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THE PRODUCTION OF ACIDIC, *O*-ACYLATED CYCLOSOPHORANS [CYCLIC (1→2)-β-D-GLUCANS] BY *Agrobacterium* AND *Rhizobium* SPECIES

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ABSTRACT

Acidic cyclosophorans [cyclic (1→2)-β-D-glucans] containing methylmalonic acid, or succinic acid, or both, were isolated by DEAE-cellulose chromatography from culture filtrates and cells of some strains of *Agrobacterium radiobacter*, *Rhizobium phaseoli*, and *R. trifolii*. The evidence suggests that one carboxyl group of the dicarboxylic acid is in ester linkage with an hydroxyl group of a sugar unit.

INTRODUCTION

Many strains of *Agrobacterium* and *Rhizobium* produce neutral, extracellular cyclosophorans, and acidic octasaccharides corresponding to the repeating units of extracellular, acidic polysaccharides[1–3].

Cyclosophorans having[4,5] degrees of polymerization (d.p.s) from 17 to 24 and larger[6] have been reported. The distribution pattern of cyclosophorans is characteristic for each bacterial strain; some strains of *Rhizobium* produce mainly cyclosophoroheptadecaose, and all strains of *Agrobacterium* tested produce a mixture of cyclosophorononadecaose, cyclosophoroeicosaose, cyclosophoroheneicosaose, and cyclosophorodocosaose[4].

Although *Agrobacterium*[1,7] and *Rhizobium*[2,3,8] species secrete cyclosophorans into their culture media along with large proportions of acidic polysaccharides, Higashiura *et al.*[9] isolated acidic polysaccharide-negative mutants which produce only cyclosophorans in the cultures. This fact made possible the isolation and identifiation of acidic cyclosophorans.

Zevenhuizen[10,11] and Abe *et al.*[12] also prepared cell-surface cyclosophorans from *Rhizobium*. It seems that the cyclosophorans play an important role in the symbiosis of *R. trifolii*[12] with its leguminous host (clover), and in the virulence of *A. tumefaciens*[13]. Sophorose also induces cellulase in *Trichoderma reesei*[14].

In the present work, we isolated extracellular and cell-surface cyclosophorans, including acidic cyclosophorans, and examined their properties.

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EXPERIMENTAL

General. — G.l.c. was performed in a Yanagimoto G180 gas chromatograph fitted with a flame-ionization detector. Methylation of the glucan was conducted as described by Hakomori[15]. The methylated sample was hydrolyzed, and analyzed as the alditol acetates on a column (2 m × 3 mm) of 0.3% of OV275–0.4% of XF 1150 on Shimalite W[16]. Methyl esterification of organic acids was performed with diazomethane in dry methanol, and methyl ester was analyzed on a capillary column (50 m × 0.25 mm) of KOCL-DS (Yanagimoto, Kyoto, Japan), with a temperature program of 80–180° at 2°/min, or 80–140° at 5°/min. G.l.c.–m.s. was performed with a Shimadzu LKB-9000 mass spectrometer (70 eV), using a column (1 m × 3 mm) of Silicone OV-17 and a temperature program of 50–170° at 10°/min.

H.p.l.c. was conducted at room temperature with a TRI ROTAR (JASCO, Tokyo, Japan) fitted with a RI detector ERC-7510, and a column (20 cm × 6 mm) of ERC-NH-1171 (both from Erma Optical Works, Tokyo, Japan), using 11:9 (v/v) acetonitrile–water as the solvent system at a flow rate of 1.0 mL/min. P.c. was conducted on Toyo No. 50 filter paper (Toyo, Tokyo, Japan) by the descending method in 10:2:15 (v/v)[17] 1-butanol–formic acid–water. Spots were detected by spraying the paper with a Bromo Cresol Green reagent. ^{1}H-n.m.r. spectra of solutions (0.5%) in Me_2SO-d_6 and $CDCl_3$ were recorded with a Bruker AM360 instrument with Me_4Si as the internal standard. Organic acid in ester linkage was estimated colorimetrically by the method of McComb and McCready[18].

Organisms and cultivation. — *Agrobacterium radiobacter* IFO 12665 and IFO 13127 were obtained from the Institute for Fermentation, Osaka, Japan. *Rhizobium phaseoli* AHU 1133 and *R. trifolii* 4S were obtained by courtesy of Prof. S. Higashi, Kagoshima University, Japan. *A. radiobacter* IFO 12665b1 and IFO 13127b were isolated as spontaneous mutants forming large amounts of (1→3)-β-D-glucan (curdlan) from stock cultures of their parent strains[1,19]. *A. radiobacter* A1-5 and *R. phaseoli* M7-7 were isolated as acidic polysaccharide-negative or diminished mutants having the ability to produce large amounts of cyclosophorans from *A. radiobacter* IFO 12665 and *R. phaseoli* AHU 1133, respectively, by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

Preparation of extracellular and cell-surface cyclosophorans. — Synthetic medium[2,9] containing 4% of D-glucose was used. The medium (95 mL) in a 500-mL, conical flask was inoculated with a culture (5 mL) grown in the same medium. Six-day culture (100 mL) was mixed with ethanol (2 vol.), and centrifuged at 9,000*g* for 30 min. The precipitate, containing cells, was supended in 65% ethanol (100 mL) at room temperature and centrifuged to wash the precipitate. The two supernatant liquors were combined, concentrated to a small volume, again mixed with ethanol (2 vol.), and centrifuged. The supernatant liquor was mixed with ethanol (4 vol.) to precipitate a low-molecular-weight material (extracellular cyclosophoran). On the other hand, a cell-surface cyclosophoran was extracted from the precipitate containing cells by treatment with 70% ethanol (200 mL) for 30 min at

~80°, according to the method of Zevenhuizen[11]. The supernatant liquor obtained by centrifugation was concentated to a small volume, mixed with ethanol (2 vol.), and centrifuged. The supernatant liquor was mixed with ethanol (4 vol.) to precipitate a low-molecular-weight material (cell-surface cyclosophoran). The resultant precipitate was collected by centrifugation, dissolved in water, and subjected to ultrafiltration through Amicon PM 10. The filtrate was concentrated to a small volume, and subjected to chromatography on Sephadex G-25, to remove salts and yellow pigments. Cyclosophorans fractionated in the void volume were collected, the solutions concentrated to a small volume, and lyophilized.

Preparation of acidic cyclosophoran. — Extracellular and cell-surface cyclosophorans were dissolved in mM potassium chloride, applied to a column of DEAE-cellulose equilibrated with mM potassium chloride, and eluted first with mM potassium chloride, and then with a linear gradient of 1 to 100mM potassium chloride. Fractions were collected, and their sugar content was measured by the phenol–sulfuric acid method. Neutral cyclosophoran was eluted with mM potassium chloride without adsorption to the column, and acidic cyclosophoran (adsorbed to the column) was eluted with the linear gradient. The fractions containing these cyclosophorans were collected, desalted, and lyophilized.

Preparation of an organic acid from acidic cyclosophoran. — A solution (0.1%) of acidic cyclosophoran was stirred in 10mM potassium hydroxide for 6 h at 20° under nitrogen, and then the base was neutralized with M sulfuric acid. The solution was concentrated to a small volume, mixed with ethanol (9 vol.), and centrifuged at 9,000*g* for 20 min, to remove deacylated cyclosophoran. The resultant supernatant liquor was concentrated to a small volume, the pH adjusted to 2 with M sulfuric acid, and then extracted continuously with ether for 48 h.

RESULTS AND DISCUSSION

Extracellular cyclosophoran (1 g) produced by *A. Radiobacter* A1-5 was separated by DEAE-cellulose chromatography into three materials (designated **P1**, **P2**, and **P3**, respectively) in yields of 768, 110, and 24 mg, as shown in Fig. 1. On the column, it is known[1,2] that a neutral material like **P1** is eluted without adsorption to the column, and that an acidic material, such as **P2** or **P3**, is adsorbed, and is then eluted at higher concentration of salt. On sugar-component and methylation analysis of each material, only D-glucose and 3,4,6-tri-*O*-methyl-D-glucose, respectively, were found, and colorimetric analysis showed occurrence of an organic acid linked in an ester linkage to **P2** and **P3**. In h.p.l.c. in a column of ERC-NH-1171, native **P2** and **P3** are not eluted, but the deacylated **P2** and **P3**, obtained by treatment at pH 12, showed almost the same elution profile as **P1** (see Fig. 2). These results suggested that **P1** is a neutral cyclosophoran, and that **P2** and **P3** are acidic cyclosophorans in which one carboxyl group of a dicarboxylic acid is linked as an ester with a hydroxyl group of a sugar unit.

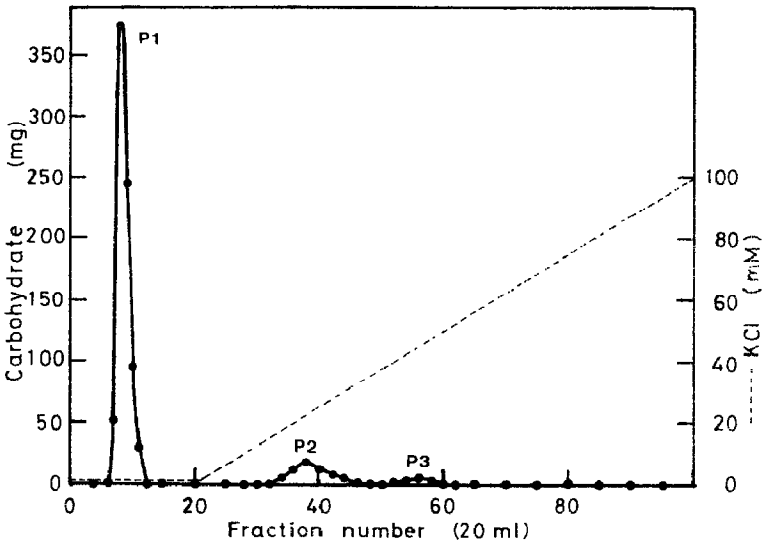

Fig. 1. DEAE-cellulose chromatography of the extracellular cyclosophoran from *A. radiobacter* A1-5. [The sample (1 g) was applied to a column (5 × 15 cm) of DEAE-cellulose equilibrated with mM potassium chloride, and material was eluted first with 400 mL of potassium chloride, and then with 1.6 L of a linear gradient of 1 to 100 mM potassium chloride. Fractions (20 mL) were analyzed for carbohydrate by the phenol-sulfuric acid method.]

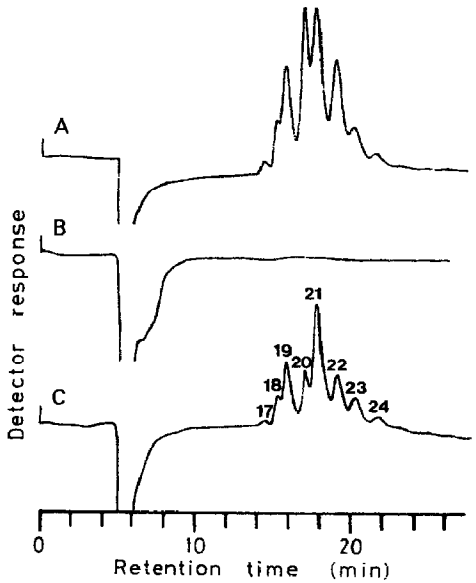

Fig. 2. H.p.l.c. profiles of neutral material **P1** (A) and acidic material **P2** (B) fractionated in Fig. 1, and deacylated **P2** (C), on a column of ERC-NH-1171. [The h.p.l.c. profile of acidic material **P3** was almost the same as that of **P2**. The numerals 17, 18, 19, 20, 21, 22, 23, and 24 indicate the numbers of D-glucosyl residues in the cyclosophorans providing the respective peaks.]

To estimate the organic acid linked to the extracellular cyclosophoran of strain A1-5, material **P2** (500 mg) was deacylated in solution at pH 12, and ~20 mg of ether-extractable substance was obtained. P.c. of the ether extract showed a single yellow spot with the R_F value of 0.85 in the solvent system. The ^{1}H-n.m.r. spectrum of the ether extract showed signals at δ 1.40, as a doublet with J 7.3 Hz, and at δ 3.36, as a quartet with J 7.3 Hz, suggesting methylmalonic acid. In g.l.c. in a capillary column of KOCL-DS, the retention time of the methyl ester derivative in the ether extract was the same as that of authentic methylmalonic acid (Aldrich Chemical Company, Inc.), as shown in Fig. 3. Furthermore, the g.l.c.–mass spectrum showed a peak at *m/z* 146 corresponding to the molecular weight of the dimethyl ester derivative, and the same fragments as the authentic sample at *m/z* 59, 72, 87, and 115. Thus, it was concluded that organic acid in **P2** was methylmalonic acid.

Furthermore, it could be estimated that **P2** [*O*-(methylmalonyl)cyclosophoran] contains one mol of the organic acid per mol of cyclic glucan, because ~20 mg of organic acid was obtained by ether extraction from 500 mg of **P2** and the position of elution of **P2** in DEAE-cellulose chromatography was almost the same as that of an acidic material containing one mol equiv. of free carboxyl group per molecule[1]. The organic acid obtained from **P3** by ether extraction could also be identified as methylmalonic acid by g.l.c., and the colorimetric value for the organic acid of **P3** was almost twice that of **P2**. From these results, it was estimated that **P3** is *O*-(methylmalonyl)cyclosophoran containing 2 equiv. of organic acid.

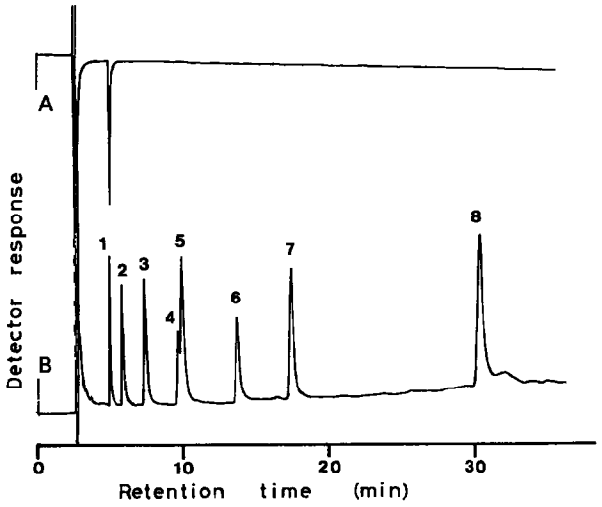

Fig. 3. G.l.c. of methyl esters of an organic acid (A) obtained from acidic material **P2** of *A. radiobacter* A1-5, and some authentic samples (B) on a column of KOCL-DS, using a temperature program of 80–180° at 2°/min. [The numerals 1, 2, 3, 4, 5, 6, 7, and 8 indicate the methyl esters of methylmalonic, malonic, succinic, citraconic, glutaric, adipic, pimelic, and mesaconic acid, respectively.]

TABLE I

PRODUCTION OF EXTRACELLULAR (EC) AND CELL-SURFACE (CS) CYCLOSOPHORANS, CURDLAN, AND ACIDIC POLYSACCHARIDE BY SOME STRAINS OF *Agrobacterium* AND *Rhizobium*

Strain	*Cyclosophorans (mg/100 mL)*		*Curdlan (mg/100 mL)*	*Acidic polysaccharide (mg/100 mL)*
	EC (AC)[a]	*CS (AC)*[a]		
A. radiobacter				
IFO 12665b1	263 (25)	170 (19)	1100	9
IFO 13127b	175 (22)	146 (20)	790	72
A1-5	559 (82)	360 (55)	0	0
R. phaseoli				
AHU 1133	35 (2.8)	9 (0.8)	0	270
M7-5	91 (8.5)	23 (1.6)	0	10
R. trifolii				
4S	34 (3.5)	12 (1.0)	0	240

[a]AC = acidic cyclosophoran. This was determined by analysis (phenol–sulfuric acid) of the fraction eluted from a DEAE-cellulose column by 50 mL of 100mM potassium chloride, after removal of the neutral material (see text).

Cell-surface cyclosophorans of *R. meliloti* and *R. trifolii* have been also reported[10–12]. Consequently cell-surface cyclosophoran was prepared from cells according to the simple method of Zevenhuizen[11] as described in the Experimental section. The production of the extracellular and cell-surface cyclosophorans from some strains of *Agrobacterium* and *Rhizobium* was investigated (see Table I). As the strains tested are acidic and octasaccharide-negative[1,2], it is possible to consider the acidic material in the low-molecular-weight fraction to be an acidic cyclosophoran. To estimate the amounts of acidic cyclosophorans in extracellular and cell-surface cyclosophorans, a sample (25 mg) was applied to a column (2.5 × 8 cm) of DEAE-cellulose equilibrated with mM potassium chloride. Neutral cyclosophoran was first eluted with 100 mL of mM potassium chloride, and then an acidic one was eluted with 50 mL of 100 mM potassium chloride. The sugar content was measured by the phenol–sulfuric acid method (see Table I). All of the acidic materials showed only D-glucose and 3,4,6-tri-*O*-methyl-D-glucose. Extracellular and cell-surface cyclosophorans contained ~7–15% of acidic cyclosophoran.

The acidic cyclosophoran (~10 mg) of each strain was deacylated, extracted continuously with ether, and transformed into the methyl ester. In g.l.c. in a capillary column of KOCL-DS, *A. radiobacter* A1-5 showed only methylmalonic acid, *A. radiobacter* IFO 12665b1 and IFO 13127b showed methylmalonic acid and succinic acid, and *R. phaseoli* AHU 1133, *R. phaseoli* M7-5, and *R. trifolii* 4S showed only succinic acid (see Fig. 4). The organic acid of cell-surface acidic cyclosophoran was almost the same as that of the extracellular one, in each strain.

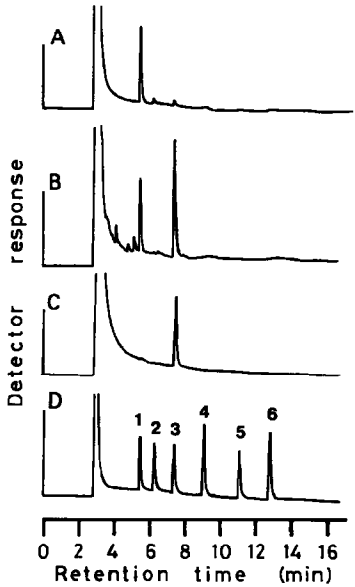

Fig. 4. G.l.c. of methyl esters of organic acids from the extracellular acidic cyclosophorans of *A. radiobacter* A1-5 (A), *A. radiobacter* 12665b1 (B), and *R. phaseoli* AHU 1133 (C), compared with standards (D), on a column of KOCL-DS. The temperature was programmed from 80 to 140° at 5°/min. [The numerals 1, 2, 3, 4, 5, and 6 indicate methyl esters of methylmalonic, malonic, succinic, glutaric, adipic, and pimelic acid, respectively.]

Occurrence of similar acidic cyclosophorans in other strains of *Agrobacterium* and *Rhizobium* may be suggested, judging from the present results and those in previous papers. In DEAE-cellulose chromatography of the low-molecular-weight fractions of *A. radiobacter* IFO 12644, *R. meliloti* J7017, *R. meliloti* IFO 13336, *R. trifolii* J60, *R. trifolii* AHU 1134, and *R. lupini* KLU, methylation analysis of the second peak (product **2** or **P2**) showed 3,4,6-tri-*O*-methyl-D-glucose and some methylated sugars corresponding to the octasaccharide repeating-unit of acidic polysaccharide[1,2]. Therefore, we previously considered that it was a complex with neutral cyclosophoran and acidic octasaccharide containing pyruvic acid[1,2], but we have now found that it is a mixture of acidic cyclosophoran and acidic octasaccharide.

Acetic acid, succinic acid, pyruvic acid, and malonic acid are known to be present in many polysaccharides and oligosaccharides produced by *Xantomonas*[20], *Agrobacterium*[21,22], *Rhizobium*[21,23], *Alcaligenes*[24], *Arthrobacter*[25], *Azotobacter*[26], *Pseudomonas*[27], *Klebsiella*[28], *Escherichia*[29,30], and *Penicillium*[31]. The present report appears to be the first to show the presence of methylmalonic acid in a glycan.

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REFERENCES

1 M. HISAMATSU, A. AMEMURA, T. MATSUO, H. MATSUDA, AND T. HARADA, *J. Gen. Microbiol.*, 128 (1982) 1873–1879.
2 A. AMEMURA, M. HISAMATSU, H. MITANI, AND T. HARADA, *Carbohydr. Res.*, 114 (1983) 277–285.
3 L. P. T. M. ZEVENHUIZEN AND A. R. W. VAN NEERVEN, *Carbohydr. Res.*, 118 (1983) 127–134.
4 M. HISAMATSU, A. AMEMURA, K. KOIZUMI, T. UTAMURA, AND Y. OKADA, *Carbohydr. Res.*, 121 (1983) 31–40.
5 A. DELL, W. S. YORK, M. MCNEIL, A. G. DARVILL, AND P. ALBERSHEIM, *Carbohydr. Res.*, 117 (1983) 185–200.
6 K. KOIZUMI, Y. OKADA, T. UTAMURA, M. HISAMATSU, AND A. AMEMURA, *J. Chromatogr.*, 299 (1984) 215–224.
7 F. C. MCINTIRE, W. H. PETERSON, AND A. J. RIKER, *J. Biol. Chem.*, 143 (1942) 491–496.
8 W. S. YORK, M. MCNEIL, A. C. DARVILL, AND P. ALBERSHEIM, *J. Bacteriol.*, 142 (1980) 243–248.
9 T. HIGASHIURA, M. IKEDA, M. OKUBO, M. HISAMATSU, A. AMEMURA, AND T. HARADA, *Agric. Biol. Chem.*, 49 (1985) 1865–1866
10 L. P. T. M. ZEVENHUIZEN AND H. J. SCHOLTEN-KOERSELMAN, *Antonie van Leeuwenhoek; J. Microbiol. Serol.*, 45 (1979) 165–175.
11 L. P. T. M. ZEVENHUIZEN, *Antonie van Leeuwenhoek; J. Microbiol. Serol.*, 47 (1981) 481–497.
12 M. ABE, A. AMEMURA, AND S. HIGASHI, *Plant Soil*, 64 (1982) 315–324.
13 V. PUVANESARAJAH, F. M. SCHELL, G. STACEY, C. J. DOUGLAS, AND E. W. NESTER, *J. Bacteriol.*, 164 (1985) 102–106.
14 M. MANDELS, F. W. PARRISH, AND E. T. REESE, *J. Bacteriol.*, 83 (1962) 400–408.
15 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
16 M. HISAMATSU, J. ABE, A. AMEMURA, AND T. HARADA, *Agric. Biol. Chem.*, 44 (1980) 1049–1055.
17 B. J. BLOCK, E. L. DURRUM, AND G. ZWEIG, *A Manual of Paper Chromatography and Paper Electrophoresis*, 2nd (rev.) edn., Academic Press, New York, 1958, p. 215.
18 E. A. MCCOMB AND R. M. MCCREADY, *Anal. Chem.*, 29 (1957) 819–821.
19 M. HISAMATSU, I. OTT, A. AMEMURA, AND T. HARADA, *J. Gen. Microbiol.*, 103 (1977) 375–379.
20 J. H. SLONEKER AND A. R. JEANES, *Can. J. Chem.*, 40 (1962) 2066–2071.
21 L. P. T. M. ZEVENHUIZEN, *J. Gen. Microbiol.*, 68 (1971) 239–243.
22 M. HISAMATSU, K. SANO, A. ANEMURA, AND T. HARADA, *Carbohydr. Res.*, 61 (1978) 89–96.
23 S. K. GHAI, M. HISAMATSU, A. AMEMURA, AND T. HARADA, *J. Gen. Microbiol.*, 122 (1981) 33–40.
24 T. HARADA, *Arch. Biochem. Biophys.*, 112 (1965) 65–69.
25 C. A. KNUTSON, J. E. PITTSLEY, AND A. JEANES, *Carbohydr. Res.*, 73 (1979) 159–168.
26 G. H. COHEN AND D. B. JOHNSTONE, *J. Bacteriol.*, 88 (1964) 329–338.
27 D. G. ORENTAS, J. H. SLONEKER, AND A. R. JEANES, *Can. J. Microbiol.*, 9 (1968) 427–430.
28 B. J. GORMUS, R. W. WHEAT, AND J. F. PORTER, *J. Bacteriol.*, 107 (1971) 150–154.
29 I. W. SUTHERLAND, *Biochem. J.*, 115 (1969) 935–945.
30 E. P. KENNEDY, *Proc. Natl. Acad. Sci. U.S.A.*, 79 (1982) 1092–1095.
31 N. NAKAMURA, J. OOYAMA, AND O. TANABE, *Nippon Nogei Kagaku Kaishi*, 35 (1961) 949–953.