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Nitrite Formation by Soil Bacteria, other than Nitrosomonas.

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(Communicated by Sir E. John Russell, F.R.S.—Received April 18, 1931.)

Introduction.

The biological nature of nitrification in soil was first established by Schlösing and Müntz (1877). Warington (1878, etc.) at Rothamsted and numerous other investigators elsewhere advanced considerably the knowledge of this process. Winogradsky (1890) first succeeded in isolating two species of bacteria, one capable of oxidising ammonia into nitrite and the other nitrite into nitrate; he further showed that nitrification in soil can take place only through the activity of a very limited group of autotrophic bacteria possessing the peculiar property of growing exclusively in an inorganic medium having a very pronounced alkaline reaction.

In ' Nature ' Cutler (1930) reported that several different bacterial strains capable of oxidising various ammonium salts to nitrite had been isolated from different sources. A description of some of these bacteria occurring in soil, together with their physiological reactions, is given in this paper.

As a result of investigations on the growth of bacteria on silica gel plates, some species were found which were capable of oxidising ammonium salts to nitrite; but which did not resemble the nitrosomonas group of organisms in that they grew freely on nutrient agar medium containing lemco and peptone. This was interesting, especially in view of the observation made by Winogradsky (1890, 1891) that the most striking characteristic of his nitrite producers, *Nitrosomonas* or *Nitrosococcus*, was their marked repugnance to organic substances.

There are, however, scattered references in the literature to organisms capable of oxidising ammonia to nitrite and nitrate, and of growing in media containing organic as well as inorganic nutrient substances.

Stützer and Hartleb in 1897 isolated *Nitrosomonas* by using magnesium-ammonium-phosphate agar plates. Silicic acid gel was also used by Stützer (1901), but did not give satisfactory results.

Beddies in 1899 reported the isolation of nitrifying organisms; these were

not very sensitive to high concentrations of organic matter and the presence of humus in the medium aided the growth.

Fremlin in 1903 isolated a nitrite-forming organism, and showed that this bacterium would grow on bouillon. In 1914, using agar, he found that the presence of broth or urine increased the activity of this micro-organism ; and recently (1930) he has again shown that it is capable of growing in the presence of organic substances.

Makrinoff (1909) isolated a nitrite-forming organism by employing gypsum plates and found that the presence of soil did not prove toxic ; a result also obtained by Gibbs (1909) when cultivating *Nitrosomonas*.

Mishustin (1926) found two spore-forming bacteria in soil which produced nitrites in media containing organic nitrogenous compounds, but not in inorganic media containing ammonium salts.

Runov (1926) obtained two species from enrichment cultures, one of these produced nitrites from organic nitrogen compounds and from ammonia in the presence of organic substances. Neither organism grew on Winogradsky's medium and the author concluded that there were many bacteria in nature capable of forming nitrites in media containing various organic substances.

The diagonistic characters given in these cases were, however, insufficient for identification purposes.

Experimental.

In the course of work on the isolation of *Nitrosomonas*, numerous bacterial colonies were picked from silica gel plates for further investigation. These were inoculated into Winogradsky's liquid medium containing excess of ammonium sulphate but no magnesium carbonate.* Periodic nitrite determination by the Griess-Ilosva method showed that these organisms were capable of oxidising ammonium sulphate into nitrite, and the quantities of nitrite formed compared very favourably with those found in a similar culture inoculated with fresh soil.

Portions of these enrichment cultures were plated on Thornton's agar and white colonies appeared after 4 days. A number of these colonies were picked off and inoculated on to Thornton's agar slopes, where a copious growth soon took place. Six strains, A, B, C, D, E and F were selected for further work.

In order to test whether the presence of sugar exerted any effect on the

* It was found from preliminary experiments that magnesium carbonate was not essential to the growth or activity of these organisms.

production of nitrite by these organisms the following mineral salt medium was made :—

	Per cent.
$(NH_4)_2SO_4$	0·1
NaCl	0·06
$CaCl_2$	0·002
$MgSO_4 7H_2O$	0·0005
KH_2PO_4	0·03

The p_H value after autoclaving was brought to 7·3 by the addition of NaOH and 0·1 per cent. sucrose was added to some of the medium.

50 c.c. portions of these two media were distributed into an equal number of 250 c.c. Erlenmeyer flasks which were inoculated with the bacteria.

The amounts of nitrite produced in each of the cultures are shown in Table I.

Table I.—Nitrite Nitrogen in milligrammes per litre produced by the different species in mineral salts medium—both in the presence and absence of sugar.

Days.	Sugar.	A.	B.	C.	D.	E.	F.	Control.
2	+	0·13	Trace	Nil	Nil	Trace	0·13	Nil
	−	0·13	,,	,,	Trace	,,	Nil	,,
5	+	0·25	0·15	0·15	Nil	0·13	Trace	,,
	−	0·13	Trace	Nil	,,	Nil	Nil	,,
8	+	0·5	0·25	,,	0·13	0·25	0·25	,,
	−	0·13	0·13	Trace	Nil	0·13	Trace	,,
12	+	0·75	0·75	0·25	0·25	0·25	0·5	,,
	−	0·25	0·5	Trace	Nil	Nil	Nil	,,
17	+	1·25	1·0	0·50	0·25	0·5	0·5	,,
	−	0·25	0·5	0·25	0·25	Nil	Trace	,,
23	+	1·0	1·0	0·75	0·50	1·0	1·0	,,
	−	0·50	0·75	0·25	0·25	0·25	Nil	—
28	+	0·5	0·5	0·25	0·75	0·75	1·0	—
	−	0·5	0·5	Nil	0·25	0·50	0·5	—

It is evident that in the presence of sugar these organisms produced more nitrite than in its absence, which is of interest considering that no growth occurs in sugar solutions (see p. below). In all the following investigations, therefore, the mineral salt medium containing 0·1 per cent. sucrose was used unless otherwise stated.

To ensure that only pure cultures were being investigated repeated platings and pickings off of single colonies were made; and the pure strains finally obtained were known as A_1, A_2, B_2, C_{21}, C_{22}, D_{33}, E and F_{11}.

The nitrite produced by each of these strains from ammonium phosphate, chloride, lactate, acetate and sulphate is shown in Table II. The nitrogen equivalent of each of the added salts amounted to 0·02 gm. per 100 c.c. medium.

Table II.—Nitrite formed by species A_1. A_2, B_2, C_{21}, C_{22}, D_{33}, E and F_{11} from various ammonium salts. (Nitrite-nitrogen in milligrammes per litre.)

Ammonium salts.	Bacteria.	Days.						
		2.	4.	6.	9.	11.	13.	16.
Phosphate	A_1	0·125	Nil	Nil	Trace	Trace	Trace	Trace
	A_2	Nil	,,	Trace	0·125	0·25	0·25	0·125
	B_2	,,	Trace	,,	Nil	Nil	Nil	Nil
	C_{21}	,,	Nil	0·25	0·5	0·25	Trace	Trace
	C_{22}	,,	,,	Nil	Nil	Nil	,,	Nil
	D_{33}	,,	,,	Trace	0·5	0·5	0·5	0·125
	E	,,	,,	0·125	0·25	0·25	Nil	Nil
	F_{11}	,,	0·125	0·25	0·5	0·5	,,	0·125
Chloride	A_1	,,	Nil	Nil	0·25	0·5	Trace	Trace
	A_2	,,	0·125	0·25	0·5	0·25	0·75	0·5
	B_2	,,	Trace	Nil	Nil	Nil	Trace	0·125
	C_{21}	,,	,,	,,	,,	,,	,,	Nil
	C_{22}	Trace	Nil	,,	,,	Trace	0·25	0·25
	D_{33}	Nil	0·125	0·125	0·25	0·25	0·25	0·25
	E	,,	Nil	Nil	Nil	Nil	Nil	Trace
	F_{11}	,,	,,	,,	Trace	,,	,,	Nil
Lactate	A_1	,,	Trace	0·125	0·75	0·75	0·75	0·5
	A_2	,,	0·125	0·5	0·5	0·5	0·75	0·5
	B_2	,,	Trace	0·25	0·5	0·25	Nil	Nil
	C_{21}	,,	,,	Trace	Nil	Nil	,,	,,
	C_{22}	,,	Nil	0·125	Trace	Trace	Trace	Trace
	D_{33}	Trace	0·125	0·5	0·5	0·25	0·25	0·25
	E	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	F_{11}	,,	,,	,,	,,	,,	,,	Trace
Acetate	A_1	,,	,,	Trace	,,	,,	,,	Nil
	A_2	Trace	,,	0·125	0·25	0·25	0·25	0·125
	B_2	Nil	,,	Nil	Nil	Nil	Nil	Nil
	C_{21}	,,	,,	,,	Trace	Trace	,,	,,
	C_{22}	,,	,,	0·125	Nil	Nil	,,	,,
	D_{33}	,,	,,	Nil	,,	0·25	0·25	Trace
	E	,,	,,	,,	,,	Trace	Trace	Nil
	F_{11}	,,	0·125	0·125	,,	Nil	Nil	,,
Sulphate	A_1	,,	Trace	Nil	0·25	Trace	,,	,,
	A_2	,,	0·125	0·125	0·25	0·25	,,	,,
	B_2	,,	Nil	0·125	0·25	0·5	0·5	0·25
	C_{21}	Trace	Trace	Nil	0·125	Trace	Trace	Nil
	C_{22}	Nil	Nil	,,	0·125	0·125	Nil	,,
	D_{33}	Trace	,,	,,	0·5	0·5	0·25	Trace
	E	,,	0·125	0·25	0·75	0·5	Nil	,,
	F_{11}	Nil	Trace	0·25	0·5	0·25	,,	,,

It will be seen that ammonium phosphate, chloride, lactate and sulphate can be oxidised into nitrite by these bacteria, but with ammonium acetate the oxidation is not very marked. The yields of nitrite are greatest with lactate and sulphate of ammonia, the former giving somewhat better results than the latter excepting in the cases of E and F_{11}.

It is interesting to note that in the majority of the cultures the amounts of nitrite diminished gradually, and the following experiments were carried out in order to test if the organisms were capable of utilising nitrite.

Sulphate of ammonia was replaced by sodium nitrite in the mineral salt medium containing 0·1 per cent. sucrose. The amount of nitrite in the medium corresponded to 1·8 mgm. of nitrogen per litre, and 50 c.c. portions of the medium were distributed into 250 c.c. Erlenmeyer flasks which were subsequently inoculated.

The results of the nitrite estimations are given in Table III.

Table III.—Nitrite Utilisation by A_1, A_2, B_2, C_{21}, C_{22}, D_{33}, E and F_{11}. (NO_2-N) originally present: 1·8 mgm. per litre.)

Days.	NO_2-N in milligrammes per litre.								
	A_1.	A_2.	B_2.	C_{21}.	C_{22}.	D_{33}.	E.	F_{11}.	Control.
0	1·8	1·8	1·8	1·8	1·8	1·8	1·8	1·8	1·8
2	1·8	—	—	1·8	1·8	1·8	1·8	Trace	1·8
3	—	Trace	Trace	—	—	—	0·05	—	1·8
4	0·8	—	—	1·8	0·6	1·8	—	Nil	1·8
6	—	Nil	Nil	—	—	—	Trace	—	1·8

No nitrate was formed in any of the cultures, so it was clear that none of the eight organisms could oxidise nitrite into nitrate.

It will be observed that A_2, B_2, E and F_{11} utilised the whole of the nitrite originally present in the medium. A_1 and C_{22} appeared to assimilate it partially, while C_{21} and D_{33} did not absorb nitrite at all in the time under consideration.

It is well known that nitrate-forming bacteria can function only in a medium containing nitrite, but it is very curious that nitrite-forming organisms should absorb any nitrite that they themselves have produced from ammonium salts. It seems probable therefore that, although essentially nitrite formers, they also can use nitrite for their metabolism. Certain soil Actinomycetes are capable of utilising nitrite when it is present in low concentrations.

With a view to ascertaining the effect of soil on the nitrifying powers of these

bacteria, the production of nitrite was studied in sterilised soil treated with sulphate of ammonia and inoculated with each species.

For this purpose air-dried Barnfield soil receiving farmyard manure was employed. The soil was passed through a 2-mm. sieve and sterilised by heating in an air oven for 1 hour at 190° C. 300 gm. portions of this soil were moistened with 60 c.c. of an aqueous solution of 0·1 per cent. ammonium sulphate and were distributed into 2-litre conical flasks previously sterilised. The soil was inoculated with 1 c.c. of bacterial suspension. 20 gms. of soil were periodically withdrawn, shaken up with 100 c.c. of distilled water and the nitrite estimated in the extract by the Griess-Ilosva method.

The nitrite formed in each case is shown in Table IV.

Table IV.—Production of Nitrite from Ammonium Sulphate in Soil by Bacteria A_1, A_2, B_2, C_{21}, C_{22}, D_{33}, E and F_{11}. NO_2-nitrogen in milligrammes per kilo of soil.

Days.	A_1.	A_2.	B_2.	C_{21}.	C_{22}.	D_{33}.	E.	F_{11}.	Control.
2	1·0	Nil	Nil	Nil	Nil	Trace	Trace	Nil	Nil
3	1·7	,,	,,	Trace	Trace	0·6	0·6	Trace	,,
4	—	—	—	0·7	—	0·6	0·6	,,	,,
5	2·0	Trace	Trace	—	0·25	—	—	0·8	,,
6	1·7	,,	,,	1·3	Trace	1·3	1·3	1·7	,,
8	0·9	0·6	Nil	Trace	Nil	1·9	1·7	1·7	,,
9	—	—	—	,,	—	1·9	1·3	—	,,
10	—	—	—	,,	—	Trace	1·3	—	,,
11	0·4	0·5	Nil	0·4	Trace	,,	1·3	1·0	,,

It is seen that A_1, A_2, C_{21}, D_{33}, E and F_{11} are definitely capable of oxidising ammonium sulphate into nitrite in soil. In none of the cultures was nitrate found.

Effect of Aeration.

Aeration has a favourable influence upon most soil organisms, and it may be assumed to be particularly beneficial to oxidising organisms. The influence of aeration on some of the strains isolated was therefore investigated.

A_2 and D_{33} were selected for this purpose, since they showed such marked differences in their assimilation of nitrite. Two flasks each containing 100 c.c. of the mineral salt medium containing 0·1 per cent. ammonium sulphate were inoculated with A_2 and D_{33} respectively. Air from which the carbon dioxide and nitrite had been removed was aspirated through the cultures at the rate of about 6 litres in 24 hours. The vessels were kept immersed in a thermo-

stat maintained at 23° C. and samples of the cultures for nitrite and bacterial determinations were taken by means of sterilised rubber tubing.

The results obtained are given in Table V.

Table V.—Effect of Aeration on the Oxidation of Ammonia to Nitrite by Strains A_2 and D_{33}. (Nitrite-nitrogen is expressed in milligrammes per litre and the bacterial numbers in millions per cubic centimetres.

Days.	A_2.		D_{33}.	
	NO_2-N.	Bacterial numbers.	NO_2-N.	Bacterial numbers.
0	Nil	31·0	Nil	44·0
2	0·62	48·6	0·5	35·7
5	1·0	78·6	1·5	25·7
7	0·5	Innumerable	1·4	26·8
8	0·25	,,	1·25	32·0

Aeration evidently stimulated the production of nitrite especially during the first few days, since the amounts of nitrite given in Table V are greater than those given in Table II. The culture inoculated with A_2 showed a rapid increase in the bacterial numbers, whereas those in D_{33} tended to diminish. In A_2 the nitrite diminished with the age of the culture, but with D_{33} no such diminution was noticed; this was in accordance with the results already obtained (Table III), that while A_2 assimilated nitrite vigorously, D_{33} did not.

Experiments with Mixed Cultures.

Investigations were next carried out with mixed cultures in which the ammonia was produced from asparagin by the ammonifying bacterium " YB " isolated from soil in this laboratory.

The composition of the medium was :—

	Per cent.
K_2HPO_4	0·1
$MgSO_4\,7H_2O$	0·02
$CaCl_2$	0·01
NaCl	0·01
$FeCl_2$	Trace
Asparagin	0·05

The nitrogen equivalent of the asparagin added was 53 mgm. per litre, and the production of ammonia and nitrite was followed. The ammonia was estimated by Nessler's method ; and the results are given in Table VI.

Table VI.—Production of Ammonia and Nitrite from Asparagin by Mixed Bacterial Cultures. (Ammonia and nitrite expressed as nitrogen in milligrammes per litre.)

Strains.	3 days.		6 days.	
	Nitrite.	Ammonia.	Nitrite.	Ammonia.
YB + A_2	0·5	40	0·85	50·0
A_2	Trace	30	Nil	37·5
YB + C_{21}	0·4	12·5	0·4	20·0
C_{21}	0·25	Trace	Nil	15·0
YB + D_{33}	0·8	37·5	0·75	37·5
D_{33}	0·4	12·5	0·25	10·0
YB + E	0·4	25	0·4	25·0
E	0·2	12·5	0·25	15·0
YB + F_{11}	0·5	25	0·5	25·0
F_{11}	Trace	Trace	0·13	15·0

These experiments show that A_2, C_{21}, D_{33}, E and F_{11} can all produce ammonia from asparagin though not to the same extent as YB. The nitrite formed from the ammonia that they themselves produce is, however, very low in comparison with the nitrite formed in association with YB. These nitrite organisms when alone can take an active part in breaking up the asparagin ; but during the process they possibly expend most of their energy and are able only to take a feeble part in oxidising this ammonia further into nitrite. When, on the other hand, they are associated with YB, the asparagin-ammonia transformation being mainly brought about by the latter bacteria, the nitrifiers are free to oxidise the ammonia produced into nitrite. Again there was no trace of nitrate in any of the cultures.

Description of Strains.

The characterisation of the strains according to the group number as arranged by the Society of American Bacteriologists is given as follows :—

Group number.	Characterisation.	
200	Endospores not produced	All.
10	Aerobic (strict)	All.
1	Gelatine liquefied	A_2, C_{22}, D_{33}, F_{11}.
2	Gelatine not liquefied	A_1, B_2, C_{21}, E.
0·4	No growth with dextrose*	All.
0·04	No growth with lactose	All.
0·004	No growth with saccharose	All.
0·0002	Nitrates reduced without gas	A_1, A_2, B_2, C_{21}, E, F_{11}.
0·0003	Nitrates not reduced	C_{22}, D_{33}.
0·00000	Non-chromogenic	All.
0·000003	Diastatic action on starch absent ..	All.
0·0000004	No growth with glycerine	All.

Total Group Number.

A_1	212·4442034
A_2	211·4442034
C_{21}	212·4442034
C_{22}	211·4443034
D_{33}	211·4443034
E	212·4442034
F_{11}	211·4442034

The characteristics of the organisms not used for finding the group number are as follows :—

Agar Slopes. 3 *days old.*—The growth of all the organisms was abundant; echinulate, raised, smooth, glistening and opaque; viscid and no odour; medium unchanged.

Agar Stabs. 3 *days old.*—All uniform, filiform, medium unchanged.

Gelatine Stabs.—A_1, B_2, C_{21}, E, line of puncture filiform; no liquefaction. A_2, C_{22}, D_{33}, F_{11}, surface growth, liquefaction.

Colonies on Thornton's Agar.—In all cases the growth is rapid, surface smooth, internal structure amorphous. There are minor differences in the character of the edge and the elevation, the edge in A_1, A_2, B_2, C_{22}, D_{33} and E being entire, while that of C_{21} and F_{11} is undulating. The elevation of the colonies

* Besides the sugars mentioned here no growth was obtained with mannitol, maltose laevulose and galactose.

of all the strains is convex, except in the case of A_2, C_{22} and E, where it is flat.

Morphology and Staining.—These characters are given in Table VII.

Table VII.

Strains.	Gram.	Shape.	Dimensions in μ.	Acid fast.	Motility.
A_1	+	Rods	1·90 × 0·85	—	—
A_2	+	,,	1·39 × 0·77	—	—
B_2	+	,,	1·89 × 0·82	—	—
C_{21}	+	,,	1·84 × 0·81	—	—
C_{22}	+	,,	1·43 × 0·71	—	—
D_{33}	+	,,	1·80 × 0·80	—	—
E	+	,,	1·73 × 0·75	—	—
F_{11}	—	Coccus	0·93	—	—

It will be seen that some of these strains have a common group number and similar morphological characteristics, and therefore they may be classified into four groups :—

I.—A_1, B_2, C_{21} and E.
II.—A_2.
III.—C_{22} and D_{33}.
IV.—F_{11}.

Reference to Table II shows that the oxidising power is not similar in what are probably the same strains ; but it must be remembered that in these experiments uniform inoculations were not made.

From the foregoing data it will be seen that in sharp contrast with Winogradsky's nitrite-forming bacteria the organisms described are capable of growing on nutrient agar and in the presence of sugar. They can produce nitrite within a wide range of p_H values varying from 4·8 to 7·3, whereas *Nitrosomonas* or *Nitrosococcus* can function only in a distinctly alkaline medium. Morphologically, too, they are different from *Nitrosomonas*, which first forms a zooglea and then breaks up into swarming, ellipsoidal, motile bacteria.

Summary.

Four species of non-spore-forming bacteria capable of oxidising ammonia into nitrite have been isolated from Rothamsted soil and all differ widely from *Nitrosomonas* or *Nitrosococcus*.

These organisms are able to carry out this reaction in artificial media as well as in soil, and some are able to assimilate nitrite.

Rapid growth takes place on nutrient agar, and the presence of 0·1 per cent. sucrose stimulates nitrite production.

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