Mechanism of Antibacterial Selectivity of 2-Amino-1,3,4-Thiadiazole

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The mechanism of the antibacterial selectivity of 2-amino-1,3,4-thiadiazole (ATDA) was studied by the incorporation experiments of ^{14}C -nicotinamide and ^{14}C -nicotinic acid into NAD, using ATDA-sensitive *Xanthomonas oryzae* and *X. citri*, the insensitive *Erwinia carotovora* and *Pseudomonas tabaci*, and the resistant *X. oryzae* strain. In the sensitive bacteria, ^{14}C -NAD was produced from ^{14}C -nicotinamide, but not from ^{14}C -nicotinic acid. The insensitive *E. carotovora* utilized both nicotinamide and nicotinic acid as precursors of NAD, but the insensitive *P. tabaci* and the resistant strain did not utilize the two precursors. ^{14}C -NAD was also formed from ^{14}C -nicotinamide in the cell extracts from the sensitive strain of *X. oryzae*, but not in the extracts from the resistant strain. ATDA is presumably integrated to NAD through the same pathway as nicotinamide in the sensitive bacteria. In the resistant strain, however, ATDA can not be integrated to NAD because of the loss of nicotinamide affinity of the enzymes involving in the synthesis of NAD from nicotinamide or the lack of the enzymes. The insensitivity of *P. tabaci* to ATDA also is due to the same mechanism as the resistant strain. In the insensitive *E. carotovora*, ATDA may be not integrated to NAD because of the divergent pathway of nicotinamide metabolism in NAD synthesis.

INTRODUCTION

There are many interesting studies on the biological activity of 2-amino-1,3,4-thiadiazole (ATDA). Oleson *et al.*¹⁾ and Troy *et al.*²⁾ found ATDA to retard the growth of tumors in mice. Krakoff and Balis³⁾ reported its uricogenic effect. A different activity was discovered by Yakushiji and Wakae,⁴⁾ who demonstrated ATDA had a high protective effect on the bacterial leaf blight of rice caused by *Xanthomonas oryzae*. They also showed ATDA was active to some plant pathogenic bacteria *in vitro*, particularly to some bacteria belonging to *Xanthomonas* genus but not to other genera, and the antibacterial activity of ATDA was reversed by nicotinamide. Probably, ATDA is specifically or selectively active to only a few bacteria such as *X. oryzae*, *X. citri*, and so on. Such a selectivity of ATDA may be dependent on the nicotinamide metabolism connected with NAD syn-

thesis, because the compound is replaced with the nicotinamide moiety of NAD to produce the ATDA analog of NAD,⁵⁾ which is a potent inhibitor of inosine 5'-phosphate (IMP) dehydrogenase involved in the formation of guanine nucleotide from IMP.⁶⁾ Thus, there seems to be a positive correlation between the antibacterial selectivity of ATDA and the utilization of nicotinamide as a precursor of NAD in the cells.

This paper describes evidence for the mechanism of selective antibacterial activity of ATDA obtained by using ATDA-sensitive, insensitive and resistant bacterial cells.

MATERIALS AND METHODS

1. Chemicals

Nicotinamide-carbonyl- ^{14}C and nicotinic acid-carboxyl- ^{14}C were obtained from Radiochemical Centre, Amersham, and ATP and 5-phosphorylribose-1-pyrophosphate (PRPP) from Boehringer Mannheim.

2. *Organisms and Growth Conditions*

Plant pathogenic bacteria used in the experiments, namely *Xanthomonas oryzae* and *Erwinia carotovora* were provided from the Division of Plant Pathology, National Institute of Agricultural Sciences, Tokyo; *Pseudomonas tabaci* from the Morioka Branch, Horticultural Research Station; *P. lachrymans* from the Laboratory of Plant Pathology, Tokyo University of Agriculture; and *X. citri* was isolated from citrus canker in our laboratory. ATDA-resistant cells were developed by selecting the cells of *X. oryzae* with stepwise increasing concentrations of ATDA, and the finally isolated cells were resistant to 1,000 $\mu\text{g/ml}$ of ATDA. All the bacterial cells used were grown in Suwa liquid medium⁷⁾ containing 10% peptone on a reciprocal shaker at 28°C for 2 days.

3. *Test for Antibacterial Activity*

Bacteria were preincubated in the above Suwa medium, and 0.1 ml of the culture was inoculated in 10 ml of the fresh medium containing ATDA. The inoculated cells were incubated with shaking at 28°C for 20 hr. Bacterial growth was spectrometrically determined by measuring the increase in absorbance at 660 nm.

4. *Determination of Incorporation of Radioactive Precursors into NAD*

Preparation of bacterial suspensions and procedure of labeling experiments were the same as described in the previous report.⁸⁾ The final radioactivities used, namely ^{14}C -nicotinamide (61 mCi/mmol) and ^{14}C -nicotinic acid (60 mCi/mmol) were 0.12 $\mu\text{Ci/ml}$ and 0.15 $\mu\text{Ci/ml}$, respectively. One ml of the bacterial suspension incubated with radioactive precursors was added to the equal volume of cold 10% trichloroacetic acid (TCA) in order to stop the cell metabolism and to extract small molecules like NAD from the cells, and then kept for more than 30 min. The mixed sample was centrifuged at 3,000 rpm for 10 min, and the supernatant was extracted 5 times with ethyl ether to remove TCA, followed by evaporating to dry under reduced pressure. The dried sample was dissolved in 0.2 ml of distilled water, and 50 μl of the solution was spotted on a Avicel *tlc*

plate. The plate was developed with the solvent system of 7 parts of 95% ethanol plus 3 parts of 1 M ammonium acetate adjusted to pH 5.0 with HCl by the procedure of Preiss and Handler.⁹⁾ Nicotinamide or nicotinic acid and NAD regions on the plate were detected by UV light or radio *tlc* scanning, and they were separately scraped from the plate. Each of the scraped samples was analyzed for radioactivity by a liquid scintillation counter. Incorporation rates of ^{14}C -nicotinamide or ^{14}C -nicotinic acid into NAD were estimated as the percent of the radioactive counts of NAD fraction to the total radioactive counts in 50 μl of spotted samples.

5. *Cell Extraction and NAD Synthesis*

The logarithmically growing cells of *X. oryzae* were collected by centrifugation, and suspended again in a small volume of 0.1 M phosphate buffer (pH 7.0). The suspended cells were disrupted by a French pressure cell. After centrifugation the supernatant was adjusted to 10 mg/ml in protein content with the same buffer by the Biuret measurement,¹⁰⁾ and used as the crude enzyme solution for NAD synthesis. An enzyme assay was made by a modification of the procedure of Im-sande.¹¹⁾ The reaction mixture consisted of 0.1 ml of 0.01 M Tris-HCl buffer (pH 7.5), 25 μl each of 20 mM PRPP, 20 mM glutathione (reduced from) and 20 mM MgSO_4 , 50 μl each of 30 mM ATP and 0.4 M NaF, 20 μl of 10 $\mu\text{Ci/ml}$ ^{14}C -nicotinamide, 0.1 ml of the enzyme solution and water to make the final volume of 0.5 ml. The mixture was incubated at 30°C for given periods, then deproteinized by heating in a boiling water bath for 1 min. A 50 μl of the protein-free mixture was separated chromatographically, and subsequent procedure was the same as in the procedure of labeling experiments described above.

RESULTS

Antibacterial activity of ATDA was tested using five different plant pathogenic bacteria by the liquid culture method. The results are shown in Table 1. Among the bacteria used, *Xanthomonas* species such as *X. oryzae* and *X. citri* were strongly inhibited by ATDA at a low concentration of 1 $\mu\text{g/ml}$, but the other

Table 1 Antibacterial activity of ATDA.

Bacteria	Concentrations of ATDA (μ /ml)		
	1	10	100
<i>Xanthomonas oryzae</i>	91	97	98
<i>X. citri</i>	70	87	98
<i>Erwinia carotovora</i>	0	0	0
<i>Pseudomonas lachrymans</i>	2	0	0
<i>P. tabaci</i>	5	2	4

Data are expressed as percent of the inhibition of bacterial growth by ATDA.

Table 2 Incorporation of 14 C-nicotinamide into NAD in bacteria.

Bacteria	Incubation time (min)	
	15	30
<i>Xanthomonas oryzae</i>	32	57
<i>X. citri</i>	23	30
<i>Erwinia carotovora</i>	41	49
<i>Pseudomonas tabaci</i>	1	4
ATDA-resistant strain	0	1

Data are expressed as percent of the radioactive counts of NAD fraction to the total radioactive counts in samples.

species such as *E. carotovora*, *P. tabaci* and *P. lachrymans* were not inhibited even at a high concentration of 100 μ g/ml. From these results, further experiments were made by using *X. oryzae* and *X. citri* which are sensitive to ATDA, *E. carotovora* and *P. tabaci* insensitive to ATDA, and a strain of *X. oryzae* resistant to 1,000 μ g/ml of ATDA.

Table 2 shows the results of the incorporation of 14 C-nicotinamide into NAD. Three bacteria used, ATDA-sensitive *X. oryzae* and *X. citri* and the insensitive *E. carotovora* showed a remarkable activity for the synthesis of NAD from 14 C-nicotinamide, so that these bacteria can utilize nicotinamide as precursor of NAD. On the other hand, *P. tabaci* and ATDA-resistant strain were hardly able to incorporate 14 C-nicotinamide into NAD, indicating that these cells can not utilize nicotinamide for NAD synthesis or absorb it from the outside of cells to the inside. Therefore, ATDA-insensitive or resistant bacteria are different from the sensitive ones in the

Table 3 Synthesis of 14 C-NAD from 14 C-nicotinamide in the extracts from cells of ATDA-sensitive and resistant strains of *Xanthomonas oryzae*.

Strains	Incubation time (min)	
	15	30
Sensitive strain	44	49
Resistant strain	1	1

Data are expressed as in Table 2.

Table 4 Incorporation of 14 C-nicotinic acid into NAD in bacteria.

Bacteria	Incubation time (min)	
	15	30
<i>Xanthomonas oryzae</i>	0	0
<i>X. citri</i>	1	1
<i>Erwinia carotovora</i>	7	20
<i>Pseudomonas tabaci</i>	1	1
ATDA-resistant strain	0	1

Data are expressed as in Table 2.

ability of nicotinamide utilization in NAD synthesis or in the synthetic pathway of NAD from nicotinamide.

To determine the mechanism of resistance to ATDA, the formation of 14 C-NAD from 14 C-nicotinamide was examined using the cell-free extracts of ATDA-sensitive and resistant strains in *X. oryzae*. As shown in Table 3, when the cell extracts from the sensitive strain were incubated for 30 min, about 50% of 14 C-nicotinamide added was utilized for the synthesis of NAD. In the cell extracts from the resistant strain, however, the synthesis of 14 C-NAD from 14 C-nicotinamide was only 1%, which is within the range of an experimental error. These results are similar to the data of the incorporation of 14 C-nicotinamide into NAD in the intact cells. Thus, the resistance of cells to ATDA is accompanied by the structural transformation or deficiency of the enzymes involving in the synthesis of NAD from nicotinamide, but not by the alteration of membrane permeability for nicotinamide.

Table 4 shows the results of the incorporation of ^{14}C -nicotinic acid into NAD. Of the bacteria used, only one of *E. carotovora* could synthesize ^{14}C -NAD from ^{14}C -nicotinic acid, and the ^{14}C -NAD production increased with the lapse of incubation time. However, the others were not able to incorporate ^{14}C -nicotinic acid into NAD even after incubation for 30 min. Hence, nicotinic acid is utilized as a precursor of NAD in *E. carotovora*, but not in the other bacteria used.

DISCUSSION

The antibacterial activity of ATDA was studied using 5 species (in 3 genera) of plant pathogenic bacteria by the liquid culture method. ATDA was found to have a high activity against *Xanthomonas* species, but not against *Pseudomonas* and *Erwinia* species. Yakushiji and Wakae⁴⁾ reported, on the basis of the 50% inhibitory concentrations by the agar dilution method, that ATDA was highly active to 5 of 7 *Xanthomonas* species and only one of 10 *Pseudomonas* species tested. They also found ATDA had no activity to *Erwinia*, *Agrobacterium*, *Corynebacterium*, *Escherichia*, *Staphylococcus*, *Bacillus* and *Lactobacillus* species. From these results, ATDA appears to be specifically active only to several species of plant pathogenic bacteria belonging to *Xanthomonas* genus.

In order to study the mode of action and the antibacterial selectivity of ATDA, antagonistic mechanism of nicotinamide to ATDA activity

was examined. ATDA-sensitive *X. oryzae* and *X. citri*, the insensitive *P. tabaci* and *E. carotovora*, and the resistant *X. oryzae* strain capable of growing in the presence of 1,000 $\mu\text{g}/\text{ml}$ of ATDA were used in the experiments. Incorporation of ^{14}C -nicotinamide into NAD occurred in ATDA-sensitive *X. oryzae* and *X. citri* and the insensitive *E. carotovora*, but not in the other insensitive and resistant cells in the intact cells. Also, the cell extracts from either the sensitive or resistant *X. oryzae* were prepared to confirm the experimental result of intact cells under cell-free conditions. The ^{14}C -NAD was formed from ^{14}C -nicotinamide in the cell extracts from the sensitive *X. oryzae*, but not in the extracts from the resistant one. This result shows that the mechanism of resistance in the resistant cells is not due to the alteration of membrane permeability to nicotinamide, but the loss of affinity of nicotinamide with the enzymes which catalyze NAD synthesis from nicotinamide or the lack of the enzymes.

Incorporation of ^{14}C -nicotinic acid into NAD was found only in *E. carotovora*. The other species could not utilize ^{14}C -nicotinic acid as a precursor of NAD. Yakushiji and Wakae⁴⁾ reported that the inhibitory effect of ATDA on the growth of *X. oryzae* was not reversed by nicotinic acid, but by nicotinamide. Obviously, it is because that nicotinamide is incorporated into NAD in the bacterium, but nicotinic acid is not.

Since ATDA is replaced with the nicotin-

Fig. 1 Schematic representation of NAD biosynthesis pathway.

PRPP: 5-Phosphorylribose-1-pyrophosphate, Gln: Glutamine, Glu: Glutamic acid

amide moiety of NAD to produce the NAD analog,⁵⁾ the active metabolite of ATDA,⁶⁾ there seems to be a positive correlation between the antibacterial selectivity of ATDA and the ability of nicotinamide utilization as a precursor of NAD in the cells. Figure 1 shows the schematic pathway of NAD biosynthesis in organisms. Nicotinamide is incorporated into NAD through the pathway (4) or (5)–(6) in the sensitive bacteria, *X. oryzae* and *X. citri*. In these bacteria, probably, ATDA is integrated to NAD through the same pathway as nicotinamide. On the other hand, although the insensitive bacterium *E. carotovora* can utilize both nicotinamide and nicotinic acid as precursors of NAD, the growth of the bacterium was not inhibited unexpectedly by ATDA. The question is why the growth of *E. carotovora* was not inhibited by ATDA. It is thought that, in the insensitive bacterium, nicotinamide might be incorporated into NAD through another pathway, (7)–(8)–(2)–(3). Another aspect, which may be less possible, is that the enzymes catalyzing the pathway (4) or (5)–(6), might have very low affinity to ATDA in the insensitive bacterium. Also, the insensitive *P. tabaci* and the resistant *X. oryzae* strain utilized neither nicotinamide nor nicotinic acid to produce NAD. In these bacteria, ATDA can not be integrated to NAD.

It is apparent from the experimental results that the sensitivity, the insensitivity and the resistance of bacteria to ATDA are conditioned on the ability or pathway of utilization of nicotinamide in the process of NAD biosynthesis.

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要 約

2-Amino-1,3,4-thiadiazole の選択的抗細菌性の機構

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2-amino-1,3,4-thiadiazole (ATDA) はある種の植物病原細菌に対し特異的に高い抗菌性を示す興味ある薬剤である。本薬剤の選択的抗細菌性の機構について、NAD への ¹⁴C-ニコチン酸アミドおよび ¹⁴C-ニコチン酸の取り込みによって調べた。実験には ATDA に対し感受性の *Xanthomonas oryzae* および *X. citri*, 非感受性の *Erwinia carotovora* および *Pseudomonas tabaci*, 耐性の *X. oryzae* 株を用いた。その結果、感受性菌では NAD の基質としてニコチン酸アミドが利用されたが、ニコチン酸は利用されなかった。非感受性菌の *E. carotovora* では両基質とも NAD 合成に利用されたが、非感受性菌の *P. tabaci* および耐性菌では両基質ともに利用されなかった。また、*X. oryzae* の無細胞抽出液による NAD 合成系では、感受性菌は ¹⁴C-ニコチン酸アミドから ¹⁴C-NAD を生成したが、耐性菌は ¹⁴C-NAD を生成しなかった。以上の結果と ATDA が NAD のニコチン酸アミド部分と交換して NAD アナログ (ATDA の活性代謝物) を形成する事実から、感受性菌ではニコチン酸アミドが NAD にはいる経路を利用して ATDA を NAD に組み込むが、耐性菌ではその経路のある種の酵素がニコチン酸アミドに対する親和性を失ったか、あるいは損失したために、ATDA が NAD に組み込まれないものと考えられた。非感受性菌の *P. tabaci* も同様の機構により ATDA が効果を示さないものと推察された。一方、非感受性菌の *E. carotovora* では、ニコチン酸アミドが感受性菌と異なる経路を通して NAD に取り込まれるため、ATDA の NAD への組み込み経路がなく、ATDA は効果を発揮できないものと考えられた。