NEW POLYENIC ANTIBIOTICS ACTIVE AGAINST GRAM-POSITIVE AND -NEGATIVE BACTERIA

II. SCREENING OF ANTIBIOTIC PRODUCERS AND TAXONOMICAL PROPERTIES OF *GLUCONOBACTER* SP. W-315

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Antibiotic producing bacteria were selected using a new screening method. Eight strains of antibiotic producing bacteria, which required a spent medium of fungi for antibiotic production, were isolated. One of them, a potent producer of antibacterial antibiotic, designated strain W-315, had following taxonomical characteristics; aerobic, Gram-negative, rod shaped and polar flagellated. Furthermore, the organism could grow under acidic conditions (pH 4.5) and had a GC content of 64.4 mole per cent. We concluded that the strain W-315 belonged to *Gluconobacter* sp.

When this bacterium was inoculated into Czapek-Dox medium, bacterial growth and antibiotic production did not occur. The antibiotic production was also not observed even when poor growth was observed in Czapek-Dox medium supplemented with ammonium sulfate. The nutritional requirements for the antibiotic production were also discussed.

In the microbial world, a number of phenomena of microbial interaction is known. For example, *Penicillium africanum* can produce antibiotic active against *Aspergillus niger* only when both strains were cultured on the same plate[1]. Although there are many reports on similar interactions[2], we adopted these classical techniques for the screening of new antibiotics. If there are certain bacteria in soil which require some specific metabolites of other microorganisms for their production of antibiotics, it seems difficult to isolate such organisms with ordinary media for antibiotic production. The donor organisms may serve the recipients certain metabolites which act as antibiotic precursors, inducers or growth factors for antibiotics producing organisms. Therefore, we would expect to isolate new antibiotics from the organisms which require a spent medium of other microorganism for their growth and antibiotic production. Although numerous reports on procedures for antibiotic production have appeared[3], there has been a little work on antibiotic production based on the method we adopted in this study.

In this paper, we describe the screening of antibiotic producer based on microbial interaction and taxonomical properties of one of the isolated bacteria.

Materials and Methods

Organisms

Neurospora crassa IFO 6068, *Aspergillus oryzae* ATCC 15240 and *Rhizopus hangchao* IFO 4749 were used. Other microorganisms were from the collection of this laboratory.

Isolation Procedures for Antibiotic Producing Microorganisms

To isolate antifungal antibiotic producers, metabolite diffusion technique was used. Czapek-Dox agar medium (sucrose 20 g, $NaNO_3$ 3 g, K_2HPO_4 1 g, KCl 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeSO_4 \cdot$

$7H_2O$ 0.018 g, and agar 15 g in 1 liter of distilled water, pH 6.5) was used for the screening of antibiotic producers. The procedures for the screening of antifungal antibiotic producers are as follows.

The fungus was inoculated into the central part of Czapek-Dox agar plate and the soil sample suspended in a small amount of distilled water was streaked near the periphery of the plate. After incubation for 2~4 days at 30°C, the mycelium of fungus grew as a circular colony. Inhibition of fungus growth is observed when there are some antibiotics excreted and diffused into the agar medium from microbial colonies originated in the soil samples.

Ether Extraction of the Spent Medium

Each 100 ml of a spent Czapek-Dox liquid medium of *N. crassa* at the stationary phase was extracted twice with 100 ml of diethyl ether under neutral, acidic and alkaline conditions, namely pH 7.0, 2.0 and 10.0, respectively. Diethyl ether layer was then removed and evaporated *in vacuo*. Each residue was dissolved in a small amount of methanol. These solutions were diluted with basal medium (Czapek-Dox medium supplemented with 0.2% of ammonium sulfate, 1% of calcium carbonate, pH 7.2) to 100 ml. Media containing the diethyl ether extract ("ether extract") and the ether extracted spent medium ("water layer") were sterilized at 110°C for 10 minutes after adjusted to pH 7.2.

Charcoal Treatment of Spent Medium of *N. crassa*

One liter of spent medium of *N. crassa* at the stationary phase was passed through a column (3×30 cm) containing 50 g of activated granular charcoal (Wako Junyaku, guaranteed reagent grade) and charcoal adsorbed materials were then eluted with 500 ml of ammonia - ethanol (50% ethanol in 0.5 N ammonia). The eluate and the unadsorbed fraction were lyophilized, and resulting materials were dissolved in 50 ml of distilled water. The active principle which stimulated the growth and production of antibiotics of strain W-315 was not affected by lyophilization and was stable at least for 3 months when stored at −20°C. Before use, charcoal-adsorbed and -unadsorbed fractions were diluted with basal medium and water, respectively, to the original concentrations in the spent medium.

Ninhydrin Treatment of the Spent Medium of *N. crassa*

Two hundred milliliters of spent medium of *N. crassa* at the stationary phase containing 0.5 g of ninhydrin were boiled for 5 minutes. After the reaction, the ninhydrin treated solution was applied to a charcoal column (1.9×31 cm) and clear eluate was obtained.

Bacterial Growth

Bacterial growth was measured by absorbancy at 660 nm using a Hitachi photoelectric colorimeter FPW-4.

Determination of the Amount of Antibiotic

The amount of the antibiotic in culture broth was determined spectrophotometrically by absorbancy at 370 nm[14] using a Hitachi 124 spectrophotometer.

Classification of Strain W-315

For the characterization of strain W-315, the methods described by HASEGAWA[6] were used. For identification of the organism, the descriptions of BUCHANAN and GIBBONS[7], HASEGAWA[6] and SKERMAN[8] were followed.

Analysis of Base Composition of DNA

DNA was isolated according to the method of MARMUR[4] and analyzed for its thermal denaturation by the method of DE LEY and SCHELL[12] using a Shimadzu UV-300 spectrophotometer. The DNA solution was heated at the increment of 0.7°C per minute and A_{260} was estimated during heating. Tm was calculated from a half value of the increment in A_{260}. Tm was converted to GC content using the equation of MARMUR and DOTY[5].

Electron Microscopic Observation

Bacterial cells at the stationary phase were fixed with 2.5% glutaraldehyde in nutrient broth. After 1 hour, samples were washed twice with distilled water. Then, the cells were placed on collodion-coated grids and shadowed by chromium. A JEM-100B electron microscope (Japan Electrooptics Laboratory) was used for the observation of specimens.

Results

Screening of Antifungal Antibiotic Producers

Three strains of fungi, *Neurospora crassa, Aspergillus oryzae* and *Rhizopus hangchao* were used as test organisms. Each plate inoculated with one of these fungi was streaked with the soil samples. Bacterial colonies were picked up from the plate in which the inhibition of fungal growth was observed. Purification of bacteria was performed using nutrient broth agar medium. The isolated bacterial strains were retested for their antibiotic production on the plate pregrown with the corresponding fungus. From 50 soil samples, we have selected 8 strains of bacteria, W-315, H, S2, S3, S4, S5, S13 and S25, as active antibiotic producers. These strains can produce antibiotics active against the fungus only when they were grown in the spent medium of the fungus to which was tested the antibiotic production.

One of them, strain W-315, which severely inhibited growth of *N. crassa* and *A. oryzae*, was isolated from a soil sample collected in our campus. It also showed a potent antibacterial activity against Gram-positive and -negative bacteria, while strain H showed nearly a half as much antibacterial activity as that of strain W-315 when they were assayed with *E. coli* K-12. As the strain W-315 could produce antibiotic activity not only against *N. crassa* but also against *A. oryzae* when the bacterium was inoculated on the plates pregrown with these fungi, the antibiotic production was not specific against *N. crassa*. On the other hand, we could not find any antibiotic producer active against *R. hangchao*.

Properties of two antibiotics, produced by strains W-315 and H, differ from each other. Fermentation broth of strain H shows UV_{max} at 330 nm and the antibiotic of strain H was not extracted with diethyl ether from the fermentation broth. Finally, we have chosen strain W-315, which can produce antibiotic in the spent medium of *N. crassa* or *A. oryzae*.

Nutritional Factors for Antibiotic Production by Strain W-315 in the Spent Medium of *N. crassa*

In order to clarify the nutritional requirements for antibiotic production, the spent medium was treated with various methods and was tested for growth and antibiotic production. Since strain W-315 has no nitrate reduction activity, Czapek-Dox medium supplemented with ammonium sulfate and calcium carbonate was used. As shown in Table 1, the spent medium was extracted with diethyl ether at neutral, acidic or alkaline conditions. The ether extracts had no stimulating activity for antibiotic production. Charcoal adsorbed fraction had also no effect on antibiotic production (Table 1). In order to test the possibility that amino acids or peptides are factors, the spent medium was treated with ninhydrin at 100°C, then passed through the column. The eluate from ninhydrin-treated spent medium has nearly the same activity as that of untreated one (Table 1). Therefore, amino acids and peptides can not be the factor. To test the effect of vitamins and other growth factors, a small amount of yeast extract (0.01%) was added to the basal medium. At this concentration of yeast extract, bacterial growth was stimulated a little, but no antibiotic production was observed (data not shown). When 0.1% yeast extract was added to the modified Czapek-Dox medium, bacterial growth was stimulated considerably and a poor production of the antibiotic was observed (Table 2).

When a small amount of sodium propionate was added to the basal medium, bacterial growth and antibiotic production were partially stimulated as shown in Table 2. These results suggest that

Table 1. Effects of various fractions of spent medium on antibiotic production.

Treatment on spent medium	Media*2	Growth*3 (OD_{660nm})	Antibiotic*3 (A_{370nm})*4
	Basal medium	+	−
Ether extraction (neutral)	ether extract	+	−
	water layer	++	++
	water layer, with basal medium*1	++	++
〃 (acidic)	ether extract	+	−
	water layer	++	++
	water layer, with basal medium*1	++	++
〃 (alkaline)	ether extract	+	−
	water layer	++	++
	water layer, with basal medium*1	++	++
Charcoal adsorbed		+	−
Charcoal not adsorbed		++	++
Treated with ninhydrin, then passed through charcoal		++	++

Incubation was carried out using test tube (1.5 cm in diameter) containing 5 ml of medium on a reciprocal shaker inoculated with strain W-315 at 30°C for 48 hours.

*1 Mixture of each half volume of water layer and basal medium.

*2 Adjusted pH to 7.2.

*3 −: not produced, +: $OD_{660}<1.0$, ++: OD_{660} or $A_{370}>1.0$

*4 Assayed with the supernatant of culture broth.

Table 2. Production of antibiotics in modified Czapek-Dox medium supplemented with sodium propionate.

Media*1	Growth (OD_{660nm})	Antibiotic (A_{370nm})*3
Modified Czapek-Dox medium*2	5.6	0.8
〃 +0.1% Na-propionate	7.0	3.1
〃 +0.01% Na-propionate	5.6	16.2
Spent medium of *N. crassa*	13.4	32.9

Incubation was carried out on a reciprocal shaker using a 500-ml culture flask containing 100 ml of medium at 30°C for 48 hours.

*1 Adjusted pH to 7.2.

*2 Czapek-Dox medium, in which $NaNO_3$ was replaced by 0.2% of $(NH_4)_2SO_4$, and further supplemented with 0.1% of yeast extract.

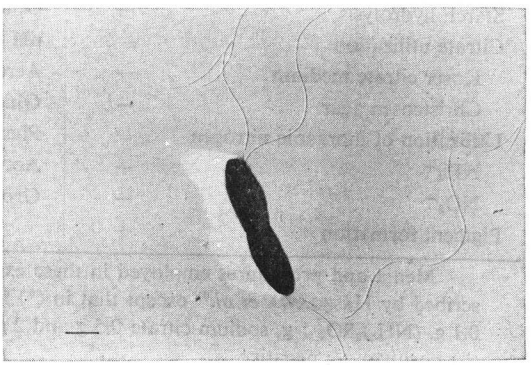
*3 Assayed with the supernatant of culture broth.

sodium propionate can be utilized for the production of this antibiotic. Glycerol, lactose and corn steep liquor did not stimulate the antibiotic production.

Morphological Characteristics

Morphological observation was carried out using optical and electron microscopes with cells of strain W-315 cultured overnight in nutrient broth. The following results were obtained; rod-shaped cells, $0.5\sim0.9\times1.6\sim2.3$ μm, occurring singly, motile, possessing 1 or 2 polar flagella,

Fig. 1. Electron micrograph of strain W-315.
Bar represents 1 μm.



Gram-negative, not acid-fast, no endospore and no pleomorphism. A typical electron micrograph of strain W-315 is shown in Fig. 1.

Culture Characteristics

Observation was carried out with strain W-315 grown on various media for 1~30 days. The following results were obtained.

(1) Colonies on nutrient agar plates incubated for 3 days at 30°C: Moderate growth, circular, raised in center, entire, butyrous, homogeneous, glistening humid and transparent with smooth surface and creamy white to creamy gray in color.

(2) Nutrient agar slants incubated for 3 days at 30°C: Moderate growth, membranous, linear and creamy gray in color.

(3) Nutrient broth incubated for 3 days at 30°C: Moderate growth, turbid homogeneously and viscous precipitate.

(4) Gelatin stab incubated for 30 days at 30°C: Liquefaction in layered form.

(5) Litmus milk incubated for 30 days at 30°C: Positive peptonization with weakly alkaline reaction.

(6) Colonies on Pseudomonas P agar (Difco Lab., Michigan) incubated for 2 days at 15, 25 or 30°C: Abundant growth and producing yellow green pigment at all temperatures tested.

Physiological Characteristics

The physiological characteristics of strain W-315 are summarized in Tables 3 and 4.

Taxonomy

It is well known that most bacteria having characteristics of aerobic, Gram-negative, rod shaped and polar flagellated belong to Pseudomonadaceae or its closely related families. The strain W-315 showed the same characteristics as those of Pseudomonadaceae as mentioned above. In the 8th

Table 3. Physiological properties of strain W-315.

KNO_3 reduction	−	Catalase	+
Denitrification	−	Urease	−
MR test	+	Oxidase	+
VP test	−	Anaerobic growth	−
Indole formation	−	O-F test	oxidative
H_2S formation	−	Maximum temperature for growth	39°C
Starch hydrolysis	−	Optimum temperature for growth	25~30°C
Citrate utilization		pH for growth	4~9
Koser citrate medium	−	Aerobiosis	aerobic
Christensen agar	−	Gluconate oxidation	+
Utilization of inorganic nitrogen		Phenylpyruvate from phenylalanine	−
NH_4^+	+	Accumulation of poly-β-hydroxybutyrate	+
NO_3^-	−	Growth in minimal medium (Davis medium)*	+
Pigment formation	+		

Media and procedures employed in these experiments were carried out according to the methods described by HASEGAWA *et al.*[6] except that in (*) the Davis medium containing K_2HPO_4 2 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, $(NH_4)_2SO_4$ 1 g, sodium citrate 0.5 g and 2 g of glucose in 1 liter of distilled water, pH 7.0 was used.
+: positive, −: negative

Table 4. Utilization of carbohydrate by strain W-315.

Carbon source	Acid formation	Gas formation	Carbon source	Acid formation	Gas formation
L-Arabinose	+	−	Trehalose	+	−
D-Xylose	+	−	D-Sorbitol	±	−
D-Mannose	+	−	D-Mannitol	±	−
D-Fructose	+	−	Inositol	±	−
D-Galactose	+	−	Glycerol	−	−
Maltose	−	−	Starch	−	−
Sucrose	−	−	Raffinose	−	−
Lactose	−	−	Rhamnose	−	−
D-Glucose	+	−			

Utilization of carbohydrate by strain W-315 was determined by the methods described by STANIER *et al.*[18] and HASEGAWA *et al.*[6] Acid formation was determined with brom cresol purple as an indicator after 7 days of incubation and gas formation was detected by bubble formation in a half-solidified medium described by STANIER *et al.*[18] after 7 days of incubation.
+: positive, ±: slightly positive, −: negative

Table 5. Characteristics differentiating the genera *Gluconobacter*, *Acetobacter* and *Pseudomonas* in relation to strain W-315.

	*Gluconobacter**	*Acetobacter**	*Pseudomonas**	Strain W-315**
Flagellation	Polar or none	Peritrichous or none	Polar	Polar
Growth at pH 4.5	+	+	−	+
Oxidation of:				
Ethanol to acetic acid at pH 4.5	+(M)	+	−	+
Lactate to CO_2	−	+	+	−
Krebs cycle member	−	+	+	−
Glycerol to dihydroxyacetone (ketogenesis)	+	±	−	+
Quinones Q_{10}	+	−		−
GC content (mole per cent)	60~64	55~64	58~70	64.4

*; From the 8th Edition of "BERGEY's Manual of Determinative Bacteriology", p. 217~277[7].
**; Tested by the methods described by KOMAGATA *et al.*[11] and HASEGAWA *et al.*[6]
M; Moderate.
±; Some (less than 90%) strains positive, others negative.
+ and −; In *, 90% or more strains positive (+) or negative (−)
in **, positive (+) or negative (−).

Edition of BERGEY's Manual of Determinative Bacteriology[7], four genera of family Pseudomonadaceae are described, *i.e. Pseudomonas, Xanthomonas, Zoogloea* and *Gluconobacter*. The genera *Xanthomonas* and *Zoogloea* are clearly differentiated from strain W-315 because the former shows complex growth requirements and the latter can not grow at pH 4.5, respectively. Furthermore, strain W-315 showed oxidase positive reaction, GC content of DNA of 64.4 mole per cent and positive growth at pH 4.5, as shown in Table 3. It is known that *Acetobacter* and *Gluconobacter* can grow at pH 4.5 while *Pseudomonas* does not under the same conditions. Therefore, we compared important characteristics among genera *Gluconobacter, Acetobacter, Pseudomonas* and strain W-315 as shown in Table 5. Since, inability to grow at pH 4.5 is one of the most important characters which differentiate genus *Pseudomonas* from other genera, strain W-315 does not belong to genus *Pseudomonas*. Other properties,

such as ability to oxidize ethanol to acetic acid at pH 4.5, inability to oxidize lactate to carbon dioxide and ability to oxidize glycerol to dihydroxyacetone (ketogenesis), also support this conclusion.

Acetobacter can oxidize lactate to carbon dioxide and also can oxidize Krebs cycle members, while strain W-315 has no such activity. As shown in Table 5, strain W-315 has the same characteristics as those of *Gluconobacter*, except for the absence of ubiquinone Q_{10}. Ubiquinone of strain W-315 is considered to be Q_7 or Q_8 by a preliminary experiment. This is the only difference between known *Gluconobacter* species and our strain W-315 as shown in Table 5. From these results, we concluded that strain W-315 belongs to genus *Gluconobacter*.

Discussion

We have isolated a novel antibiotic producing bacterium, strain W-315. Procedures for screening employed in this work were based on an assumption that there are some microorganisms which can produce antibiotics only when they consume metabolites of other organisms. By this screening, we have isolated and selected 8 strains as active antibiotic producers from 50 soil samples. Strain W-315 showed potent lytic action on mycelium of *N. crassa* when they are cultured in a mixed culture. Culture supernatant of this mixed culture, after dialysis against phosphate buffer (1 mM, pH 7.2), could solubilize the mycelium of *N. crassa* and liberated several soluble carbohydrates from the mycelium. The action of this enzyme(s) may contribute, at least partially, to the antifungal activity detected in the process of screening.

We have not yet characterized the factors in the spent medium of *N. crassa* effective for antibiotic production. Since not only the spent medium of *N. crassa* but also that of *A. oryzae* were shown to donate factor(s) for antibiotic production, the factor(s) may be produced by various genera of fungi. The factor(s) in the spent medium of *N. crassa* lost its activity gradually when the medium was kept in a refrigerator. When the spent medium of *N. crassa* was heated at 120°C for 15 minutes or exposed to daylight, the activity of the factor(s) in promoting growth of W-315 decreased and the antibiotic production also decreased which showed different UV absorption profile. Probably, the factor(s) for antibiotic production in the spent medium of *N. crassa* may act as a precursor(s) for biosynthesis of the antibiotics.

The biosynthesis of macrolide rings of the polyene antibiotics occurs *via* the polyketide pathway by repeated (head to tail) condensation of acetate and propionate units[9]. Propionyl-CoA and methylmalonyl-CoA are shown to be the starting materials in the biosynthesis of polyene macrolide. As shown in Table 2, sodium propionate supplemented in the modified Czapek-Dox medium stimulated the production of the antibiotic. This result suggests that the antibiotic is also synthesized from propionic acid in this organism. The factor in the spent medium, however, is different from propionic acid since the ether extract of the spent medium under acidic condition has no stimulating activity for antibiotic production.

At the beginning of our taxonomical study, we could not find any flagellum and motility of this organism under an optical microscope. Moreover, this organism showed a lytic action on mycelium of fungus. Therefore, we assumed that this organism belonged to non-fruiting, fungicidal *Myxobacter*[10]. Repeated examination of this organism by the electron microscope revealed that it had polar flagella which were easily detouched from the cells. The fragile nature of the flagella is the reason, we suppose, why we could not observe them under the optical microscope in stained preparations. Ultimately, we have found that strain W-315 is aerobic, Gram-negative, rod shaped, polar flagellated, oxidase positive with GC content (of DNA) of 64.4 mole per cent. These properties suggest that strain W-315 belongs to either genus *Pseudomonas* or *Gluconobacter*. However, strain W-315 has "intermediate" characteristics, which is close to one bridging property of 2 or 3 other genera.

There are many reports about "intermediate" strains among the genera of *Gluconobacter-Pseudomonas-Acetobacter*[11] and it is still a matter of controversy how to classify the "intermediate" strains. Strictly speaking, strain W-315 must belong to the "intermediate" type, but it has close characteristic

to *Gluconobacter* except for the absence of Q_{10}. In the present paper, we propose that strain W-315 belongs to *Gluconobacter* sp. rather than to classify it as a new genus because of the presence of discrepancies in the discription of "intermediate" strains. New taxon might be necessary for this strain in the future, when "intermediate" strains would be divided into their own genera.

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