A reassessment of the genetic determinants, the effect of growth conditions and the availability of an electron donor on the nitrosating activity of *Escherichia coli* K-12

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Anaerobic, but not aerobic, cultures of *Escherichia coli* K-12 catalysed the rapid nitrosation of the model substrate 2,3-diaminonaphthalene when incubated with nitrite. Formate and lactate were effective electron donors for the nitrosation reaction, which was inhibited by nitrate. Optimal growth conditions for the expression of nitrosation activity by various strains and mutants were determined. Highest activities were found with bacteria that had been grown anaerobically in a minimal medium rather than in Lennox broth, with glycerol and fumarate rather than glucose as the main carbon and energy source, and in the presence of a low concentration of nitrate. Bacteria harvested in the early exponential phase were more active than those harvested in later stages of growth. Well-characterized mutants defective in the synthesis of one or more anaerobically induced electron transfer chains were screened for nitrosation activity under these optimal growth conditions: only the respiratory nitrate reductase encoded by the *narGHJ* operon was implicated as a major contributor to nitrosation activity. Due to the limited sensitivity of the assays currently available, a minor contribution from the two alternative nitrate reductases or even other molybdoproteins could not be excluded. The role of formate in nitrosation was complex and was clearly not limited simply to that of an electron donor in the bacterial reduction of nitrite to nitric oxide: at least two further, chemical roles were inferred. This extensive study of more than 400 independent cultures of *E. coli* K-12 and its derivatives resolved some, but not all, of the apparently conflicting data in the literature concerning nitrosation catalysed by enteric bacteria.

Keywords: nitrosation, *Escherichia coli*, regulation, nitrite reduction, nitrosamine formation

INTRODUCTION

The formation of *N*-nitroso compounds by bacteria in the gastro-intestinal tract is widely believed to be a major cause of gastric cancer and the role of nitrite as the precursor of nitrosamines and nitrosamides is well established (Hill *et al.*, 1973; Kunisaki & Hayashi, 1979; Leach *et al.*, 1979, 1987; Leach, 1995; Reed *et al.*, 1981; Ralt & Tannenbaum, 1981). Many types of bacteria isolated from humans have been shown to catalyse nitrosation reactions in the laboratory at rates which

vary over a very wide range (Calmels *et al.*, 1985, 1987, 1988; De Bernadis *et al.*, 1983). Highest activities were reported for denitrifying bacteria, but these organisms are rarely isolated from the gastro-intestinal tract, even when the pH of gastric juice is abnormally high due to achlorhydria (Forsythe *et al.*, 1988). Consequently, despite their 10-fold lower catalytic activity, the far larger population of enteric bacteria can be predicted to contribute the majority of nitrosation activity in the human body. This has prompted many studies of the factors which determine the rates of bacterial nitrosation, as assayed with different model substrates (Calmels *et al.*, 1988; Ji & Hollocher, 1988; Kunisaki & Hayashi, 1979; O'Donnell & Edwards, 1992; Ralt *et al.*,

Abbreviation: DAN, 2,3-diaminonaphthalene.

1988). Most of these reports presented the preliminary results of what was stated to be part of a more comprehensive study, but results from the latter have failed to materialize. Consequently, many contradictory statements can be found in the literature. The closest to a consensus view to emerge is that nitrosation activity involves one or more molybdoproteins that are induced during anaerobic growth under the control of the transcription activator, FNR. The most active and hence the most important enzyme involved in nitrosation was shown to be the respiratory nitrate reductase, NarGHI (Calmels *et al.*, 1988; Ji & Hollocher, 1988; Ralt *et al.*, 1988). However, subsequent studies implicated the NADH-dependent nitrite reductase rather than nitrate reductase (O'Donnell & Edwards, 1992). The most recent study confirmed that spontaneous mutagenesis due to nitrosamine formation was dependent upon a molybdoprotein, but apparently eliminated a significant role for the respiratory nitrate reductase in this process (Taverna & Sedgwick, 1996).

Since the preliminary studies were completed, it has been established that *Escherichia coli* synthesizes not one but three biochemically and genetically distinct nitrate reductases during anaerobic growth: the two more recently discovered enzymes are the alternative, membrane-bound enzyme, NarZ, encoded by the *narZYWV* operon and periplasmic nitrate reductase, Nap, encoded by the complex *nap-ccm* operon (Blasco *et al.*, 1992; Grove *et al.*, 1996). Genes for previously uncharacterized molybdoproteins involved in dimethyl sulphoxide, trimethylamine *N*-oxide and nitrite reduction, as well as three formate dehydrogenase activities have been cloned and sequenced. In contrast to the earlier studies, the availability of a much wider range of well-characterized mutants has provided the opportunity to investigate whether all three nitrate reductases, the alternative, formate-dependent nitrite reductase, other molybdoproteins involved in formate oxidation or in the reduction of trimethylamine *N*-oxide and dimethyl sulphoxide can also contribute to the overall rate of bacterial nitrosation.

In all of the previous studies, bacteria were grown overnight to stationary phase before nitrosation activities were determined. In most cases, only a single medium composition, often containing an extremely high, non-physiological concentration of nitrate, was used (but see Calmels *et al.*, 1988 for a partial exception). We have therefore determined how nitrosation activity varies during exponential and stationary phases of growth, during aerobic or anaerobic growth, and during anaerobic growth in the presence or absence of different electron acceptors. The results present a much clearer view of some of the many factors which determine the nitrosation activity of enteric bacteria.

METHODS

***E. coli* strains and growth conditions.** The *E. coli* K-12 strains used in this study are listed in Table 1.

Unless otherwise stated, bacteria were grown anaerobically at

37 °C by transferring 5 ml of an aerated, overnight culture into 200 ml minimal salts supplemented with 5% (v/v) Lennox broth (LB), 0.4% (w/v) glycerol and 40 mM fumarate in an unshaken 250 ml conical flask. Sterile sodium nitrate or sodium nitrite was also added to the medium where noted. In some experiments, the minimal salts medium was replaced by full strength LB and 0.4% (w/v) glucose replaced the glycerol and fumarate as the main carbon and energy source. For aerobic cultures, bacteria were grown to the early-exponential phase in 200 ml medium in a well-aerated 2 litre conical flask and harvested at an optical density at 650 nm (OD_{650} measured using a Phillips PU8720 UV-vis recording spectrophotometer and 1 cm light path cuvettes) of less than 0.4 (corresponding to a biomass of less than 0.15 g dry mass l^{-1}).

Preparation of washed bacterial suspensions. Bacteria in the exponential phase of growth were harvested by centrifugation at 7000 r.p.m. for 10 min at 4 °C, washed twice with PBS (50 mM Na_2HPO_4 , 50 mM KH_2PO_4 , pH 7.3) and resuspended in PBS to a density of 7–10 mg bacterial dry mass ml^{-1} (corresponding to an OD_{650} of 0.6–0.8 for a 30-fold diluted sample). Harvested bacteria were stored on ice for a maximum of 3 h until assayed.

Determination of the nitrosation rate. Nitrosation was measured as described by Ralt *et al.* (1988). The analysis is based on the formation of a fluorescent triazole, 1*H*-naphthol [2,3-*d*] triazole, following nitrosation of the secondary amine 2,3-diaminonaphthalene (DAN).

A series of 1.5 ml closed Eppendorf tubes containing (final concentration) bacterial suspension (0.753 mg dry bacteria ml^{-1}), DAN (0.46 mM) and electron donor (usually 20 mM formate) were equilibrated to 37 °C and the reaction was started by the addition of a solution of sodium nitrite (to 25 mM). The final volume in each tube was 0.6 ml. At 5 min intervals for 30 min tubes were plunged into slush ice to stop the reaction and bacteria were sedimented by centrifugation. Part of the supernatant (0.3 ml) was transferred to tubes containing 2.7 ml PBS and the fluorescence was determined using a Perkin Elmer 1000 fluorescence spectrophotometer at an excitation wavelength of 375 nm and emission wavelength of 450 nm. As 1*H*-naphthol [2,3-*d*] triazole could not be obtained commercially, a calibration curve for the fluorimeter was constructed using 7-amino-4-methylcoumarin, which emits fluorescence at the same wavelength. To convert fluorescence measurements to quantities of DAN converted, a further calibration curve was established by incubating excess bacteria with a series of limiting quantities of DAN until the reaction had proceeded to completion. This revealed that 10 units of fluorescence correspond to 1 nmol 1*H*-naphthol [2,3-*d*] triazole under the stated conditions. In subsequent sections, 1 unit of nitrosation activity corresponds to 1 nmol 1*H*-naphthol [2,3-*d*] triazole formed (mg dry bacteria) $^{-1}$ min $^{-1}$.

Nitrate reductase activity. Bacteria which had been grown overnight in 500 ml Lennox broth supplemented with 0.4% glucose and 20 mM potassium nitrate were harvested by centrifugation (10000 g; 5 min), resuspended in 5 ml 50 mM potassium phosphate buffer, sedimented by centrifugation as above and resuspended in 5 ml fresh phosphate buffer. Samples were transferred to a 10 ml water-jacketed reaction vessel and a nitrate electrode coupled to an Ion Meter (Spectronic Analytical Instruments) was used to determine the rate of nitrate reduction at 30 °C in the presence or absence of 40 mM sodium formate.

Table 1. Strains of *E. coli* K-12 used and their source

Strain	Genotype or phenotype	Source
DSS401	<i>dms</i> derivative of MC4100	M. Chippaux & G. Giordano, CNRS, Marseilles, France
JCB387	Δ <i>nir</i> derivative of strain RV	Darwin <i>et al.</i> (1993)
JCB322	<i>Chl</i> ^R derivative of JCB387	Griffiths & Cole (1987)
JCB323	<i>Chl</i> ^R derivative of JCB387	Griffiths & Cole (1987)
JCB606	<i>dipZ</i> derivative of JCB387	Crooke & Cole (1995)
JCB3876	<i>fdhF</i> derivative of JCB387	Darwin <i>et al.</i> (1993)
JCB3877	<i>fdnG</i> derivative of JCB387	Darwin <i>et al.</i> (1993)
JCB3878	Δ (<i>nrf</i> - <i>fdhF</i>) derivative of JCB387	Darwin <i>et al.</i> (1993)
JCB3881	<i>sel</i> ::Kan ^R derivative of JCB387	Darwin <i>et al.</i> (1993)
JCB3882	<i>fdnG fdhF fdo</i> derivative of JCB387	Darwin <i>et al.</i> (1993)
JCB38700	Δ <i>nir narG</i> derivative of JCB387	This work
JCB38701	Δ <i>nir narZ</i> derivative of JCB387	This work
JCB38702	Δ <i>nir napA</i> derivative of JCB387	This work
JM712	<i>pro his trp</i> Δ <i>lac</i>	M. Jones-Mortimer, Mold, Clwyd, UK
LCB628	<i>tor</i> derivative of MC4100	M. Chippaux & G. Giordano, CNRS, Marseilles, France
MC4100	<i>araD</i> Δ (<i>argF-lac</i>) <i>ptsF deoC flbB relA rpsL</i>	Casadaban & Cohen (1979)
RV	Prototroph; parent of JCB strains	T. Young, University of Birmingham, UK
RV5265	<i>narG</i> derivative of strain RV	This work

RESULTS

Nitrosation of DAN by *E. coli* and its inhibition by nitrate

Initial attempts to detect nitrosation of DAN by anaerobically grown cultures of several *E. coli* strains were unsuccessful. Calmels *et al.* (1988) reported that nitrate in the reaction mixture strongly inhibits *in vitro* nitrosation. To test the possibility that inability to detect nitrosation activity was due to the carry-over of nitrate from the growth medium, *E. coli* strain RV was grown anaerobically in LB supplemented with 0.4% glycerol, 40 mM fumarate and 100 mM nitrate, harvested, washed twice with PBS and resuspended in PBS. Nitrosation activity was readily detected when bacteria had been washed and assayed in the absence of nitrate or growth medium, either in the presence or absence of formate, with rates significantly higher than the chemical rate (Fig. 1). Higher rates were detected when bacteria were assayed with the electron donor formate. Nitrosation activity was strongly inhibited by the growth medium and to a lesser extent in the presence of 10 mM nitrate. It can be concluded that carry-over of nitrate from the growth medium into reaction mixtures to be assayed was responsible for the failure to detect nitrosation activity in previous experiments.

The rate of nitrosation was assayed in the presence or absence of the potential electron donors lactate, ethanol, glucose, succinate, pyruvate and formate. The highest rate, 310 units nitrosation activity [nmol 1*H*-naphthol

[2,3-*d*] triazole formed (mg dry bacteria)⁻¹ h⁻¹], was observed with formate. Rates in the presence of lactate were 75% and with glucose around 50% of the maximum activity (210 and 140 units, respectively), but rates in the presence of succinate, ethanol and pyruvate were comparable to the endogenous rate in the absence of an electron donor. In subsequent experiments bacteria were assayed in the presence and absence of 20 mM formate as the electron donor.

Nitrosation rates and formate-dependence of nitrosation activity in bacteria at various stages of growth

In most of the previous studies of nitrosation catalysed by *E. coli*, bacteria were grown overnight to stationary phase in media containing 100 mM nitrate, a concentration far higher than that encountered *in vivo* which rarely, if ever, exceeds 1 mM (Forsythe *et al.*, 1988). In initial experiments to determine the effect of growth phase on the nitrosation rates and formate-dependence of nitrosation, *E. coli* strain RV was grown anaerobically in minimal salts supplemented with 0.4% glycerol, 40 mM fumarate and, in parallel experiments, with either 5 mM or 100 mM nitrate. Bacteria in the middle- and late-exponential and stationary phases of growth were collected by centrifugation, washed twice, resuspended in PBS and then assayed with or without formate. The highest formate-dependent rate of nitrosation was catalysed by bacteria which had been

Fig. 1. Nitrosation of DAN by *E. coli* and its inhibition by nitrate. *E. coli* strain RV was grown anaerobically in 250 ml flasks filled with Lennox broth supplemented with 0.4% glycerol, 40 mM fumarate and 100 mM nitrate. Bacteria in the stationary phase were harvested, washed and assayed for nitrosation activity in the presence or absence of formate and nitrate. ■, Formate, no nitrate; ○, no formate, no nitrate; □, formate plus nitrate; ●, formate plus growth medium.

harvested in the middle of the exponential phase of growth. The formate-dependent activity of bacteria harvested in the late exponential phase was around 70% of the maximum 450 ± 37 units of activity observed during the middle of the exponential phase of growth. The nitrosation rates of bacteria harvested in the stationary phase of growth were more variable, typically in the range 50–70% of the fully induced rate during exponential growth. Endogenous rates of bacteria harvested in all three stages of growth were consistently far lower than rates in the presence of formate (47 ± 1 units for exponential-phase cultures; 16 ± 3 units for late-exponential-phase cultures and 18 ± 1 units for stationary-phase cultures).

In a further series of experiments, nitrosation activities of independent cultures of bacteria which had been grown in different batches of culture medium and harvested either in the middle of the exponential phase or in the stationary phase of growth were compared (Table 2). Not only were stationary-phase bacteria less active, but both the rates detected and the effect of formate on nitrosation activity were more variable than those of exponential-phase bacteria. In all subsequent experiments bacteria were harvested in the middle of the exponential phase of growth to achieve optimal nitrosation activity.

The effect of culture conditions on the induction of nitrosation activity in *E. coli* strain RV

To determine the optimal growth conditions for the development of nitrosation activity, *E. coli* strain RV was grown anaerobically in either Lennox broth or minimal medium in the presence of either 0.4% glycerol and 40 mM fumarate or 0.4% glucose as the main sources of carbon and energy. Some cultures were supplemented with sodium nitrate or nitrite. Bacteria were harvested in the exponential phase of growth, washed and assayed for nitrosation activity in the presence or absence of formate.

Rates of nitrosation by bacteria which had been grown in rich media were consistently lower than those grown in minimal media, irrespective of whether the carbon and energy source was glucose or glycerol and fumarate (Table 3). Furthermore, the rate of nitrosation in the presence of formate was significantly or far higher than the chemical rate. With one exception, nitrosation rates were also far higher in the presence of formate than in its absence. However, the endogenous rate of nitrosation was consistently higher than the rate in the presence of formate when bacteria had been grown in minimal medium with glycerol and fumarate but in the absence of nitrate or nitrite. Highest formate-dependent rates of nitrosation were found with bacteria that had been grown in minimal medium supplemented with glycerol, fumarate and a low concentration of nitrate. Nitrosation activity was not induced during growth in the presence of nitrite. No nitrosation activity could be detected in bacteria which had been grown aerobically.

Table 2. Nitrosation activities of independent cultures of bacteria harvested either in the middle of the exponential phase or in the stationary phase of growth

Nitrosation activity is expressed as nmol 1*H*-naphthol [2,3-*d*] triazole formed (mg dry bacteria)⁻¹ h⁻¹.

Growth phase	Formate in assay	No. of cultures	Mean	Range	Standard deviation
Exponential	—	7	6	4–12	4.2
	+	7	329	242–402	70
Stationary	—	4	21	<1–50	21
	+	5	212	107–373	102

Table 3. Effect of composition of the growth medium on nitrosation activity by the wild-type *E. coli* strain RV

Culture conditions*			Nitrosation activity†	
Medium	Carbon source	Supplements	Endogenous	Formate-dependent
LB	Glucose (0.4%)	NaNO ₃ (100 mM)	<10	350 ± 3.5
LB	Glycerol (0.4%) + fumarate (40 mM)	NaNO ₃ (100 mM)	<10	350 ± 13
MM	Glucose (0.4%)	NaNO ₃ (100 mM)	22 ± 2	86 ± 9
MM	Glycerol (0.4%) + fumarate (40 mM)	No supplement	434 ± 31	232 ± 26
MM	Glycerol (0.4%) + fumarate (40 mM)	NaNO ₃ (5 mM)	11 ± 9.5	399 ± 11.4
MM	Glycerol (0.4%) + fumarate (40 mM)	NaNO ₃ (100 mM)	20 ± 2.9	253 ± 6
MM	Glycerol (0.4%) + fumarate (40 mM)	NaNO ₂ (2.5 mM)	<10	199 ± 2.5

* Bacteria were grown anaerobically in either minimal salts medium (MM) or Lennox broth (LB) supplemented as shown, washed and assayed anaerobically in the presence of 20 mM sodium nitrite with or without 20 mM formate as the electron donor.

† Nitrosation activity is expressed as nmol 1*H*-naphthol [2,3-*d*] triazole formed (mg dry bacteria)⁻¹ h⁻¹. The chemical rate, 8.5 ± 1, was deducted from all values. Values are means ± SD of two to four assays.

Nitrosation activity in *E. coli* mutants deficient for a combination of nitrate and nitrite reductases

Although the respiratory nitrate reductase encoded by the *narGHJI* operon was implicated as the major source of nitrosating activity (Ralt *et al.*, 1988; Ji & Hollocher, 1988; Calmels *et al.*, 1988), two subsequent studies have led to opposite conclusions. O'Donnell & Edwards (1992) concluded from studies of a *nir* mutant, strain LCB82, that the NADH-dependent nitrite reductase was a major contributor. In contrast, on the basis of comparisons of mutagenesis caused by nitrosamine formation by a *narG* mutant and a *moa* mutant which is unable to synthesize the molybdenum cofactor essential for all nitrate reductase activity, Taverna & Sedgwick (1996) concluded that a molybdoprotein was involved, but the respiratory nitrate reductase was not essential. A possible explanation for the conflict between these studies is that since 1988 it has been recognized that *E. coli* synthesizes multiple nitrate and nitrite reductases encoded by operons that have only recently been cloned and sequenced (Blasco *et al.*, 1992; Darwin *et al.*, 1993; Grove *et al.*, 1996; Hussain *et al.*, 1994). The growth conditions used previously were not optimal either for the induction of nitrosation activity or for the expression of the operons for these alternative reductases, so the possibility that nitrosation activity is catalysed by these alternative enzymes, either as well as or instead of Nir and NarG, has not been assessed. As Nap and Nrf activities are both expressed optimally during anaerobic growth with a limiting concentration of nitrate or nitrite, conditions under which NarZ and Nir are also expressed (Darwin *et al.*, 1993; Grove *et al.*, 1996), a series of well-characterized mutants defective in genes known to be essential for the synthesis of all known anaerobically induced electron transfer pathways in *E. coli* were grown anaerobically in the presence of 5 mM nitrate and assayed under optimal conditions to identify which enzymes are directly implicated in the nitrosation reaction.

The rate of nitrosation by strain JCB387, which lacks only the major, NADH-dependent nitrite reductase, was similar to that of the parental strain, RV (Table 4). As this is a deletion mutant, the possibility that the sirohaem-dependent but molybdopterin-independent nitrite reductase is a major contributor to nitrosation activity can be excluded (O'Donnell & Edwards, 1992). Strain JCB387 was the parent of many of the strains used in this study. Genes for the alternative, formate-dependent nitrite reductase have been deleted from strain JCB3878; this strain was also as active as the parental strain in the nitrosation assay. In contrast, no nitrosation activity was detected in two chlorate-resistant mutants, strains JCB322 and JCB323, which lack all molybdoproteins due to their inability to synthesize the molybdopterin cofactor. This agrees with previous reports that nitrosation activity requires the activity of one or more molybdoproteins (Calmels *et al.*, 1987; Ralt *et al.*, 1988; Ji & Hollocher, 1988). Note that fumarate reductase activity and the presence of fumarate in the medium were essential for the growth of these *moa* strains; this excludes the formal but unlikely possibility that fumarate reductase contributes significantly to nitrosation activity.

Three anaerobically expressed electron transfer chains are known to require *c*-type cytochromes to transfer electrons from the quinone pool to the terminal reductase. Two of these reductases, Nap and the trimethylamine-*N*-oxide reductase, Tor, are molybdoproteins. Strain JCB606 is unable to synthesize any of the five known *c*-type cytochromes in *E. coli* and is therefore Nrf⁻, Nap⁻ and Tor⁻. The nitrosation activity of strain JCB606 was the same (or possibly slightly higher) than that of the parental strain.

Finally, mutants defective in each of the three known nitrate reductases were constructed. Only the mutant lacking the major, respiratory nitrate reductase encoded by the *narGHJI* operon was severely defective in nitrosation activity (Table 4). Furthermore, no nitro-

Table 4. Genetic determinants of nitrosation activity by *E. coli*

Strain	Relevant genotype	Nitrosation activity*	
		Endogenous	Formate-dependent
RV	Parental strain	11 ± 9.5	399 ± 11.4
JCB387	Δnir	< 10	304 ± 10.5
JCB322	$\Delta nir moa^\dagger$	< 10	< 10
JCB323	$\Delta nir moa^\dagger$	< 10	< 10
JCB606	$\Delta nir dipZ$	< 10	433 ± 23
JCB3878	$\Delta nir nrf$	< 10	333 ± 20
JCB38700	$\Delta nir narG$	< 10	< 10
RV5265	$narG$ in RV	< 10	< 10
JCB38701	$\Delta nir narZ$	< 10	450
JCB38702	$\Delta nir napA::\Omega$	< 10	539
Isogenic strains in the MC4100 background			
MC4100	Wild-type	< 10	205
LCB628	tor	< 10	125
DSS401	dms	< 10	165

* Nitrosation activity is expressed as nmol 1H-naphthol [2,3-*d*] triazole formed (mg dry bacteria)⁻¹ h⁻¹. Values are means ± SD of two to four assays; where no SD is given, values are means of two independent assays.

† *moa*, chlorate-resistant strain due to a mutation in genes essential for the synthesis of the prosthetic group of nitrate reductase and other molybdoproteins, molybdopterin guanine dinucleotide.

sation activity was detected when the *narG* mutant was grown in the absence of nitrate, or with excess nitrate (data not shown). We conclude that the requirement for anaerobic growth and the stimulatory effect of nitrate during growth on the development of nitrosation activity was due to the induction of the NarGHI complex. Consistent with this conclusion and in agreement with an earlier report by Ralt *et al.* (1988), nitrosation activity increased in parallel with the induction of nitrate reductase activity when strain JCB387 was grown anaerobically in the presence or absence of 2.5 mM nitrite or 100 mM nitrate (data not shown). These combined results establish that neither Nap nor NarZ contribute significantly to the overall rate of nitrosation by *E. coli*, which is directly dependent on the presence of the membrane-bound nitrate reductase encoded by the *narGHJI* operon in *E. coli*.

Nitrosation activity of strains defective in formate dehydrogenase activity

The electron donor in this and other studies was formate, which is oxidized by three biochemically and genetically distinct formate dehydrogenases, all of which are molybdoproteins. The effects of mutations resulting in the loss of one or all of these activities was therefore investigated. The major formate dehydrogenase induced during anaerobic respiratory growth in the presence of nitrate is Fdn (Berg & Stewart, 1990; Berg *et al.*, 1991). During anaerobic growth, transcription of the *fdnGHJ* operon is activated coordinately with the respiratory

nitrate reductase operon, *narGHJI*, by two transcription factors, FNR and NarL (Rabin & Stewart, 1993; Stewart, 1993); however, the rate of formate-dependent nitrosation by the Fdn mutant, JCB3877, was similar to that of the parental strain, JCB387 (Table 5). The same was true for strain JCB3876 which lacks the formate dehydrogenase formed during fermentative growth, FDH_H; this enzyme couples formate oxidation to H⁺ reduction by the formate hydrogenlyase complex (Peck & Gest, 1957; Gest & Peck, 1955; Gray & Gest, 1965; Böhm *et al.*, 1990; Sauter *et al.*, 1992). Particularly surprising was the observation that formate still stimulated nitrosation by two mutants which lack all three known formate dehydrogenases (Table 5). One of these strains, JCB3882, carries three separate mutations in each of the three operons encoding the formate dehydrogenases; the other strain, JCB3881, is pleiotropically negative due to the deletion of a gene required for the insertion of an essential selenocysteine residue in all three formate dehydrogenase molybdoproteins (Pinsent, 1954; Lester & DeMoss, 1971; Sawers *et al.*, 1991). Two further tests were used to confirm that these mutants had been constructed correctly and had not reverted. First, they were both shown to be defective for formate hydrogenlyase activity, a reaction that requires a functional *fdhF* gene encoding FdhH. Secondly, their rates of nitrate reduction in the presence and absence of formate were compared with those of the parental strain, JCB387. The slow endogenous rate of nitrate reduction by washed suspensions of the parental strain increased more than 10-fold upon addition of formate [from 30 to

Table 5. Nitrosation activities of formate dehydrogenase mutants after growth in the presence or absence of nitrate

Strain	Culture conditions*	Relevant genotype	Nitrosation activity†	
			Endogenous	Formate-dependent
JCB387	– Nitrate	<i>E. coli</i> RV $\Delta nir B$	434	266
	+ Nitrate		220	506
JCB3876	– Nitrate	JCB387 <i>fdhF</i>	422	306
	+ Nitrate		79	413
JCB3877	– Nitrate	JCB387 <i>fdnG</i>	453	288
	+ Nitrate		224	454
JCB3881	– Nitrate	JCB387 $\Delta sel::kan$	437	320
	+ Nitrate		244	472
JCB3882	– Nitrate	JCB387 <i>fdnG fdhF fdo</i>	270	251
	+ Nitrate		146	356

* Bacteria were grown in minimal salts supplemented with 0.4% glycerol, 40 mM fumarate, 5% LB with or without 5 mM nitrate.

† Nitrosation activity is expressed as nmol 1*H*-naphthol [2,3-*d*] triazole formed (mg dry bacteria)^{–1} h^{–1}. Values are means of two independent assays.

310 nmol of nitrate reduced (mg dry bacteria)^{–1} min^{–1}]. In contrast, no formate-dependent increase in rate was detected with either of the mutants. These observations suggest that the primary role of formate in nitrosation is to supply reductant for a chemical rather than an enzymic reaction. An alternative, but unlikely possibility is that there is a fourth, currently uncharacterized formate dehydrogenase in *E. coli*. If so, this fourth enzyme must be sufficient to sustain full nitrosation activity; furthermore, it cannot be a selenoprotein.

Effect of aeration during the assay on the rate of nitrosation

Previous data indicated that bacteria grown in the absence of nitrate or nitrite catalyse a higher rate of nitrosation in the absence of formate than in its presence (Table 3). Furthermore, the endogenous rate of nitrosation by these bacteria is similar to that of bacteria grown anaerobically with nitrate and assayed in the presence of formate. Several possible explanations for this inhibition of nitrosation by formate were considered. Two of the initial stages in the nitrosation reaction are the enzyme-catalysed formation of nitric oxide followed by the spontaneous, but oxygen-dependent, chemical oxidation of the complex formed between NO and the nitrosated substrate (Ji & Hollocher, 1988). It is conceivable that formate inhibits one or other of these two stages, or at some stage competes for a key substrate such as the nitrosated intermediate or oxygen. Any explanation must also account for the differences between bacteria that are only partially induced for nitrosation and those that are more fully induced.

To test whether oxygen was limiting in the second stage of the nitrosation reaction, strain RV was grown anaerobically in minimal medium supplemented with 0.4% glycerol and 40 mM fumarate in the presence or absence of 5 mM nitrate. Bacteria were harvested in the exponential phase of growth, washed and assayed in the presence or absence of formate in 25 ml flasks which were either vigorously aerated during the assay, or left unshaken. The rates of nitrosation catalysed by bacteria that were aerated during the assay were approximately 50% of those that were not aerated. Furthermore, bacteria which had been grown in the absence of nitrate attained higher rates of nitrosation in the absence of formate than in its presence (120 units for the aerated sample in the absence of formate and 90 units in the presence of formate, compared with 230 and 200 units, respectively, for the samples that were not aerated during the nitrosation assay). In contrast, endogenous rates were suppressed and formate-dependent rates were higher when bacteria were grown in media supplemented with nitrate (120 and 67 units for the aerated samples assayed in the presence or absence of formate compared with 350 and 120 units for the unaerated assay samples, respectively). In conclusion, the consistently higher endogenous rate than the rate in the presence of formate by bacteria that had been grown in the absence of nitrate or nitrite cannot be explained simply by a formate-enhanced oxygen deficiency during the nitrosation reaction.

DISCUSSION

Although the ability of enteric bacteria to catalyse nitrosation is well-established, preliminary attempts to identify the enzymes involved have led to conflicting

conclusions. More than 400 independent cultures of well-characterized *E. coli* strains grown under a variety of conditions have enabled us to resolve some, but not all, of the apparent discrepancies in the literature. These experiments have provided the first systematic analysis of the relative roles of all known anaerobically induced electron transfer pathways in *E. coli* in nitrosation, how nitrosation activity varies with growth conditions, and the role of formate as an electron donor for the nitrosation reaction. Preliminary experiments (data not shown) established that *E. coli* strain RV, which was used for most of these experiments, expressed slightly higher nitrosation activity than *E. coli* strain M182 and threefold higher activity than strain JM712; all three of these strains have been used previously in this laboratory to study the regulation of anaerobically expressed electron transfer chains (Hussain *et al.*, 1994; Grove *et al.*, 1996).

Genetic determinants of nitrosation activity

The availability of a comprehensive range of mutants defective in each of the known anaerobic respiratory pathways of *E. coli* has enabled us to confirm previous reports that the major, respiratory nitrate reductase is essential for virtually all of the nitrosation activity (Calmels *et al.*, 1988; Ralt *et al.*, 1988; Ji & Hollocher, 1988). Although the assay used to measure nitrosation rates is the most sensitive currently available (Ralt *et al.*, 1988), the lower limit of detection is still rather high, about 1% of the optimal rate detected in fully induced bacteria. The strains used in this study were grown under conditions optimal for Nap and NarZ expression; nevertheless, we estimate that these alternative enzymes contribute less than 5% to the total rate of nitrate reduction (G. Thomas, L. Potter & J. A. Cole, unpublished results). Any gratuitous conversion of nitrite to nitric oxide by NarZ or Nap at a specific activity similar to that of the major, respiratory nitrate reductase would be too slow to be detected reliably by this assay. Consequently, although a major contribution by other enzymes can be excluded, it is not possible to state that they do not contribute at all to the overall rate of nitrosation.

Dependence of nitrosation activity on the growth conditions

Factors which influence the nitrosation activity of *E. coli* include the phase at which bacteria are harvested, whether bacteria are grown aerobically or anaerobically, the carbon source and the richness of the medium, and the electron acceptor available during anaerobic growth.

In none of the previous studies was the effect of growth phase on nitrosation activity reported, but Ralt *et al.* (1988) commented that similar activities were found with exponential-phase and overnight cultures. However, no additional carbon source was added by these authors to support growth, so it is not clear whether the cultures used had entered the stationary phase when the bacteria were harvested after overnight growth. The

nitrosating activity of several strains used in this study was twice as high in exponentially growing bacteria as in those harvested after overnight growth (typically about 16 h). Furthermore, the endogenous rate of nitrosation in the absence of an added electron donor was often much greater than that of exponential-phase cultures. We were unable to establish a correlation between this increase and either the disappearance of nitrate from the culture or with the accumulation of nitrite during growth.

A critical factor in determining the nitrosation activity of cultures was the degree of aeration. Contrary to the results of O'Donnell & Edwards (1992), virtually no activity was detected in bacteria grown in well-aerated cultures. Control experiments in this laboratory in which an oxygen electrode was suspended in 400 ml of medium in a 'well-aerated' 2 litre conical flask revealed that the culture effectively became oxygen-limited when the density approached 0.15 mg dry bacteria ml⁻¹ (corresponding to an OD₆₅₀ of only 0.4). At this density of bacteria, most anaerobically induced electron transfer chains which are activated by the FNR protein are at least partially derepressed (unpublished observations). Consequently, it is essential to harvest bacteria at an early stage in the growth cycle for effects of oxygen on metabolism to be assessed reliably. It is not clear whether this requirement was satisfied in the earlier studies.

In most of the previous studies, bacteria were grown in a rich broth supplemented with very high concentrations of nitrate. It is now clear that such conditions are far from optimal for the development of nitrosation activity, which was far higher after anaerobic growth in a minimal salts medium than in a rich broth. It was also induced strongly by nitrate, but partially repressed by glucose. Higher activities were detected in bacteria that had been grown with a low, limiting concentration of nitrate, 5 mM, than with an unphysiologically high concentration, 100 mM. Nitrite accumulated in both types of culture and was present when the bacteria were harvested, but nitrate was still present only in the culture grown with the higher nitrate concentration. Measurement of the nitrate reductase activity of some cultures confirmed earlier reports that nitrosation activity increased with increasing nitrate reductase activity, but strict proportionality between the two was not established. This implies that other factors also determine the nitrosation activity of a culture. Note particularly the very high nitrosation activity of bacteria grown in the absence of nitrate and assayed in the absence of an added electron donor (Table 3). Nitrate reductase activity was only weakly induced in these cultures.

A surprising role for formate in bacterial nitrosation

Nitrosation of model substrates such as DAN proceeds in two distinct stages, the enzymic reduction of nitrite to nitric oxide, followed by a series of chemical reactions in which O₂ is thought to form the direct nitrosylating species, N₂O₃ and N₂O₄ (Ji & Hollocher, 1988). Electrons are required for the first reaction, and formate

is recognized as a preferred electron donor for the respiratory nitrate reductase (Ruiz-Herrera & DeMoss, 1969; Enoch & Lester, 1975). Other *E. coli* fermentation products as well as NADH generated during catabolism can also donate electrons to nitrate reductase. The role of formate in enhancing the rate of nitrosation, and the ability of lactate, pyruvate or glucose to replace formate, all suggest that the primary role of the electron donor is to provide electrons for nitrite reduction to NO by nitrate reductase. If so, mutants defective in formate dehydrogenase activity should be defective in formate-dependent nitrosation. This was not observed (Table 5). This suggests that formate must fulfill a chemical role in nitrosation, possibly acting as a reducing agent. Such a role would be consistent with the observation of Ji & Hollocher (1988) that formate has little effect on the yield of triazole if bacteria are incubated with nitrite and DAN under strictly anaerobic conditions. Note, however, that the role of formate cannot be to remove oxygen biochemically because formate dehydrogenase activity is also essential for electron transfer from formate to oxygen (Pinsent, 1954). Also puzzling is why formate inhibits nitrosation by bacteria that have been grown in the absence of nitrate, even in mutants defective in the fermentative formate dehydrogenase, FDH_H. No single explanation can be offered to explain all of these observations. Rather, they suggest that formate plays several, conflicting roles in the multiple stages of DAN nitrosation, acting both to enhance and inhibit one or more of the chemical reactions involved after the enzyme-catalysed production of NO. The ability of formate to donate electrons via any of the three *E. coli* formate dehydrogenases and either menaquinone or ubiquinone to the respiratory nitrate reductase is only of secondary significance.

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