MICROBIAL FORMATION OF CARCINOGENIC DIMETHYLNITROSAMINE

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Microorganisms possessing high nitrate reductase activity generally produce carcinogenic dimethylnitrosamine at neutral pH when incubated with dimethylamine and nitrate. Cell suspensions of several microorganisms enhanced nitrosation of dimethylnitrosamine in the presence of nitrite; however, involvement of an enzyme was not detected.

The formation of nitrosamine in natural ecosystems in the presence of high concentrations of secondary amines and nitrite has been demonstrated by Ayanaba *et al.* (1) and Pancholy (2). Secondary amines occur widely among plant and animal kingdoms (3), which ultimately find their way into the soil. During decomposition, certain pesticides (e.g. thiram, eptam, and vernam) are known to produce secondary amines (4). There exists the possibility that nitrite concentrations may attain high levels at localized microsites to effect nitrosation of secondary amines.

The occurrence and formation of dimethylnitrosamine (DMNA) has been intensively studied because of its high toxicity, carcinogenicity, high water solubility, and ubiquitous occurrence of the precursor amines (5, 6). Soluble DMNA, synthesized in soil, may leach downward into the natural water table and eventually pose a health hazard to wild animals, livestock, and human beings. Sander (7) first pointed out that bacteria can cause the nitrosation reaction at neutral pH. A microbial contribution in the synthesis of nitrosamines has been shown (8). The present investigation was designed to study the role of microorganisms in the synthesis of DMNA at neutral pH.

MATERIALS AND METHODS

Nitrate-utilizing microorganisms were isolated from six different sources (two soils, lake sediments, sewage, and sheep and cattle feed lots) by using nitrate-agar medium (glucose 2% KNO_3 0.2%, K_2HPO_4 1.06%, KH_2PO_4 0.53%, $MgSO_4$ 0.01%, and agar 1.5%). Eight isolates of bacteria, one actinomycete and four fungi were obtained this way. Two bacteria (DM-A) and (DM-B) that could utilize dimethylamine (DMA) as the sole source of both carbon and nitrogen were isolated from the sewage and sheep feed lot by an enrichment culture technique. One g of soil was introduced into 100 m*l* of DMA-isolation medium containing dimethylamine hydrochloride (DMAHCl) 0.058%; K_2HPO_4 1.06%; KH_2PO_4 0.53%, and $MgSO_4$ 0.001%. Duplicate flasks were prepared for each soil. The flasks were incubated at 30 C for 10 days. One to two loopfuls of the enrichment solution were then streaked on an agar plate of the same composition. Representative colonies were purified by repeated streaking on the same medium.

Enterobacter cloacae and *E. aerogenes,* obtained from North Carolina Biological Supply, and *Escherichia coli* from the University of Oklahoma microbiology culture collection were also utilized.

In order to get a large quantity of bacterial inoculum, the selected isolates were first streaked on an agar plate of the same composition. Glucose at 0.5% level was added to the DMA-isolation medium. The plates were incubated for 40 hr at 30 C, and the bacterial growth was scraped off and used to inoculate a broth of the same composition.

The flasks were incubated for 40 hr on a shaker. The bacterial cells were harvested by centrifugation at 6000 *g* for 15 min. The pellets were washed twice with 0.1*M* phosphate buffer, pH 7.0, and resuspended in an appropriate volume of the same buffer.

Initial inocula of the fungi and the actinomycetes were built up on a nitrate-agar medium, and then they were grown in nitrate-broth at 30 C without shaking for 10 days until the mats covered the surface of broth. After decanting off the fluid the

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fungal mat was washed twice with the phosphate buffer and homogenized, and almost the whole quantity was used as an inoculum. The actinomycete inoculum was prepared in the same way as the bacterial inoculum, starting from the isolation.

Three ml of the bacterial suspension were introduced into 27 m*l* of nitrosating solution (KNO_3 2.5 g, glucose 2.0 g, DMAHCl 0.29 g, and 0.1*M* phosphate buffer pH 7.0, 100 m*l*). The control flask received an equal amount of the buffer. The flasks were incubated at room temperature on a rotary shaker at 110 rpm up to 96 hr; nitrite and DMNA were determined quantitatively at 24 hr intervals.

An aliquot of the supernatant from the nitrosating solution was pipeted out for the determination of nitrite content by the colorimetric method of Montgomery and Dymock (9). In the analysis for DMNA the remaining portion of the sample was steam distilled (10) and DMNA measured by the photochemical method of Daiber and Preussman (11).

To determine whether microorganisms can directly enhance nitrosation of DMA in the presence of nitrite, and differ in nitrosating ability, cell suspensions of 16 microorganisms were incubated in the nitrosating solution containing DMAHCl 0.29 g, $NaNO_2$ 0.25 g, glucose 0.10 g, and phosphate buffer pH 7.0, 100 m*l*. The method of inoculation and the incubation conditions were the same as before. In the case of bacteria DMNA was measured at 0, 24, 48, 72, and 96 hr and at intervals of 0, 1, 3, 7, and 10 days in the case of fungi and actinomycetes.

In order to test whether cell-free extract of *E. coli* could enhance nitrosation, the bacterium was grown for 48 hr on a shaker in a large flask with continuous aeration. The cells were harvested by centrifugation and twice washed with phosphate buffer solution. They were dispensed in four small centrifuge tubes, each containing approximately 2 g of bacterial cells, and after resuspending in 2.5 m*l* of buffer were exposed to ultrasonic vibration for 10 min each. The cells were centrifuged at 10,000 *g* for 15 min; the clear supernatant was pooled and divided into 2 equal parts, one of which was kept in a boiling water bath for 20 min. Two m*l* of this extract were incubated with 8 m*l* of nitrosating solution. Control flasks received equal amounts of buffer. Analyses were done after 0, 1, 2, and 4 hr of incubation.

RESULTS AND DISCUSSION

Of the 18 microorganisms tested, 11 bacteria, one actinomycete and four fungi produced nitrite from nitrate in levels ranging from 0.3 to 180 mg/*l* under the test conditions. Formation of DMNA was recorded in the cultures of five bacteria, one actinomycete, and one fungus (Tables 1 and 2). The organisms yielding high levels of nitrite also produced DMNA. The results are in agreement with those of Hill and Hawksworth (12). Among the microorganisms investigated, *E. cloacae* had the highest nitrate reductase activity. The yield of both nitrite and DMNA in the cultures of *E. cloacae* attained maximum at 96 hr (Table 1). The production of nitrite and DMNA with *E. aerogenes* reached their peaks at 24 and 96 hr respectively; at 96 hr the pH of 4.29 was highly favorable for the nitrosation reaction.

Members of *Enterobacteriaceae* contain nitrate reductase and may therefore be considered to be potential contributors to nitrosamine formation. Contrary to other reports (1) the strain of *E. coli* used in this experiment yielded negligible nitrite and no DMNA; this suggests the existence of strain variation in this respect.

DMNA can be nitrosated to some extent even at neutral pH (Tables 3 and 4). Sander (8) obtained similar results working with diphenylamine.

DMNA production in the inoculated flasks containing various microbial cultures was higher than in uninoculated flasks. Among these, the highest yield was obtained in the culture of *E. cloacae* after 48 hr and 72 hr, after which the amount decreased. The loss of DMNA during the later period of incubation is not understood.

The data presented here indicated that, in addition to altering pH and reducing nitrate, the microorganisms can directly enhance nitrosation under neutral pH in a system containing both DMA and nitrite.

TABLE 1. *Formation of NO_2^- and DMNA by bacteria in a medium containing DMA and NO_3^-*

Bacteria	Incubation period (hr)											
	0			24			48			72			96		
	pH	NO_2^-	DMNA	pH	NO_2^-	DMNA[a]	pH	NO_2^-	DMNA[a]	pH	NO_2^-	DMNA[a]	pH	NO_2^-	DMNA[a]
None	7.0	0.0	0.0	7.1	0.0	0.025	7.1	0.0	0.028	7.2	0.0	0.28	7.1	0.0	0.028
E. aerogenes[b]	7.0	0.0	0.0	6.4	32.0	0.028	6.2	23.5	0.039	4.4	12.5	0.056	4.9	15.5	0.129
E. cloacae[b]	7.0	0.0	0.0	6.6	120.0	0.036	6.2	130.0	0.129	6.2	120.0	0.109	6.1	180.0	2.016
E. coli	7.0	0.0	0.0	7.1	0.0	0.025	7.2	0.0	0.028	7.1	0.0	0.028	7.1	4.2	0.028
Bact. F	7.0	0.0	0.0	6.5	17.0	0.028	6.1	2.5	0.073	6.0	0.5	0.073	4.9	0.0	0.098
Bact. DM-A	7.0	0.0	0.0	6.8	42.5	0.036	6.5	31.3	0.036	6.4	3.3	0.073	6.5	10.5	0.728
Bact. DM-B	7.0	0.0	0.0	6.5	3.0	0.028	6.5	1.7	0.036	6.5	1.8	0.098	6.4	26.5	0.092

[a]Average of duplicates (mg/*l*).
[b]Enterobacter.

TABLE 2. *Formation of NO_2^- and DMNA by fungi and an actinomycete in medium containing DMA and NO_3^-*

Micro-organisms	Incubation period (days)											
	0			1			3			7			10		
	pH	NO_2^-	DMNA	pH	NO_2^-	DMNA[a]	pH	NO_2^-	DMNA[a]	pH	NO_2^-	DMNA[a]	pH	NO_2^-	DMNA[a]
None	7.0	0.0	0.0	7.1	0.0	0.028	7.1	0.0	0.036	7.0	0.0	0.036	7.3	0.0	0.036
Actinomycete	7.0	0.0	0.0	6.8	5.5	0.165	6.5	22.5	0.073	6.4	26.0	0.109	4.8	0.5	0.073
Fungus A	7.0	0.0	0.0	6.9	1.4	0.109	6.8	97.5	0.109	6.6	132.5	0.756	6.5	3.8	0.073
Fungus B	7.0	0.0	0.0	7.1	0.3	0.028	7.2	0.0	0.036	7.3	0.0	0.036	7.5	3.5	0.036

[a]Average of duplicates (mg/*l*).

TABLE 3. *Formation of DMNA by bacteria incubated with DMA and NO_2^-*

Bacteria	Incubation period (hr)									
	0		24		48		72		96	
	pH	DMNA[a]	pH	DMNA[a]	pH	DMNA[a]	pH	DMNA[a]	pH	DMNA[a]
None	6.9	0.140	6.9	0.238	6.9	0.252	7.0	0.252	6.9	0.252
E. aerogenes[b]	7.0	0.135	6.9	0.252	6.9	0.302	6.0	0.336	n.d.	n.d.
E. cloacae[b]	6.9	0.140	7.0	0.294	7.0	0.364	7.1	0.330	7.2	0.252
Bact. F	7.0	0.135	7.0	0.224	7.1	0.252	7.1	0.294	7.2	0.252
Bact. DM-A	6.9	0.138	6.9	0.269	6.9	0.322	7.0	0.322	7.0	0.238

[a]Averages of duplicates (mg/*l*).
[b]Enterobacter.

TABLE 4. *Formation of DMNA by fungi and an actinomycete incubated with DMNA and NO_2^-*

Micro-organisms	Incubation period (days)									
	0		1		3		7		10	
	pH	DMNA[a]	pH	DMNA[a]	pH	DMNA[a]	pH	DMNA[a]	pH	DMNA[a]
None	6.9	0.138	7.0	0.238	6.9	0.280	6.9	0.350	6.8	0.350
Actinomycete	7.0	0.142	6.8	0.308	6.8	0.308	6.6	0.350	6.4	0.407
Fungus A	6.9	0.130	6.9	0.285	6.9	0.308	6.9	0.280	6.9	0.148
Fungus B	7.0	0.140	7.1	0.276	7.2	0.311	7.4	0.380	n.d.	n.d.

[a]Averages of duplicates (mg/*l*).

In the present investigation the cells of *E. cloacae* incubated with DMA and nitrite produced DMNA, but the cell-free extract failed to enhance the reaction. These results do not agree with those of Ayanaba and Alexander (1).

In conclusion, microorganisms contribute to the nitrosation reaction by reducing nitrate to nitrite, lowering the pH in the system, and possibly creating microsites with high concentrations of precursors by adsorbing nitrite and secondary amines. The occurrence of the nitrosation reaction in natural soil environment appears highly probable from the experimental data available to date.

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