Nitrosamine Formation by Denitrifying and Non-denitrifying Bacteria: Implication of Nitrite Reductase and Nitrate Reductase in Nitrosation Catalysis

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Biochemical, microbiological and genetic studies were done to characterize the mechanism of bacterial formation of *N*-nitrosomorpholine (NMOR) from morpholine and nitrite at neutral pH. In *Escherichia coli* and *Proteus morganii*, the nitrosating activity was markedly induced when bacteria were cultured under anaerobiosis in minimal medium containing nitrate, while in the presence of nitrite there was no induction. However, induction of the nitrosating activity in *Pseudomonas aeruginosa* occurred in anaerobic cultures in the presence of either nitrate or nitrite. The nitrosation capacity was also examined in various *E. coli* K12 mutants whose structural gene of either nitrate reductase or nitrite reductase was deleted. Nitrosation was not linked to the three (NADH-, formate- and glucose-dependent) nitrite reductases but was directly dependent on the presence of a nitrate reductase.

INTRODUCTION

Various bacteria catalyse nitrosation of secondary amines to form *N*-nitrosamines at neutral pH (Kunisaki & Hayashi, 1979; Suzuki & Mitsuoka, 1984; Calmels *et al.*, 1985; Leach *et al.*, 1985), but the mechanism has not been entirely elucidated. We previously studied several biochemical and microbiological factors which affect the nitrosation of morpholine. We also investigated the induction of such nitrosation activity in *Escherichia coli* strain A10 and in some of its mutants, deficient in either nitrate reductase or nitroreductase. These results suggested that a molybdenoenzyme, possibly nitrate reductase, is involved in the catalysis of nitrosation in *E. coli* (Calmels *et al.*, 1987).

We have now studied the effect of different culture conditions on the induction of nitrosating activity in species from four bacterial genera: *E. coli*, *Proteus morganii*, *Pseudomonas aeruginosa* and *Paracoccus denitrificans*. Several genetically, well-characterized mutants of *E. coli* were also examined in order to test whether their ability to catalyse the nitrosation of morpholine with nitrite was linked to specific loci. The aim of these studies was to gain a better understanding of the mechanisms and relevance of bacterial nitrosation likely to occur in man, since endogenous formation of carcinogenic *N*-nitroso compounds in humans in the presence of bacteria has been associated with an increased risk of cancer at sites such as the stomach and the urinary bladder (Mirvish, 1983; Hicks *et al.*, 1977).

METHODS

Chemicals. $NaNO_2$, $NaNO_3$, morpholine hydrochloride, *N*-nitroso-methyl-n-pentylamine were obtained from Merck; *N*-nitrosomorpholine (NMOR), methyl viologen and DL-dithiothreitol were obtained from Sigma; and *N*-(1-naphthyl)ethylenediamine dihydrochloride was obtained from Fluka. All other chemicals were of analytical grade and obtained from either Merck or Sigma.

Abbreviation: NMOR, *N*-nitrosomorpholine.

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Organisms and culture. E. coli A10, isolated from human intestine (Suzuki & Mitsuoka, 1984) was kindly provided by Dr S. Suzuki (Institute of Physical and Chemical Research, Saitama, Japan). *Pr. morganii* and *Ps. aeruginosa* were isolated from a human urinary infection and from a human wound, respectively (Calmels *et al.*, 1985). *Pc. denitrificans* (CIP 71.11) was obtained from the culture collection of the Institut Pasteur, Paris, France. Specific mutants of *E. coli* K12 listed in Table 1 were described previously (Abou-Jaoude *et al.*, 1978, 1979; Pascal *et al.*, 1981) and were kindly provided by Dr M. Chippaux (CNRS, Marseille, France). All bacteria except *E. coli* K12 mutants were grown in minimal medium (five times diluted haemocult broth, Institut Pasteur, Lyon, France) under aerobiosis (100 ml culture broth in 1 litre Erlenmeyer flasks at 37 °C overnight with shaking) or under anaerobiosis (100 ml stoppered bottles at 37 °C overnight without shaking). Mutants of *E. coli* K12 were grown at 37 °C overnight in non-diluted rich haemocult broth. The other bacteria were cultured under aerobiosis or anaerobiosis in minimal media; when specified, $NaNO_3$ (10 mM), $NaNO_2$ (5 mM) or tungstic acid (sodium salt) (2 mM) were added to the growth medium (Table 2). After washing with 0·9% (w/v) saline, the bacteria were resuspended in ice-cold 0·9% saline. Protein was determined by the Lowry method using bovine serum albumin fraction V (Sigma) as a standard.

Assay for nitrosation. This was done in a reaction mixture containing 0·1 M-Tris/HCl buffer pH 7·2, 25 mM-morpholine, 25 mM-$NaNO_2$ and the bacterial suspension (final volume 5 ml). The reaction mixture was incubated with shaking in a water bath at 37 °C for 1 h. The reaction was stopped by adding 20 ml 1% (w/v) NaOH to 1 ml of the reaction mixture. Control experiments were done in parallel by replacing the bacterial suspension with 0·9% saline.

Specific nitrosation activity was defined as nmol NMOR formed (mg protein)$^{-1}$ h^{-1}, after subtraction of the control value obtained in a cell-free assay.

Analysis of nitrosamines. After stopping the nitrosation reaction, a suitable amount of the internal standard *N*-nitroso-methyl-n-pentylamine was added to the reaction mixture. Volatile nitrosamines were extracted three times in 30 ml dichloromethane. The combined dichloromethane extracts were dried by filtration on anhydrous Na_2SO_4 and concentrated to 1 ml in a Kuderna–Danish evaporator at 47 °C. A sample was analysed for volatile nitrosamines on a Perkin–Elmer Sigma 3B gas chromatograph (Norwalk) interfaced with a Thermal Energy Analyzer, model 502 (Thermo-electron Corp.). A 2 m × 3 mm i.d. column packed with 5% (w/w) FFAP (Alltech) on chromosorb WHP (80–100 mesh) was used, at a column temperature of 190 °C; the injection-port temperature was 220 °C; the flow rate of the carrier gas (argon) was 30 ml min^{-1}.

Assays of bacterial nitrate reductase and nitrite reductase. Nitrate reductase was assayed by measuring reduction of nitrate to nitrite with methyl viologen as the electron donor (McGregor *et al.*, 1974). The assay was done in a final volume of 2 ml reaction mixture, containing 250 mM-sodium phosphate buffer (pH 7·1), 50 mM-$NaNO_3$, 50 µM-DL-dithiothreitol, 0·005% methyl viologen and the bacterial suspension. To start the reaction, 0·1 ml of a solution containing 0·8% $NaHCO_3$ and 0·8% sodium dithionite was added to the assay mixture which was gently mixed until it reached a uniform blue colour. After incubation for 5 min at room temperature, the reaction was stopped by mixing the assay mixture rapidly in a vortex mixer until the blue colour disappeared. Nitrite was then determined by the method of Nicholas & Nason (1957). Specific activity of nitrate reductase was expressed as µmol nitrite formed (mg protein)$^{-1}$ h^{-1} at 23 °C.

Rates of nitrite reduction were assayed as previously reported (Abou-Jaoude *et al.*, 1979) in open test-tubes containing 5 ml 40 mM-Tris/HCl buffer pH 8, 40 mM-glucose and 0·7 ml of the bacterial suspension in 0·9% saline. The reaction was started by the addition of 0·5 mM-$NaNO_2$. Tubes were incubated at 37 °C and at intervals samples were tested for nitrite concentration as described above. Specific activity was expressed as µmol nitrite reduced (mg protein)$^{-1}$ h^{-1} at 37 °C.

RESULTS AND DISCUSSION

Nitrosation activity in E. coli mutants deficient for either nitrate or nitrite reductase

Pleiotropic chlorate-resistant mutants of *E. coli* are unable to form NMOR, suggesting that a molybdenoenzyme, possibly nitrate reductase, is involved in bacterial nitrosation (Calmels *et al.*, 1987). For futher confirmation we examined several mutants, in which the structural gene of either nitrate reductase or nitrite reductase was deleted, for their ability to catalyse nitrosation of morpholine and to reduce nitrate or nitrite (Table 1). Although the parental strain, LCB 320, which had been grown in rich medium, formed NMOR – range of activity 170–210 nmol NMOR (mg protein)$^{-1}$ h^{-1} – neither strain LCB 22 (deleted for *nirR*, the regulatory gene controlling the expression of *narGHI*, the nitrate reductase structural gene) nor strain LCB 333 (deleted for *narGHI*) exhibited nitrosating activity. Strain LCB 79, also deficient for nitrate reductase activity as a result of the insertion of *lac* structural genes in the *narGHI* gene, did not nitrosate morpholine while the parental strain, MC 4100, had a nitrosation activity in the range

Table 1. *Nitrosating activity of various mutants of E. coli K12*

Bacterial strains were cultured in rich media and assayed twice for their nitrate reductase and nitrosating activities. The experiments were repeated twice with two separate cultures, except for the parent strains that were tested several times for both activities.

Strain	Genotype	Phenotype	Nitrate reductase activity [μmol NO_2^- formed (mg protein)$^{-1}$ h^{-1}]	Nitrosating activity [nmol NMOR (mg protein)$^{-1}$ h^{-1}]
LCB 320	*thi-1 thr-1 leu-6 lacY rpsL* 175	Parent strain	12·0–14·0	170–210
LCB 22	As LCB 320 but *ana-1 nir-2.2*	Deficient for positive regulation of nitrate and nitrite reductase	1·8; 1·5	ND
LCB 900	As LCB 320 but *ana-1*	Deficient for alcohol and aldehyde dehydrogenase	12·0; 10·4	100; 83
LCB 82	As LCB 900 but *nirD*	Deficient for nitrite reductase	9·0; 6·7	77; 84
LCB 84	As LCB 900 but *nirF*	Deficient for nitrite reductase	8·9; 7·5	385; 344
LCB 85	As LCB 900 but *nirE*	Deficient for nitrite reductase	7·8; 6·1	270; 327
LCB 190	As LCB 900 but *nirG*	Deficient for nitrite reductase	7·6; 7·1	ND, 6
LCB 197	As LCB 900 but *nirH*	Deficient for nitrite reductase	9·8; 11·2	393; 176
LCB 333	As LCB 900 but Δ*narGHI*	Deficient for nitrate reductase	1·5; 1·6	ND
MC 4100	*lac rpsL* 175	Parent strain	10·1; 10·4	45–78
LCB 79	As MC 4100 but *nar–lac*	Deficient for nitrate reductase	1·2; 1·7	ND

ND, Not detected.

of 45–78 units. LCB 333 and LCB 79 had residual nitrate reductase activities (Table 1), but showed significant nitrite reductase activities of 1·2 and 2·4 units [μmol nitrite reduced (mg protein)$^{-1}$ h^{-1}] respectively.

Five mutants derived from the *E. coli* strain LCB 900, all of which are deficient for nitrite reductase, were further examined. All strains had nitrate reductase activity in the range of 6·1 to 11·2 units. Only one of these strains, LCB 190, did not show significant nitrosation activity; the others, strains LCB 82, 84, 85 and 197 had appreciable activity. All these nitrite-reductase-deficient strains have recently been biochemically characterized (MacDonald *et al.*, 1985): the nitrosating strain LCB 82 (*nirD*) exhibits a strong formate-dependent nitrite reductase activity, but lower glucose-dependent and no NADH-dependent nitrite reductase activities, as compared to the parental strain (LCB 900). Therefore, we can assume that the NADH-dependent nitrite reductase in *E. coli* is not involved in nitrosation catalysis. Strain LCB 197 (*nirH*), which exhibits a high nitrosation activity, also has high glucose-dependent and NADH-dependent nitrite reductase activities. Although the *nirH* gene is required for electron transfer from formate to nitrite (MacDonald *et al.*, 1985), it appears not to be involved in the bacterial nitrosation reaction. Strains LCB 190 (*nirG*), LCB 84 (*nirF*) and LCB 85 (*nirE*) are defective in anaerobic glucose metabolism rather than specifically in nitrite reduction (MacDonald *et al.*, 1985). Under our conditions, the nitrosating activity of the latter two strains was not affected by the low residual glucose-dependent nitrite reduction still observed in these mutants. In contrast, strain LCB 190 (*nirG*) did not, unexpectedly, exhibit a nitrosating activity. On the basis of published data, this effect cannot be interpreted but this mutation is genetically not well-characterized and may affect multiple steps in glucose metabolism, and indirectly also the nitrosation reaction. Based on our comparison between nitrosation activity and the reported nitrite reductase activities in these five mutants, we can state that the nitrosation in *E. coli* is not likely to be linked to any of the three (NADH-, formate- and glucose-dependent) nitrite reductase activities.

Effect of culture conditions on the induction of nitrosation

In previous studies on *E. coli* A10, we observed that low nitrate concentrations in the reaction mixture strongly inhibited *in vitro* nitrosation, but that the presence of nitrate during culture and

Table 2. *Nitrosation, nitrate reductase and nitrite reductase activities in two non-denitrifying bacteria, E. coli A10 and Pr. morganii, after incubation under various culture conditions*

Values are expressed as the mean ± SD of three to five assays.

Culture conditions*	Nitrosating activity [nmol NMOR (mg protein)$^{-1}$ h^{-1}]		Nitrate reductase activity [μmol NO_2^- (mg protein)$^{-1}$ h^{-1}]		Nitrite reductase activity [μmol NO_2^- (mg protein)$^{-1}$ h^{-1}]	
	E. coli A10	*Pr. morganii*	*E. coli* A10	*Pr. morganii*	*E. coli* A10	*Pr. morganii*
Aerobiosis						
MM	ND	ND	3·6 ± 0·3	4·6 ± 1·3	ND	ND
MM + $NaNO_3$ (10 mM)	ND	ND	5·4 ± 0·2	4·5 ± 0·7	ND	ND
MM + $NaNO_2$ (5 mM)	ND	ND	6·1 ± 1·3	4·8 ± 0·2	ND	ND
Anaerobiosis						
MM	90 ± 6	48 ± 10	9·1 ± 2·1	7·2 ± 2·4	3·0 ± 1·4	ND
MM + $NaNO_3$ (10 mM)	422 ± 62	1083 ± 134	24·4 ± 5·2	15·9 ± 2·3	ND	ND
MM + $NaNO_3$ (10 mM) + tungstic acid (2 mM)	ND	ND	5·0 ± 1·0	4·1 ± 0·7	1·9 ± 0·3	ND
MM + $NaNO_2$ (5 mM)	ND	ND	6·2 ± 0·7	4·5 ± 0·9	3·8 ± 1·7	ND
MM + $NaNO_2$ (5 mM) + tungstic acid (2 mM)	ND	ND	5·7 ± 1·4	4·4 ± 1·25	2·2 ± 0·3	ND

ND, Not detected.
* MM, minimal medium.

anaerobiosis was required for the induction of a bacterial nitrosating activity (Calmels *et al.*, 1987). In contrast, Leach *et al.* (1987) reported that the nitrosating abilities of two *E. coli* isolates were suppressed when they were cultured anaerobically in a medium containing nitrate; on the other hand, denitrifying bacteria such as *Ps. aeruginosa* required anaerobic growth with nitrate or nitrite in order to induce the nitrosation activity. In order to clarify the effects of the growth conditions on the induction of nitrosation, two non-denitrifying bacteria (*E. coli* A10 and *Pr. morganii*, Table 2) and two denitrifying bacteria (*Ps. aeruginosa* and *Pc. denitrificans*, Table 3) were tested; their abilities to nitrosate morpholine and to reduce nitrate or nitrite were measured after overnight culture at 37 °C under aerobiosis or anaerobiosis in minimal medium supplemented with different compounds. Both *E. coli* and *Pr. morganii*, when grown under anaerobiosis in the presence of 10 mM-$NaNO_3$ exhibited high nitrosating activities (Table 2). However, this induction was inhibited by the addition of 2 mM-tungstic acid (sodium salt) to the minimal medium. Tungsten, a molybdenum analogue, is a known inhibitor of molybdeno-enzymes such as nitrate reductase (Campbell *et al.*, 1985). In these two micro-organisms, the induction of nitrosating activity correlated well with the induction of nitrate reductase activity, but not with the induction of the nitrite reductase activity. In *Pr. morganii*, no nitrite reductase activity could be detected although it exhibited a higher ability to form NMOR than *E. coli*. In addition, the highest rate of nitrite reductase activity in *E. coli* was observed after anaerobic growth in the presence of nitrite where no induction of the nitrosating activity was detectable. In the presence of nitrite with or without tungstic acid, *E. coli* exhibited a residual nitrate reductase activity which was still rather high (25% of the wild-type) (Table 2). The level of activity may either not be enough to catalyse the nitrosation reaction, or, alternatively, nitrite may induce another type of nitrate reductase which is not involved in the nitrosation reaction.

In contrast, the conditions required for the induction of nitrosating activity in *Ps. aeruginosa* and *Pc. denitrificans* were distinctly different (Table 3). When grown aerobically in minimal medium with or without nitrate or nitrite, these bacteria did not exhibit any significant nitrosating activity. Under anaerobic conditions and in the presence of nitrite rather than of nitrate, *Ps. aeruginosa* exhibited the highest activity. Thus the mechanism for the induction of the nitrosating enzyme in enterobacteria seems to be different from that in *Ps. aeruginosa*. In contrast, the nitrosating activity of *Pc. denitrificans* was induced under anaerobiosis, in the presence of nitrate. When nitrite was added during growth, the induction of the nitrosation activity in *Pc. denitrificans* was inhibited by 80% (Table 3); under the same conditions, activity

Table 3. *Nitrosation, nitrate reductase and nitrite reductase activities in two denitrifying bacteria, Ps. aeruginosa and Pc. denitrificans, after incubation under various culture conditions*

Values are expressed as the mean ± SD of three to five assays.

Culture conditions*	Nitrosating activity [nmol NMOR (mg protein)$^{-1}$ h^{-1}]		Nitrate reductase activity [μmol NO_2^- (mg protein)$^{-1}$ h^{-1}]		Nitrite reductase activity [μmol NO_2^- (mg protein)$^{-1}$ h^{-1}]	
	Ps. aeruginosa	*Pc. denitrificans*	*Ps. aeruginosa*	*Pc. denitrificans*	*Ps. aeruginosa*	*Pc. denitrificans*
Aerobiosis						
MM	1·0 ± 1·7	ND	6·3 ± 2·8	5·7 ± 0·3	ND	ND
MM + $NaNO_3$ (10 mM)	5·3 ± 8·0	0·5 ± 0·5	8·1 ± 2·6	17·1 ± 1·3	ND	ND
MM + $NaNO_2$ (5 mM)	0·7 ± 1·3	0·4 ± 0·3	9·9 ± 2·6	9·8 ± 0·1	ND	ND
Anaerobiosis						
MM	110 ± 64	4 ± 5	3·5 ± 0·8	6·7 ± 1·4	ND	ND
MM + $NaNO_3$ (10 mM)	1171 ± 387	107 ± 133	6·0 ± 1·3	13·9 ± 5·4	1·0 ± 0·8	1·6 ± 0·5
MM + $NaNO_3$ (10 mM) + tungstic acid (2 mM)	176 ± 36	19 ± 27	2·9 ± 0·6	4·7 ± 1·6	ND	ND
MM + $NaNO_2$ (5 mM)	1532 ± 734	20 ± 20	4·9 ± 2·4	8·1 ± 3·5	0·57 ± 0·55	2·26 ± 0·6
MM + $NaNO_2$ (5 mM) + tungstic acid (2 mM)	1218 ± 320	10 ± 12	4·05 ± 2·1	5·4 ± 2·1	0·67 ± 0·8	1·8 ± 1·2

ND, Not detected.
* MM, minimal medium.

in *E. coli* and *Pr. morganii* was completely inhibited (Table 2). Thus it appears that even within the group of denitrifying bacteria, the nitrosating activity is not induced by the same mechanism(s). However, in *Pc. denitrificans* the ability to catalyse nitrosation correlated with the ability to reduce nitrate to nitrite, as previously observed in *E. coli* and *Pr. morganii.* Also the nitrosation activity of *Ps. aeruginosa* was strongly induced by culturing the bacteria in minimal medium containing either nitrate or nitrite. Moreover, tungstate inhibited the nitrate-mediated induction of both the nitrosating activity and the nitrate reductase, whereas it had no significant effect when these two activities were induced by nitrite (Table 3); these data suggest the possible involvement of a nitrite-reducing enzyme in the formation of nitrosamines by *Ps. aeruginosa*, when this nitrosating activity is induced by nitrite. The possibility that different nitrosating enzymes are induced when bacteria are cultured in the presence of either nitrate or nitrite is supported by a previous study (Garber & Hollocher, 1982), in which it was proposed that a dissimilatory nitrite reductase in denitrifying bacteria such as *Ps. aeruginosa* is responsible for the catalysis of the nitrosation reaction, the latter being measured by the catalysis of nitrosyl transfer from nitrite to azide, hydroxylamine and water.

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