Bacterial strains were isolated from a sandy soil, St Fergus, Aberdeenshire, using the protocol of Yin and colleagues (2002). Batch cultures were incubated anaerobically (He-flushed; < 1% O_2 v/v) for 120 h at 27°C and mineral N concentrations (NO_3^--N, NO_2^--N and NH_4^+-N) were determined at 24 h intervals, up to 120 h. NO_3^--N, NH_4^+-N and NO_2^--N concentrations were measured colorimetrically by flow injection analysis (FIA STAR 500). Values in parentheses are ± one standard deviation.

Nitrite, ammonium and nitrous oxide production by nitrate ammonifiers in batch cultures of different C-to-NO_3-N ratios

Citrobacter sp. (isolate 45) and *Bacillus* sp. (isolate 4) both reduced NO_3^- when grown anaerobically. The ratio of C-to-NO_3-N in the medium at the beginning of the incubation influenced the concentration of products of NO_3^- reduction at 120 h. Total recovery (mineral N + N_2O-N) was negatively related to C-to-NO_3-N ratio, and for C-to-NO_3-N ratios of 10-, 25- and 50-to-1 recovery was greater for *Bacillus* sp. than for *Citrobacter* sp. (Fig. 1). At low C-to-NO_3-N ratio (5-to-1) NO_2^- was the major product of nitrate reduction for both *Citrobacter* sp. (69% of NO_3^- reduced to NO_2^-) and *Bacillus* sp. (63% of NO_3^- reduced to NO_2^-). At higher C-to-NO_3-N ratios (10-, 25- and 50-to-1) no NO_2^- accumulated in the *Citrobacter* sp. culture, with NH_4^+ being the only form of mineral N recovered (Fig. 1A). In contrast, *Bacillus* sp. accumulated NO_2^- in the medium even at C-to-NO_3-N ratio of 25-to-1, where 50% of applied NO_3^- was reduced to NO_2^- (Fig. 1B).

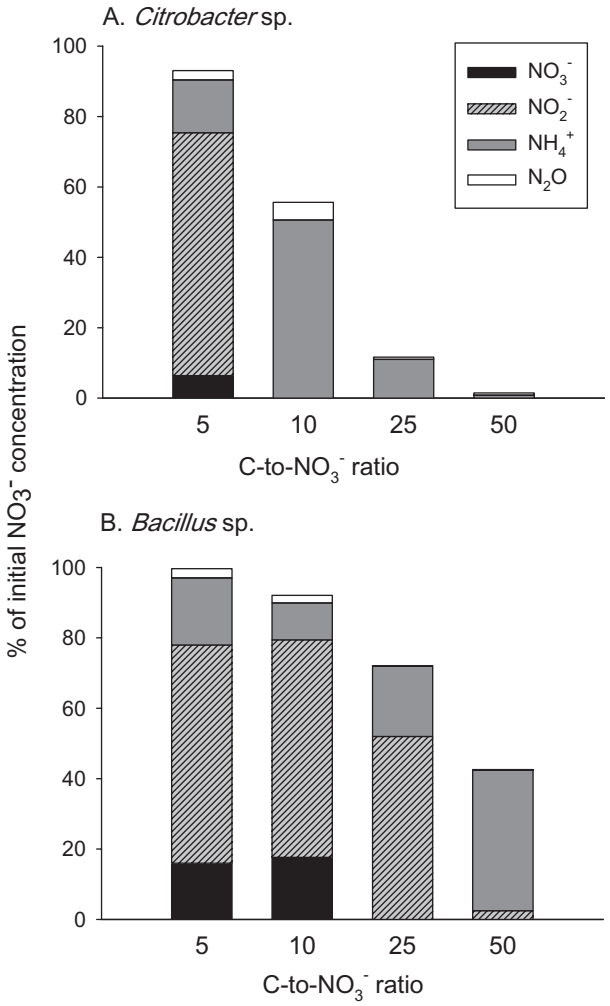

Fig. 1. Effect of C-to-NO_3^--N ratio on nitrate reduction products by pure cultures of soil-isolated nitrate-ammonifying bacteria on modified P medium with glycerol (20 mM) and NO_3^--N (1–10 mM) to create C-to-NO_3-N ratios of 5-, 10-, 25- and 50-to-1. After 120 h gas samples (12 ml) were taken from the headspace of additional (undisturbed) serum bottles, and analysed (1 ml) for N_2O concentration on a Clarus 500 gas chromatograph with ECD and Elite-Q PLOT column (Perkin Elmer, USA), with injector, oven and detector temperatures of 115°C, 35°C and 300°C respectively. (A) *Citrobacter* sp.; (B) *Bacillus* sp.

The percentage of NO_3^- reduced to N_2O by both isolates was lower during growth on media with higher C-to-NO_3-N ratios (25- and 50-to-1) than in media with low C-to-NO_3-N ratio (Fig. 1). In media with lower C-to-NO_3-N ratios (5- and 10-to-1) up to 2.7% or 5.0% of NO_3^- was reduced to N_2O by *Bacillus* sp. and *Citrobacter* sp. respectively (Fig. 1). However, at higher C-to-NO_3-N ratios (25- and 50-to-1) only up to 0.1% or 0.7% of NO_3^- was reduced to N_2O by *Bacillus* sp. and *Citrobacter* sp. respectively. To maximize the generation of a transmembrane proton gradient at low C-to-NO_3^- ratios under electron-donor-limiting conditions it makes bioenergetic sense when using Nar to maximize NO_3^- reduction to NO_2^- and minimize the use of scarce electrons to reduce NO_2^- to NH_4^+. This is exactly what is observed under C-to-NO_3^- ratios of 5-to-1 for *Citrobacter* sp. and up to 25-to-1 for *Bacillus* sp.

Chemostat cultures of the soil isolates under carbon sufficiency or nitrate sufficiency

The aim of this part of the study was to quantify N_2O production in chemostat cultures operated in a steady state under defined conditions of carbon sufficiency/nitrate limitation (i.e. high C-to-NO_3-N) and carbon limitation/nitrate sufficiency (i.e. low C-to-NO_3-N). Comparisons were made between our soil-isolated strains (*Citrobacter* sp. – isolate 45, *Bacillus* sp. – isolate 4), *E. coli* wild-type strains DH5α, RK4353, MG1655 and BL21 and *B. subtilis* 1A01. An illustrative experimental run is shown in Fig. 2 for *Citrobacter* sp. After 24 h of aerobic growth the air supply was turned off and cultures shifted from O_2 to NO_3^- respiration. This resulted in a decrease in biomass and protein concentration until a new steady state was reached after approximately three to four vessel volume changes (70–90 h). During the transient non-steady state phase, between 24 and 70 h, the NO_3^--N concentration in the reactor vessel decreased and a concomitant increase in NO_2^--N concentration was observed, reflecting the respiratory reduction of NO_3^- to NO_2^- (Fig. 2). Under nitrate-sufficient conditions this increase in NO_2^--N concentration was accompanied by significant production of N_2O up to ~300 μM N_2O-N at 100 h for *Citrobacter* sp. and ~55 μM N_2O-N by *Bacillus* sp. (Table 2). In contrast, N_2O production in nitrate-sufficient cultures of *B. subtilis* 1A01 (Table 2) and different *E. coli* wild-type strains (Table 3) was much lower. The N_2O production by *B. subtilis* 1A01 (Table 2) was ~100 nM, but for *E. coli* strains it ranged from ~30 to ~600 nM (Table 3). Under nitrate-limited conditions the concentration of N_2O produced by both *Bacillus* sp. and *Citrobacter* sp. was lower than under nitrate sufficiency (Table 2).

This observation that N_2O is a more significant product of nitrate ammonification at low C-to-NO_3^- ratios or under nitrate sufficiency is probably a consequence of the accumulation of NO_2^- under these conditions, which likely leads to N_2O production via a range of enzymatic routes in the cell through the one-electron reduction of NO_2^-, as recently suggested for the nitrate reductase, NarG (Gilberthorpe and Poole, 2008) to the cytotoxic radical NO and the subsequent reductive detoxification to N_2O via systems, such as the flavohaemoglobin or flavorubredoxin cytoplasmic NO reductases that are widespread in dissimilatory nitrate-reducing bacteria (Gilberthorpe and Poole, 2008).

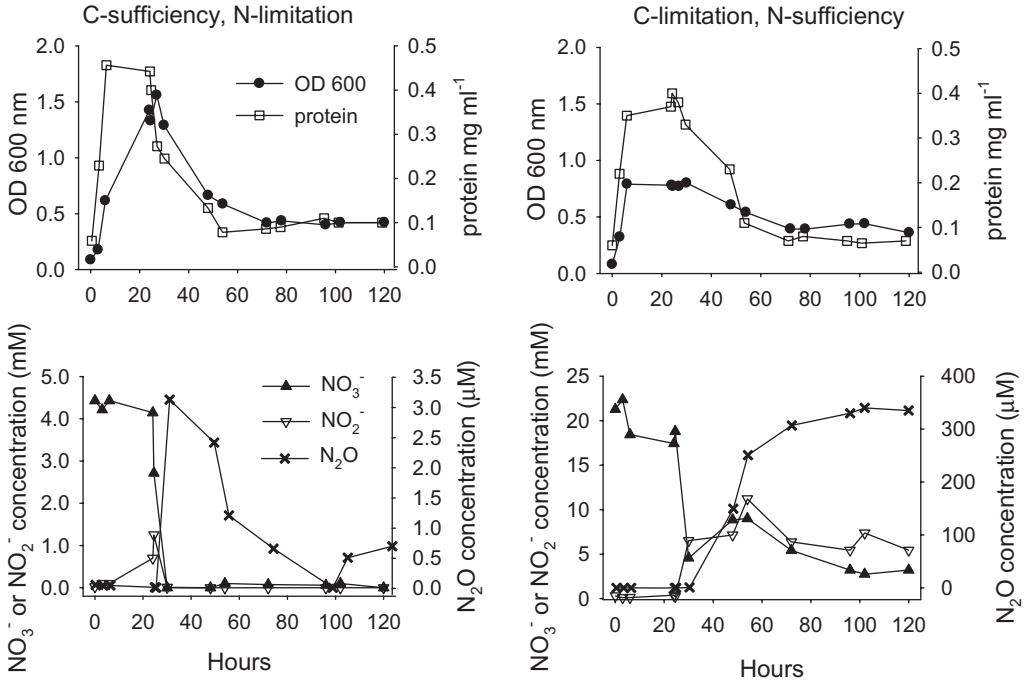

Fig. 2. Specimen typical continuous culture of *Citrobacter* sp. optical density (600 nm) protein concentration (mg ml^{-1}), concentrations of NO_3^--N (mM), NO_2^--N (mM) and N_2O-N (μM), under C sufficiency N limitation, and C limitation N sufficiency. Data derived from triplicate experiments are shown in the tables with standard errors.

Potential for N_2O production during nitrate ammonification in soil

The benefit in studying soil-isolated strains is that we provide a more robust assessment of quantity and composition of N products during nitrate ammonification, and response to differing C-to-NO_3^- ratios, than would be possible *in situ* in soil where nitrate ammonification occurs within a mixed microbial community in a heterogeneous soil matrix, and in possible competition with denitrification

Table 2. Nitrogen compound, optical density and protein concentration in glycerol-sufficient nitrate-limited continuous cultures (BioFlo 3000 chemostat, New Brunswick Scientific, USA, 1.2 l volume, pH 7.0) of *Bacillus subtilis* 1A01 and the soil isolates *Citrobacter* sp. and *Bacillus* sp. after four fermenter volume changes to reach steady state.

Strain	NO_3^- c (mM)	NO_2^- p (mM)	N_2O p (μM)	OD_{600}	Protein (mg ml^{-1})
Nitrate-limited cultures					
Citrobacter sp.	4.8 ± 0.05	0.002 ± 0.001	0.17 ± 0.03	0.41 ± 0.07	0.08 ± 0.00
Bacillus sp.	4.4 ± 0.45	1.9 ± 1.1	3.6 ± 1.5	0.30 ± 0.01	0.04 ± 0.02
Bacillus subtilis 1A01	4.2 ± 0.45	4.6 ± 0.02	0.01 ± 0.001	0.22 ± 0.03	0.09 ± 0.02
Nitrate-sufficient cultures					
Citrobacter sp.	16.4 ± 0.52	4.1 ± 0.51	339.3 ± 41.0	0.1 ± 0.005	0.00 ± 0.02
Bacillus sp.	18.9 ± 2.6	6.4 ± 4.5	55.8 ± 61.7	0.2 ± 0.0007	0.1 ± 0.02
Bacillus subtilis 1A01	17.9 ± 0.01	18.0 ± 4.1	0.1 ± 0.10	0.2 ± 0.01	0.1 ± 0.004

The main carbon source was glycerol (5 mM for N-sufficient cultures and 20 mM for N-limited cultures) and the main terminal electron acceptor was sodium nitrate (22 mM for N-sufficient cultures and 5 mM for N-limited cultures). Bacteria were cultivated at 37°C either aerobically in Luria–Bertani medium for initial growth (Sambrook *et al*., 1989) or anaerobically in modified MS medium (Pope and Cole, 1982) for the determination of N_2O production. One hundred millilitres of MS medium was inoculated with 5 ml of LB culture and aerobically incubated overnight at 37°C (for *E. coli* strains) or 30°C for soil isolates. Fifty millilitres of this culture was used to inoculate the chemostat. After 24 h of aerobic growth the air supply was shut down and a feed of MS medium was started (dilution rate D = 0.042 h^{-1} for *E. coli* and *Citrobacter*, or D = 0.018 h^{-1} for *Bacillus* sp. and *Bacillus subtilis* 1A01). Headspace gases were sampled, stored and analysed for N_2O concentration. Concentrations of NO_3^--N, NH_4^+-N and NO_2^--N were measured colorimetrically by flow injection analysis (FIA STAR 500) and protein concentration was determined using the Coomassie Blue method (Bio-Rad). Abbreviations: c, consumed; p, produced.

Table 3. Nitrogen compound, optical density and protein concentration in glycerol-limited continuous cultures (as described for Table 2) of different *E. coli* wild-type strains (DH5α, RK4353, MG1655 and BL21) after four fermenter volume changes.

Strain	NO_3^- c (mM)	NO_2^- p (mM)	N_2O p (nM)	OD_{600}	Protein (mg ml^{-1})
DH5α	16.6 ± 2.5	8.6 ± 2.43	27 ± 4.0	0.14 ± 0.01	0.09 ± 0.02
RK4353	21.4 ± 0.33	17.3 ± 0.02	397 ± 29	0.21 ± 0.01	0.11 ± 0.02
MG1655	15.9 ± 0.97	16.6 ± 0.18	607 ± 19	0.15 ± 0.01	0.08 ± 0.004
BL21	22.5 ± 0.99	17.0 ± 0.50	355 ± 35	0.22 ± 0.01	0.10 ± 0.02

Initial nitrate concentration 22 mM. Abbreviations: c, consumed; p, produced.

and plant uptake of N. While the potential for soil-isolated bacterial strains (*Enterobacter*, *Bacillus*, *Citrobacter* spp.) to produce N_2O during NO_3^- or NO_2^- reduction to NH_4^+ has previously been demonstrated (Smith and Zimmerman, 1981; Smith, 1982), our batch and chemostat experiments provide the first evidence of the influence of C and NO_3^- availability on this N_2O production. We demonstrate that NO_2^- and N_2O production by *Bacillus* and *Citrobacter* strains is greater under low C-to-NO_3^- conditions, with up to 5% (*Citrobacter* sp.) of NO_3^--N being reduced to N_2O under these conditions.

The greater reduction of NO_3^- to NH_4^+ by *Bacillus* sp. at high C-to-NO_3^- ratio confirms findings from the soil environment (e.g. Silver *et al*., 2001; 2005; Schmidt *et al*., 2011), and fits with the traditional consensus of nitrate ammonification occurring in C rich environments. However, from our soil-isolated strains we demonstrate the potential for greater N_2O production from this process under high NO_3^- conditions. Based on the responses of these soil-isolated strains we recommend the following for consideration in soil investigations: (i) a re-assessment of the conditions under which nitrate ammonifier N_2O production occurs in soil, (ii) quantification of NO_2^-, as well as NH_4^+ concentrations, as being indicative of nitrate ammonification, (iii) recognition of the potential for nitrate ammonification to occur in fertilized, or high NO_3^- environments, and (iv) acknowledgement of the possibility denitrification is not the only dissimilatory nitrate-reducing process producing N_2O in soil.

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