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Production of 3-Nitrosoindole Derivatives by *Escherichia coli* during Anaerobic Growth[▽]

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When *Escherichia coli* K-12 is grown anaerobically in medium containing tryptophan and sodium nitrate, it produces red compounds. The reaction requires functional genes for trytophanase (*tnaA*), a tryptophan permease (*tnaB*), and a nitrate reductase (*narG*), as well as a natural drop in the pH of the culture. Mass spectrometry revealed that the purified chromophores had mass/charge ratios that closely match those for indole red, indoxyl red, and an indole trimer. These compounds are known products of chemical reactions between indole and nitrous acid. They are derived from an initial reaction of 3-nitrosoindole with indole. Apparently, nitrite that is produced from the metabolic reduction of nitrate is converted in the acid medium to nitrous acid, which leads to the nitrosation of the indole that is generated by tryptophanase. An *nfi* (endonuclease V) mutant and a *recA* mutant were selectively killed during the period of chromophore production, and a *uvrA* strain displayed reduced growth. These effects depended on the addition of nitrate to the medium and on tryptophanase activity in the cells. Unexpectedly, the killing of a *tnaA*$^+$ *nfi* mutant was not accompanied by marked increases in mutation frequencies for several traits tested. The vulnerability of three DNA repair mutants indicates that a nitrosoindole or a derivative of a nitrosoindole produces lethal DNA damage.

Under hypoxic conditions, nitrate replaces oxygen as the preferred electron acceptor in *Escherichia coli*. The nitrate is first reduced to nitrite by periplasmic and cytoplasmic nitrate reductases, and then nitrite is reduced directly to ammonia by nitrite reductases (22). The nitrite, which is an intermediate, is in equilibrium with molecular nitrous acid (HNO_2), which can condense with the loss of water to form N_2O_3 (dinitrogen trioxide, nitrous anhydride). N_2O_3 is a harmful nitrosating agent that reacts with many cellular constituents, including DNA (57). The N nitrosation of the exocyclic amines of the nucleobases in DNA results in mutagenic deamination (46) and cross-linking of strands (26). Nitrosation of ring nitrogens in DNA destabilizes the glycosylic bond, leading to depurination (36). The nitrosation of secondary amines and amides can turn them into mutagenic alkylating agents (42, 51). The cell is protected from potential damage by high nitrite reductase activity, by a membrane pump that extrudes excessive nitrite, and by the low pK of HNO_2, which results in an unfavorable equilibrium for its production at neutral pH (22). Nevertheless, nitrosative mutagenesis does occur under these conditions (56). It results mainly from the apparent production of a small amount of nitric oxide as a by-product of cytoplasmic nitrite reductase (12). The nitric oxide is auto-oxidized to N_2O_3 when the culture is exposed to air (56).

For study of nitrosative mutagenesis under hypoxic conditions, *E. coli* was grown in sealed tubes in a medium containing nitrate as an electron acceptor (56). In this study, we report our observation that some strains formed red pellets after they grew to saturation, whereas other cultures remained almost white. We show that the red cultures produced tryptophanase and therefore indole, whereas the white cultures had an unsuspected tryptophanase mutation. We purified the red compounds and identified them as condensation products of indole that were probably derived from 3-nitrosoindole, and we studied the toxicity and possible mutagenicity of the growth conditions.

MATERIALS AND METHODS

Strains, media, and growth conditions. The strains used are listed in Table 1. Luria-Bertani (LB) media (38) were used for routine growth. Anaerobic growth medium consisted of LB broth that was supplemented with 40 mM sodium fumarate, 0.4% glycerol, and 100 mM $NaNO_3$. It was filter sterilized to avoid destruction of tryptophan by autoclaving. The cultures were placed in screw-cap tubes or bottles that were overfilled before capping so that they had no air space. Vogel-Bonner medium E (53) was used as a minimal medium. It was supplemented with 0.4% glucose or 0.1% lactose, 10 μg/ml of thiamine, and 100 μg/ml of required L amino acids. Antibiotics were used at the following concentrations: streptomycin, 200 μg/ml; kanamycin, 50 μg/ml; chloramphenicol, 20 μg/ml; and ampicillin, 100 μg/ml.

Tryptophanase assays. Stationary-phase cells were washed by centrifugation and resuspended in 20 mM potassium phosphate (pH 7.8). Assays were performed with supernatants of sonic extracts using reaction conditions that were described previously (50), except that 12 mM tryptophan replaced S-*o*-nitrophenyl-L-cysteine as the substrate, and indole production was measured with Kovac's reagent (31). Protein concentrations were determined by the method of Bradford (8) with the Pierce Coomassie Plus protein reagent.

Isolation and analysis of indole derivatives. Strain RV was inoculated into anaerobic growth medium and grown to saturation at 37°C in a filled 1-liter bottle with no air space. The cells were pelleted by centrifugation. The supernatant was extracted with 1 ml of chloroform. The cell pellet was resuspended in 2 ml of water and extracted with 5 ml of diethyl ether. The samples were concentrated under a stream of air to about 50 to 100 μl each and applied to a thin-layer chromatography (TLC) cellulose sheet (Brinkmann MN300), which was then developed with hexane-ethyl acetate-acetic acid (70:30:1, vol/vol/vol) (39). For analysis by mass spectrometry, the spots were eluted from multiple chromatograms with diethyl ether and purified twice more by TLC. Mass spectrometry was performed at the Emory University Mass Spectrometry Center

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TABLE 1. Bacterial strains used

Strain	Relevant genotype[a]	Source or reference[b]
BW386	*recA56 srlD300*::Tn*10*	Laboratory collection
BW1177	KD1092 *trpA58 nfi-1*::*cat*	41
BW1824	CC106 *tna*$^+$ *dgoA*::Tn*10dkan*	This study
BW1996	KD1092 *uvrA277*::Tn*10*	P1(N3055) × KD1092[c]
BW1997	KD1092 *uvrA277*::Tn*10* Δ*tnaA739*::*kan*	P1(N3055) × BW2021[c]
BW2018	RV Δ*tnaA739*::*kan*	P1(JW3686-7) × RV
BW2019	RV *tnaB*::mini-Tn*5-lac-tet/1*	P1(MT113) × RV
BW2020	RV *narG205*::Tn*10*	P1(RK5268) × RV
BW2021	KD1092 Δ*tnaA739*::*kan*	P1(JW3686-7) × KD1092
BW2022	KD1092 Δ*tnaA739*::*kan nfi-1*::*cat*	P1(JW3686-7) × BW1175
BW2023	KD1092 *recA56 srlD300*::Tn*10*	P1(BW386) × KD1092[c]
BW2024	KD1092 Δ*tnaA739*::*kan recA56 srlD300*::Tn*10*	P1(BW386) × BW2021[c]
CC106	*tnaA* Δ(*gpt-lac*)*5* (F′ *lacZCC106*)[d]	14
JCB387	RV Δ*nirB*	15
JW3686-7	Δ*tnaA739*::*kan*	3
KD1092	*trpA58*	16
MT113	*tnaB*::mini-Tn*5-lac-tet/1*	4
N3055	*uvrA277*::Tn*10*	CGSC[e]
RK5268	*narG205*::Tn*10*	48
RV	Δ*lac tna*$^+$	15

[a] All strains are derivatives of *E. coli* K-12 and are F$^-$ λ$^-$ unless stated otherwise. *cat*, *kan*, and *tet* are genes specifying resistance to chloramphenicol, kanamycin, and tetracycline, respectively.

[b] Transductions with phage P1 are described as follows: P1(donor) × recipient.

[c] Tetracycline-resistant transductants were tested for UV sensitivity (*recA*).

[d] The *tnaA* mutant genotype was found in this study (see Results).

[e] CGSC, Coli Genetic Stock Center, Yale University (http://cgsc.biology.yale.edu).

using samples in acetonitrile or acetonitrile-water (1:1) and a Thermo Electronics LTQ-FTMS machine at 100k resolution. Xcalibur and SciFinder software were used to determine the probable atomic compositions and identities of compounds based on their mass/charge (*m*/*z*) ratios.

Mutation frequencies. Stationary-phase bacteria were diluted to a concentration of 2×10^5 cells/ml in anaerobic growth medium and incubated anaerobically at 37°C for 24 h in multiple screw-cap glass tubes (13 by 100 mm). To score mutations that resulted in tryptophan independence, the cells were washed by centrifugation in 10 mM $MgSO_4$, spread on minimal medium lacking tryptophan, and incubated for 2 days at 37°C. The other markers were scored after further aerobic growth to allow segregation. About 2×10^7 cells from each 24-h anaerobic culture were transferred to 2 ml of fresh LB broth, grown to saturation on a roller overnight at 37°C, washed twice by centrifugation in 10 mM $MgSO_4$, and spread on selective media. Before selective plating, samples were removed from two cultures of each strain to determine the viable cell count by dilution and plating on LB agar. The selective media were LB agar containing 200 μg/ml of streptomycin and minimal agar medium containing either 40 μg/ml of 6-azauracil or 100 μg/ml of L-valine.

Other methods. Generalized transduction with phage P1 *dam rev6* was performed as previously described (47). Random insertional mutagenesis was performed by transposition of mini-Tn*10dkan* (element 103) from a bacteriophage λ vector (33). An insertion in the *dgoA* gene was identified by inverse PCR (52), using an endonuclease AluI digest of chromosomal DNA. The Tn*dkan* primers that were used were CCACCTTAACTTAATGATTTTTACCA and GGTCTG CCTCGTGAAGAAGG. The PCR product was sequenced by the Macrogen Corp. (Seoul, Korea), using the same primers. Plasmid isolation (7) and plasmid transformation (11) were performed as previously described. Digital photographs were imported into Adobe Photoshop, and their background casts were adjusted to white by overall changes in the color balance.

RESULTS

Locus for chromophore production. We observed that some *E. coli* strains, such as KL16 (5), RV (15), and AB1157 (5), produced a red cell pellet when they were grown in our nitrate-containing anaerobic growth medium, whereas the Cupples-Miller mutation indicator strains (13, 14), such as CC106 (Fig. 1), did not do this. To localize the chromophore, which we

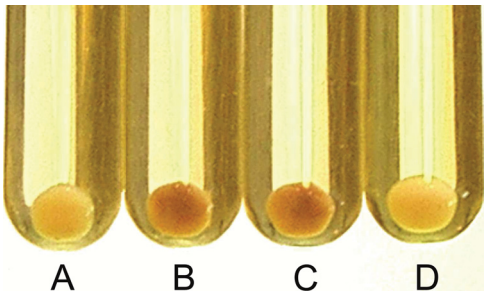


FIG. 1. Pellets of bacterial cultures grown anaerobically in the presence of 100 mM nitrate. The photograph shows the lower portions of tubes containing centrifuged saturated cultures. The strains used were CC106, a *tna* mutant (tube A); BW1824, a *tna*$^+$ transductant of CC106 (tube B); RV (tube C); and BW2018, a *tnaA* mutant of RV (tube D).

initially believed to be a cytochrome, we tried to release periplasmic proteins with a chloroform-saturated buffer (1). Surprisingly, the red color was taken up almost completely by the chloroform layer, suggesting that it is not associated with a protein and that the material is located outside the plasma membrane. Its visible absorption spectrum had a broad peak extending from 430 nm to 640 nm, with a maximum at 525 nm (Fig. 2).

The chromophore-producing gene was located by generalized transduction. First, strain RV (red phenotype) was mutagenized by random insertion of a mini-Tn*10dkan* transposon. Then, P1 phages were grown on the pooled Kan^r derivatives and used to transduce (white) Cupples-Miller strain CC106 to Kan^r. Transductants were picked and grown in sealed microwell plates in the anaerobic growth medium to which 20 mM sodium citrate was added to inhibit phage growth. Of the 380

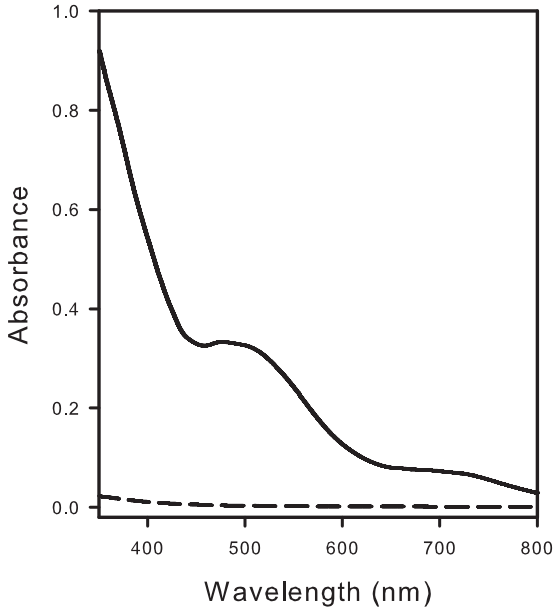


FIG. 2. Visible spectra of $CHCl_3$-extracted cell pellets. Equal amounts of saturated cultures were used (see Materials and Methods). The strains used were RV (*tnaA*$^+$) (solid line) and BW2018 (*tnaA* mutant) (dashed line).

transductants that were tested, 50 (13%) developed a red color. These transductants were presumed to have received a gene from strain RV that was required for chromophore production and that was cotransduced with the mini-transposon. The transductants were then used as transductional donors in backcrosses with strain CC106. One donor strain produced 97% cotransduction of kanamycin resistance and the red phenotype. Its transposon was inserted into *dgoA*, as determined by DNA sequencing of an inverse PCR product. The *tnaA* and *tnaB* genes, which are about 15 kb from *dgoA* (http://www.ncbi.nlm.nih.gov/nuccore/NC_000913), were likely to be responsible for the red chromophore. Both of these genes affect the production of indole from tryptophan. *tnaA* encodes tryptophanase, which breaks down tryptophan to produce indole, pyruvate, and NH_3 (37). *tnaB* encodes the major tryptophan permease (59). Indole is known to react with nitrous acid, a by-product of nitrate metabolism, to produce 3-nitrosoindole, which condenses with other indole molecules to produce red compounds (30).

Genetic defect in the Cupples-Miller strains. An attempt was made to see if strain CC106 is a *tnaA* mutant or a *tnaB* mutant. *tnaA* is directly upstream of *tnaB* in the same operon (37). The specific activity of tryptophanase in this strain was 20% that in BW1824, a red transductant. Although this result suggests that there is a defect in *tnaA*, it is also consistent with the presence of a mutation affecting the permease (TnaB). If the entry of tryptophan into the cell is blocked, tryptophanase may not be fully induced. Complementation testing was performed with the ASKA GFP Plus plasmids (32) bearing the *tnaA* or *tnaB* gene. Transformants of CC106 were grown anaerobically in medium containing chloramphenicol (20 μg/ml) and the inducing agent isopropyl-β-D-1-thiogalactopyranoside (1 mM). Only the strain carrying the *tnaA* plasmid produced red cell pellets. Therefore, strain CC106 is a *tnaA* mutant, as are, presumably, its chromophore-deficient relatives (13, 14).

Requirements for chromophore production. Saturated aerobic cultures were diluted 10^4-fold into anaerobic growth medium and grown anaerobically at 37°C. Glycerol in the medium served as the major electron donor. Nitrate, when present, was the major electron acceptor, and fumarate was provided as an alternative. The cell density reached a maximum at 12 h, at which time the *tna*$^+$ cells were still not red and the pH of the unbuffered medium was 6.0 to 6.4. Color development occurred between 16 and 20 h as the pH dropped to 5.4 to 5.8. Similar drops in pH occurred in both *tnaA*$^+$ and *tnaA* mutant cultures. The requirements for chromophore production are summarized in Table 2. They include functional *tnaA*, *tnaB*, and *narG* (nitrate reductase) genes, hypoxic conditions, nitrate and tryptophan in the medium, and an acid pH (i.e., an unbuffered medium). Nitrite reductase (NirB) is not required. To see if chromophore production required exogenous tryptophan, the tryptone and yeast extract in the growth medium were replaced by vitamin assay Casamino Acids (Difco), a digest of casein in which tryptophan is destroyed by acid hydrolysis. This medium required addition of tryptophan or indole for chromophore production (Table 2). Apparently, the tryptophan produced by de novo synthesis was not sufficient to generate a noticeable red color, a conclusion that is reinforced by a requirement for tryptophan transport (*tnaB*$^+$) as well.

TABLE 2. Requirements for production of the red chromophores

Conditions	Red pellet
Complete[a]	+
−*tnaA*	−
−*tnaB*	−
−*narG*	−
−*nirB*	+
+O_2[b]	−
−NO_3^-	−
20 mM NO_3^-	+
−NO_3^- + 20 mM NO_2^-	−
+0.1 M 3-(*N*-morpholino)propanesulfonic acid/NaOH buffer (pH 7.8)	−
Tryptophan-free medium[c]	−
+tryptophan (0.5 mM)	+
+indole (0.5 mM)	+

[a] Cultures were grown to saturation at 37°C in sealed, filled tubes using an initial concentration of 100 to 200 cells/ml. After 20 h the cultures were centrifuged, and the pellets were observed. The complete conditions for chromophore production included a *tna*$^+$ strain (RV) grown anaerobically in glycerol-fumarate medium containing 100 mM $NaNO_3$. Additional strains used were BW2018 (*tnaA*), BW2019 (*tnaB*), BW2020 (*narG*), and JCB387 (*nirB*).

[b] The culture was aerated by shaking in an open flask.

[c] Vitamin assay Casamino Acids (Difco) was substituted for tryptone and yeast extract in the growth medium.

The hypoxia was probably needed to induce the major nitrate reductase (NarGHI), which produces nitrite (22). A low pH shifts the equilibrium from free nitrite toward molecular nitrous acid and its anhydride (N_2O_3), which reacts with indole (57). The resulting 3-nitrosoindole produces red condensation products (30). Unexpectedly, nitrite could not replace nitrate even though nitrite is presumed to be an intermediate. Although the nitrite could be used only at concentrations below 20 mM (above this concentration it was lethal), nitrate produced a red color even at these concentrations (see Discussion).

Identification of the indole derivatives. The major products of the nitrosation of indole in vitro are shown in Fig. 3. 3-Nitrosoindole, which exists primarily in the oxime form, eventually forms dimeric and trimeric condensation products (43, 44). To verify that the chromophores are indole derivatives, they were extracted from the cells and separated by TLC as described in Materials and Methods. Four migrating colored spots were observed (Fig. 4), which were similar to those produced when indole was incubated with nitrous acid. Analysis of the culture supernatant yielded similar results (not shown). Material was eluted from each spot, repurified by TLC, and analyzed by mass spectrometry. The compositions of compounds predicted to have *m/z* ratios closest to those found corresponded to the compositions of indole derivatives (Table 3). Spots A and B contained primarily (>85%) indole red and indoxyl red, respectively. Spot C contained an indole trimer and indoxyl red. The indoxyl red, which gave the spot its red color, was probably produced after chromatography by reversal of the reaction that formed the trimer (58). The *m/z* ratios are the same for 2,2-bis(3′-indolyl)indoxyl (the trimer shown in Fig. 3) and its isomer, trisindoline (called β-diindyl-isatin in reference 34), in which the positions of the oxo and diindolyl substituents are reversed. However, only the former compound is known to be produced by treatment of indole with nitrous

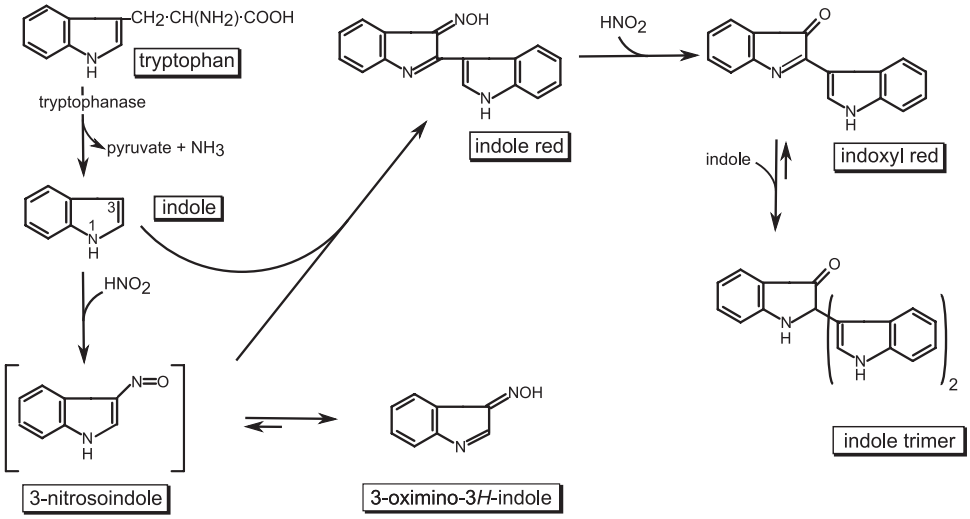


FIG. 3. Formation of 3-nitrosoindole and derivatives (based on data from references 2, 29, 30, and 43).

acid (43) and is therefore shown in Fig. 3. When material in spot X was rechromatographed for mass spectrometry, it produced two spots (not shown), the R_f and *m/z* values of which corresponded to those of indole red and indoxyl red. The compound from which these spots were derived could not be isolated.

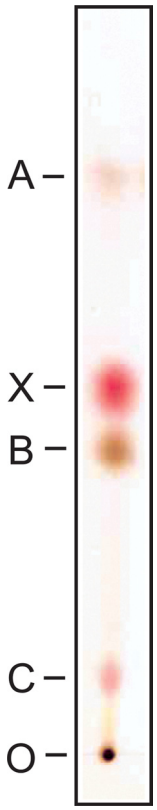


FIG. 4. TLC of chromophores in a $CHCl_3$ extract of strain RV. The unstained chromatogram was photographed in ambient light. Spot O is at the origin.

In the visible range, indoxyl red has a maximum absorbance at 522 nm in ethanol (2), which was approximately the maximum absorbance of the cell extract (Fig. 2). Indole red and 2,2-bis(3′-indolyl)indoxyl have major peaks of absorbance at 445 nm (2) and 400 nm (58), respectively. Although these compounds were extracted from a cell pellet, they may have been mostly extracellular, in the form of fine precipitates that cosedimented with the cells.

Mutation frequencies of *tna*$^+$ and *tna* mutant strains. *nfi* mutants are defective in the repair of deaminated purines and are therefore unusually susceptible to the mutagenic effects of nitrosation (41, 56). To explore the possibility that nitrosoindole derivatives might be mutagenic, we measured mutation frequencies under our anaerobic growth conditions. Both general and specific mutation indicators were used. Valine resistance and 6-azauracil resistance may each arise from a variety of different base pair changes at multiple loci in several genes (35). On the other hand, reversion of the *trpA58* allele is highly specific for an A · T→G · C transversion at one base pair (60), and mutations to streptomycin resistance occur at A · T base pairs in only two codons (19). Nitrous acid-induced mutations of these alleles to *trp*$^+$ and streptomycin resistance are greatly enhanced in *nfi* mutants (24, 41), which are defective in removal of nitrosatively deaminated adenine from DNA. Under our anaerobic growth conditions, however, the *tnaA*$^+$ and *tnaA* mutant strains displayed no marked differences in the mutation frequencies for the four markers tested (Table 4).

In previous work (41), a *tnaA*$^+$ *nfi trpA58* mutant (BW1177) had a median spontaneous reversion frequency for *trpA58* of

TABLE 3. Identification of indole derivatives by mass spectrometry

Postulated derivative	*m/z* predicted	*m/z* found	TLC spot[a]
Indoxyl red	247.08659	247.08612	A
Indole trimer	364.14444	364.14379	B
Indole red	262.09749	262.09706	C

[a] Spot from which the compound was isolated (see Fig. 4).

TABLE 4. Mutation frequencies of *tnaA*$^+$ and *tnaA* mutant strains grown anaerobically in the presence of nitrate

Selected trait[a]	Mutation frequency[b]	
	BW1177 (*nfi*)	BW2022 (*nfi tnaA*)
Str^r	2.6×10^{-9}	7.1×10^{-9}
AU^r	7.6×10^{-7}	9.9×10^{-7}
Val^r	1.0×10^{-7}	1.6×10^{-7}
Trp$^+$	$<2.0 \times 10^{-9}$	$<2.0 \times 10^{-9}$

[a] Str^r, streptomycin resistance; AU^r, resistance to 6-azauracil; Val^r, valine resistance; Trp$^+$, tryptophan independence.
[b] Each value is the median for at least five independent cultures that were grown as described in Materials and Methods.

7×10^{-10} under aerobic conditions, a value that is comparable to the value which we obtained in this study during anaerobic growth (Table 4). However, the absence of any Trp$^+$ colonies in this study raised doubts about the selective media and about the revertibility of the strain. The correctness of the media and the absence of hidden auxotrophic markers were confirmed by placing a drop of a tryptophan solution (25 mg/ml) on the center of an experimental plate that had failed to yield revertants; after 24 h, there was diffuse growth in the region of the tryptophan. Mutability was confirmed by applying 5 μl of the mutagenic alkylating agent methyl methanesulfonate to the center of plates on which either strain BW1177 or strain BW2022 was spread. After 48 h, numerous Trp$^+$ or valine-resistant colonies were clustered around the areas of killing produced by the mutagen.

Toxicity due to indole nitrosation. Although the mutation frequencies did not differ greatly, the levels of cell survival did. The growth of *tnaA* and *nfi* mutants was studied during prolonged anaerobic incubation in the presence of 100 mM nitrate (Fig. 5A). The growth densities of the *tnaA*$^+$ strains were less than those of the *tnaA* mutants. After 18 h of growth, there was a marked decline in the titer of the *tnaA*$^+$ *nfi* mutant but not in the titer of the *tnaA nfi* double mutant. Moreover, the surviving *tnaA*$^+$ *nfi* cells were not normal. After 28 h of incubation in broth culture, the *tnaA*$^+$ strains produced much smaller colonies on agar than their *tnaA* mutant counterparts produced and required an additional day of growth before they could be counted.

At 28 h, *nfi* mutant strains BW1177 and BW2022 (Fig. 5A) reached about same the optical density at 600 nm (within 20%), and microscopic observation revealed no evidence of aggregation. These results, together with a decline in the number of CFU between 18 and 28 h, indicate that the *tnaA*$^+$ strain was selectively killed; a difference in growth rates cannot explain the progressive loss of viable cells.

Even though there was no enhanced mutagenicity, the *tnaA*$^+$ cells required a DNA repair enzyme, endonuclease V, to avoid cell death. To verify that DNA damage occurred, other DNA repair mutations were studied. The *recA* recombinase is needed for recombinational (postreplication) repair; *recA* mutants have increased sensitivity to almost any DNA-damaging agent (18). The *recA* mutant also lost viability after 18 h of anaerobic growth in the presence of nitrate, which was largely prevented by a mutation in the *tnaA* gene (Fig. 5B).

A *uvrA* mutant was studied similarly. *uvrA* mutants are defective in the repair of bulky adducts in DNA. Even the *tnaA*-deficient *uvrA* mutants had reduced survival (Fig. 5C) compared to the wild type (Fig. 5C). However, the presence of a *tnaA* mutation had no noticeable effect on the rate of death after 18 h (Fig. 5C). Despite this high background of cell death unrelated to indole production, the *uvrA tnaA*$^+$ strain grew to about 1/10 the cell density of its *tnaA* mutant counterpart. The inocula were checked and found to contain similar numbers of viable cells. Moreover, when cultures of *nfi*, *recA*, or *uvrA* cells were grown anaerobically for 28 h in the absence of nitrate,

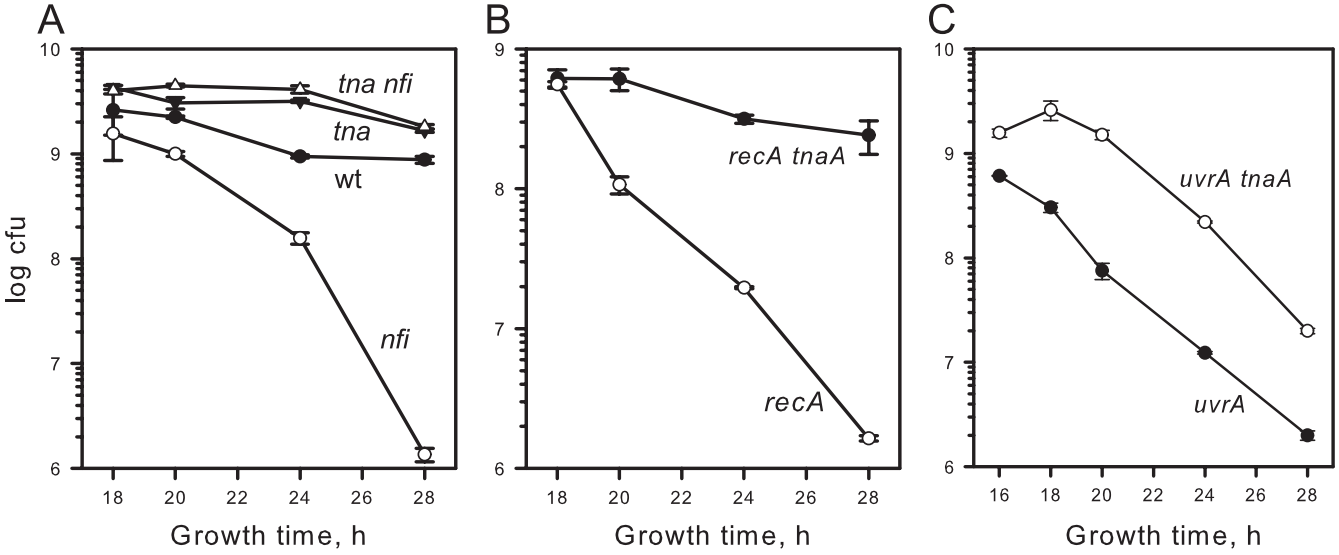


FIG. 5. Susceptibility of DNA repair mutants to the lethal effects of indole production (*tnaA*$^+$ genotype) in the presence of nitrate. For each strain tested, multiple tubes were inoculated with 2×10^5 bacteria per ml and incubated anaerobically for the indicated times, and then the preparations were plated on LB medium to determine the concentration of viable cells. The strains (and relevant genotypes) were as follows: KD1092 (wild type [wt]), BW1177 (*nfi*), BW2021 (*tnaA*), and BW2022 (*tnaA nfi*) (A); BW2023 (*recA*) and BW2024 (*recA tnaA*) (B); and BW1996 (*uvrA*) and BW1997 (*uvrA tnaA*) (C).

there were only small differences in titer between the *tnaA*$^+$ and *tnaA* mutant derivatives (≤20%). Therefore, the observed lethal DNA damage required both indole formation and the presence of nitrate, and it occurred mostly after 18 h of growth, concomitant with a drop in pH and the production of red derivatives of 3-nitrosoindole.

DISCUSSION

In 1887, it was reported that a broth culture of *Vibrio cholerae* turned red when it was acidified (9). This "cholera red" reaction, which provided a method to identify the organism, is based on the production of indole and nitrite in cultures and the subsequent formation of nitrosoindole derivatives upon addition of acid (6). In our anaerobic cultures of *E. coli*, a red color developed during growth and depended on simultaneous production of indole (from tryptophan), nitrite (from nitrate), and acid. Thus, the reaction was blocked by mutations affecting indole production (*tnaA*) or nitrate reduction (*narG*) or by the presence of a neutralizing buffer. Paradoxically, although nitrate was presumably needed only for the production of the nitrite, it could not be replaced by 20 mM nitrite. This result was probably due to inhibition of the color reaction by excess nitrite; when nitrite is added to saturated, acidified cultures of *E. coli*, it produces a red color only if its concentration is less than 0.001% (0.14 mM) (6).

Indoxyl red and the trimer 2,2-bis(3′-indolyl)indoxyl may be produced by reactions other than nitrosation. They have been generated in vitro by oxidation of indole, using peracetic acid (58), by radiation (29), and by catalysis (10, 20). The reactions proceed through a 3-oxo-indole rather than a 3-nitrosoindole intermediate. *Haemophilus influenzae* (49) and *Pseudomonas aureofaciens* (25) have also been shown to produce the trimer. In *Haemophilus*, the synthesis was shown to depend on the presence of both nitrate and nitrate reductase. It was conjectured that nitrate reductase might catalyze the oxidation of indole using nitrate as the electron acceptor. Our results suggest a simpler alternative, nitrosation. In our *E. coli* cultures, we found indole red, which contains an isonitroso (oximino) group, which is a nitroso tautomer and cannot be introduced by oxidation.

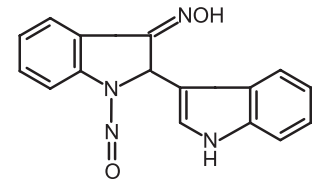
The preferred site of nitrosation of unsubstituted indole is the C-3 position (40), which leads to the condensation reactions shown in Fig. 3. However, if the C-3 position of indole is already occupied by a substituent, then the N-1 position is nitrosated (40). The properties of 3-nitrosoindole should not be confused with those of the *N*-1-nitrosoindoles, which have been extensively studied. For example, the plant growth hormone indole-3-acetonitrile and similar compounds occurring in food form mutagenic *N*-1-nitroso derivatives (54). They damage DNA mainly by transnitrosation, which leads to deamination and depurination (36). However, less is known about the effects of 3-nitrosoindole derivatives. A recent survey of the chemical and biological literature (MedLine, Beilstein, and Gmelin databases) revealed no publications on the possible reactions of 3-nitrosoindoles with DNA or its components.

In earlier work in our lab (41, 55, 56), it was found that an *nfi* mutant of *E. coli* is unusually susceptible to nitrosative mutagenesis. It exhibited an elevated rate of transition mutations induced either by exposure to nitrous acid in vitro or by anaerobic growth in the nitrate-containing anaerobic medium used here. However, the source of the endogenous nitrosating agent (N_2O_3 or nitrous anhydride) was not molecular HNO_2 derived from nitrite but rather NO (nitric oxide) produced in small amounts as a by-product of nitrite reductase. The mutations did not occur during anaerobic growth but afterward, when the culture was exposed to air and the NO was autooxidized to N_2O_3 (56). In contrast, in the present study, HNO_2 had to be the proximate source of the N_2O_3 used for the production of nitrosoindole; the source could not be NO because the chromophores were generated in the absence of oxygen.

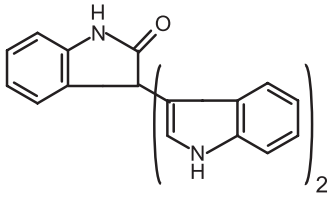
The *trpA58* allele, which was used in our mutagenesis studies, is a specific indicator of A · T→G · C transitions (60). Its HNO_2-induced reversion was enhanced by an *nfi* mutation, which blocks the repair of nitrosatively deaminated adenine (hypoxanthine) in DNA (41). The wild-type and *nfi* mutant strains (KD1092 and BW1177) that were used in the current study were the strains that were used previously. In the previous study, when strains KD1092 and BW1177 were treated directly with HNO_2 so that ≥3% survived, the *nfi* mutation resulted in a 500-fold increase in *trpA58* reversion (41). In the current study, during anaerobic growth, the opposite occurred. Survival decreased about 1,000-fold without a marked increase in mutations. Therefore, the killing of the *tnaA*$^+$ *nfi* mutant (Fig. 5A) was not mediated by mutagenic transnitrosation.

The *nfi-1*::*cat* mutation used in this study is an insertion mutation that does not affect the expression of nearby genes (23). Thus, the sensitivity of the mutant to the effects of nitrosoindole production is directly attributable to the loss of endonuclease V activity. The hypothesis that this sensitivity is due to a DNA repair defect was confirmed by the finding that a deficiency in two other DNA repair proteins (UvrA and RecA) also affected growth or survival during nitrosoindole production. Therefore, 3-nitrosoindole or its products must damage DNA. In vivo, endonuclease V (Nfi) has been shown to recognize only deaminated purines (hypoxanthine or xanthine), which are mutagenic lesions in DNA (24, 41). In vitro, however, this enzyme cleaves not only near these lesions (27, 61, 62) but also near any DNA lesion that distorts the secondary structure such that a single-stranded or open region is adjacent to a bihelical region. Among its substrates, for example, are DNAs that have been damaged by OsO_4, 7-bromomethylbenz[*a*]anthracene, UV light, or acid (17, 21) and DNAs with base mismatches, flaps, stem-loop structures, or pseudo-Y structures (63). Although it has not been demonstrated that these secondary activities are sufficient in vivo to be of any consequence, our results suggest that endonuclease V may be needed to repair lesions other than deaminated purines, such as the unknown lesions generated during nitrosoindole production.

Because of the disparity between the mutation rates and the survival of the DNA repair mutants, the death of these mutants cannot be attributed to accumulation of lethal base pair changes. It must be due to replication-blocking lesions. These lesions may include very bulky DNA adducts and intra- and interstrand cross-links. UvrA is part of a protein complex that excises an oligonucleotide containing a bulky adduct, whereas RecA is a recombinase that enables the patching of almost any lesion (18). Both of these proteins are involved in the repair of



"Dinitrosoindole"



Trisindoline

FIG. 6. Other indole derivatives (see Discussion).

cross-linked DNA (18). Nitrous acid itself may produce cross-links directly (26, 45), but its mutagenicity for *nfi* mutants outweighs its lethality (41). Therefore, it is unlikely that the lethality of 3-nitrosoindole or its derivatives is due to cross-links generated by transnitrosation. Alternatively, it would be possible for an indole compound itself to constitute the cross-link if it were bifunctional, i.e., if it has two reactive groups. Although none of the compounds shown in Fig. 3 have this feature, one such compound, a dinitroso-diindole termed "dinitrosoindole" (Fig. 6), has been isolated after treatment of indole with nitrous acid (28). This compound may not have been detected in our cultures because its partially saturated structure may have rendered it almost colorless. Although our evidence implies that the DNA-damaging agents were derivatives of nitrosoindole, they may not have been derivatives of 3-nitrosoindole, specifically.

It should be noted that trisindoline (Fig. 6), an isomer of the trimer shown in Fig. 3, has been shown to have antibacterial activity. In trisindoline, the substituents at the 2 and 3 positions are reversed compared with those of our trimer. Trisindoline is produced by a marine *Vibrio* (34), and in filter disk assays it inhibited the growth of *E. coli*, *Bacillus subtilis*, and *Staphylococcus aureus* (34).

A high ratio of toxicity to mutagenesis is a very useful property for antineoplastic and antimicrobial compounds. Therefore, 3-nitrosoindole and its derivatives and their analogs warrant further study. Of immediate interest is research on their possible chemical interactions with DNA.

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