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Note

Formation of an oligosaccharide, the repeating unit of succinoglucan, by *Alcaligenes faecalis* var. *myxogenes**

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A mutant strain 22-33 of *Alcaligenes faecalis* var. *myxogenes* 22 has been found to produce the repeating unit of succinoglucan in a synthetic medium enriched with 0.1% yeast extract, in a yield of about 370 mg per 100 ml. Strain 22 also produced the oligosaccharide in the absence of yeast extract when a reagent such as penicillin, or more especially bacitracin, was added to the synthetic medium. When analysed chemically and enzymically with a specific, intracellular β-D-glucanase of *Flavobacterium* M64, the oligomer appeared similar to the oligomer derived from succinoglucan by hydrolysis with succinoglucan depolymerase from strain M64. It is proposed that this oligomer is the repeating unit of succinoglucan.

Alcaligenes faecalis var. *myxogenes* strain 10C3, which was isolated from soil, produces an extracellular, acidic polysaccharide, succinoglucan[1,2], utilizing ethylene glycol as well as D-glucose. Strain 10C3 also produces some curdlan in addition to succinoglucan, whereas mutant strain 22 derived from 10C3 produces only succinoglucan. Succinoglucan depolymerase induced by *Flavobacterium* M64 hydrolyzes succinoglucan to repeating units[3]. Strains 10C3 and 22 cannot hydrolyze succinoglucan appreciably. Recently, some strains of *Agrobacterium* have also been shown to form a water-soluble, acidic polysaccharide resembling succinoglucan[4]. The present paper describes the formation of repeating units of succinoglucan by mutant strains of *Alcaligenes faecalis* var. *myxogenes* 10C3.

MATERIALS AND METHODS

Organisms. — *Alcaligenes faecalis* var. *myxogenes* 22 (ref. 5) and a mutant strain 22-33 derived from the strain by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine were used.

Cultures. — A synthetic chemical medium[6] containing 4% of D-glucose with

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and without 0.1% yeast extract was used for production of succinoglucan and the oligosaccharide **1**.

Volumes of 95 ml of the medium in 500-ml conical flasks were inoculated with 5 ml of seed culture that had been grown in the same medium. The cultures were shaken reciprocally at 120 strokes per min at 30°.

Preparation and determination of succinoglucan, oligosaccharide **1**, *and cells.* — The culture broth was mixed with sodium chloride to 1% concentration and then with two volumes of ethanol. The precipitate containing succinoglucan and cells was removed by centrifugation, and the supernatant solution was concentrated to one tenth of its volume by vacuum evaporation, again mixed with two volumes of ethanol, and centrifuged to remove a trace of succinoglucan. The supernatant layer was subjected to ultrafiltration with Diaflo (Amicon Co.), which cuts off materials having molecular weights $> 10{,}000$. The filtrate was mixed with 4 volumes of ethanol and the insoluble material was collected by centrifugation, washed with 1:4 (v/v) water–ethanol, dissolved in a small amount of water, and deionized with Amberlite IR-120 (H^+) and IR-45 (CO_3^{2-}) resins. Four volumes of ethanol were then added to the solution and the resultant precipitate was collected by centrifugation, dehydrated with acetone, and dried *in vacuo* at 40°. This product was the oligosaccharide **1** (short-chain glucan). The amounts of succinoglucan and cells were determined by the procedure described previously[5].

*Preparation of intracellular, specific β-*D*-glucanase.* — Intracellular, specific β-D-glucanase[7] was prepared from a culture of *Flavobacterium* M64, capable of growing in a medium containing succinoglucan as the sole carbon source.

Preparation of repeating units (**2**) *from succinoglucan by hydrolysis with succinoglucan depolymerase.* — Repeating units (**2**) were prepared from succinoglucan by hydrolysis with succinoglucan depolymerase of *Flavobacterium* M64 as described previously[3].

Determination of sugar and organic acids. — Sugar and organic acid components of oligosaccharides and succinoglucan were determined as described previously[4].

High-voltage paper electrophoresis. — High-voltage paper electrophoresis was carried out in a Fujiriken apparatus with a hexane-cooled tank at 40 volts per cm. The electrophoretic buffer system was 20:20:960, (v/v) pyridine–acetic acid–water, pH 4.7. Sugars on the paper were detected with silver nitrate reagent.

Digestion of oligosaccharides (**1** *and* **2**) *with the specific intracellular β-*D*-glucanase from* Flavobacterium *M64.* — The oligosaccharide (1 mg) was dissolved in 200 μl of 0.1M acetate buffer (pH 5.8) and incubated with 100 μl of a solution of intracellular β-D-glucanase (3 units) from strain M64 for 5 h at 40°. The mixture was then boiled for 3 min and passed through a column of Amberlite IR-120 (H^+ resin) to remove cations. The effluent was concentrated by vacuum evaporation, applied to Toyo-Roshi No. 50 paper, and developed with 6:4:3 (v/v) 1-butanol–pyridine–water. Sugars on the paper were detected with silver nitrate reagent.

RESULTS AND DISCUSSION

Formation of oligosaccharide **1**. — Previously, we showed that *Alcaligenes faecalis* var. *myxogenes* strain 22 derived from strain 10C3 produces large amounts of succinoglucan only in a synthetic chemical medium[6] and in a yeast extract–D-glucose medium[8]. Recently, using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, we have obtained mutant strains from the organism that produce materials giving a positive reaction on paper chromatograms with silver nitrate reagent. Many mutant strains were found to produce oligosaccharides, and the oligosaccharides produced by many different mutants seem to be similar, or identical, to each other; we have provisionally termed them SCG (short-chain glucan, **1**). Among the mutants, we selected strain 22-33. The formation of succinoglucan and the oligosaccharide **1** by strains 22 and 22-33 in chemically defined medium, with and without 0.1% yeast extract, are shown in Table I. Prolonged incubation was required for maximal formation of the oligosaccharide by strain 22-33. Strain 22-33 produced much more of the oligomer in the complex medium than in the synthetic medium, although strain 22 produced little in either medium. The products of succinoglucan and the oligomer by strain 22 became maximal after ~6 days, whereas those of strain 22-33 were maximal after ~9 days.

The ability of strain 22-33 to produce succinoglucan seemed to be changed by mutation from strain 22. It seemed probable that the cell surface changes because of mutation, resulting in excretion of the oligosaccharide **1**. Thus, the effects of various

TABLE I

FORMATION OF OLIGOSACCHARIDE **1** AND SUCCINOGLUCAN IN SYNTHETIC MEDIA BY *Alcaligenes faecalis* VAR. *myxogenes* 22 AND 22-33

	Incubation time (days)	*Production (mg/100 ml) of* **1** *and succinoglucan by*	
		Strain 22	*Strain 22-33*
Without yeast extract			
1	3	11	11
	6	16	55
	9	17	85
Succinoglucan	3	950	145
	6	1050	725
	9	1060	764
With 0.1% yeast extract			
1	3	58	95
	6	67	166
	9	71	366
Succinoglucan	3	980	50
	6	1150	480
	9	1190	640

reagents that change the cell surface, such as penicillin, bacitracin, and sodium dodecyl sulfate on the production of succinoglucan and the oligomer by strain 22 were examined by using a synthetic chemical medium without yeast extract, as shown in Table II. Addition of certain amounts of penicillin, and more especially bacitracin, caused an increase in the amount of the oligomer produced, but a decrease in succinoglucan production. Recently, the syntheses of colanic acid and its sugar–lipid intermediate were shown to be inhibited by bacitracin[9], which binds specifically with C_{55}-polyisoprenol pyrophosphate[10]. It is thus probable that the increase in the oligosaccharide **1** on addition of bacitracin is related to decrease of C_{55}-polyisoprenol in the system for biosynthesis of succinoglucan.

TABLE II

EFFECTS OF SOME REAGENTS ON FORMATION OF OLIGOSACCHARIDE **1** AND SUCCINOGLUCAN BY *Alcaligenes faecalis* VAR. *myxogenes* 22[a]

Compound added	*Concentration (μg/ml)*	*Production of*			
		1	*Succinoglucan (mg/100 ml)*	*Cells*	*Consumption of D-glucose (%)*
None		15	1075	175	100
Penicillin G	1.2	17	956	174	100
	6.0	37	767	150	100
Bacitracin	10	43	675	174	100
	50	87	535	151	100
	100	106	527	147	100
Sodium dodecyl sulfate	10	18	558	145	100
	20	15	415	149	100

[a]Cultures were harvested after 9 days of incubation.

Isolation and properties of oligosaccharide **1**. — About 15 g of the oligosaccharide (**1**) was obtained from the culture broth (5 l) obtained after culture of strain 22-33 in a medium containing 0.1% yeast extract for 10 days, as already described (Methods).

Elementary analysis of the oligomer gave: C, 43.80; H, 6.27. The components of **1** are shown in Table III, together with those of the repeating unit (**2**) of succinoglucan derived from succinoglucan by hydrolysis with succinoglucan depolymerase. The difference in the proportions of D-glucose, D-galactose, succinic acid, and pyruvic acid in the three preparations were within the limits of experimental error.

The degree of polymerization was estimated by determination of end groups by by the modified method[11] of Unrau and Smith (Table III). Compounds **1** and **2** seem to have a degree of polymerization of 9, but the true value cannot be known until the structure of the oligomers has been clarified. The homology of the oligomers in strains 22 and 22-33 was studied by high-voltage paper electrophoresis with **2** for comparison (Fig. 1). The three single spots on the positive side given by the three preparations appeared to coincide with each other. The products from oligosac-

TABLE III

COMPONENTS AND DEGREE OF POLYMERIZATION OF OLIGOSACCHARIDES **1** AND **2** DERIVED FROM SUCCINOGLUCAN BY HYDROLYSIS WITH SUCCINOGLUCAN DEPOLYMERASE, COMPARED WITH THOSE OF SUCCINOGLUCAN

	D-*Glucose* (%)	D-*Galactose* (%)	*Succinic acid* (%)	*Pyruvic acid* (%)	*D.p.*
1	77	10	6.6	5.8	9.1[a]
2	78	9.5	6.0	5.9	9.4[a]
Succinoglucan 10C3	78	10	6.3	5.4	1600[b]

[a]Determined as described in this paper. [b]Estimated[12] by taking the molecular weight as 3×10^5.

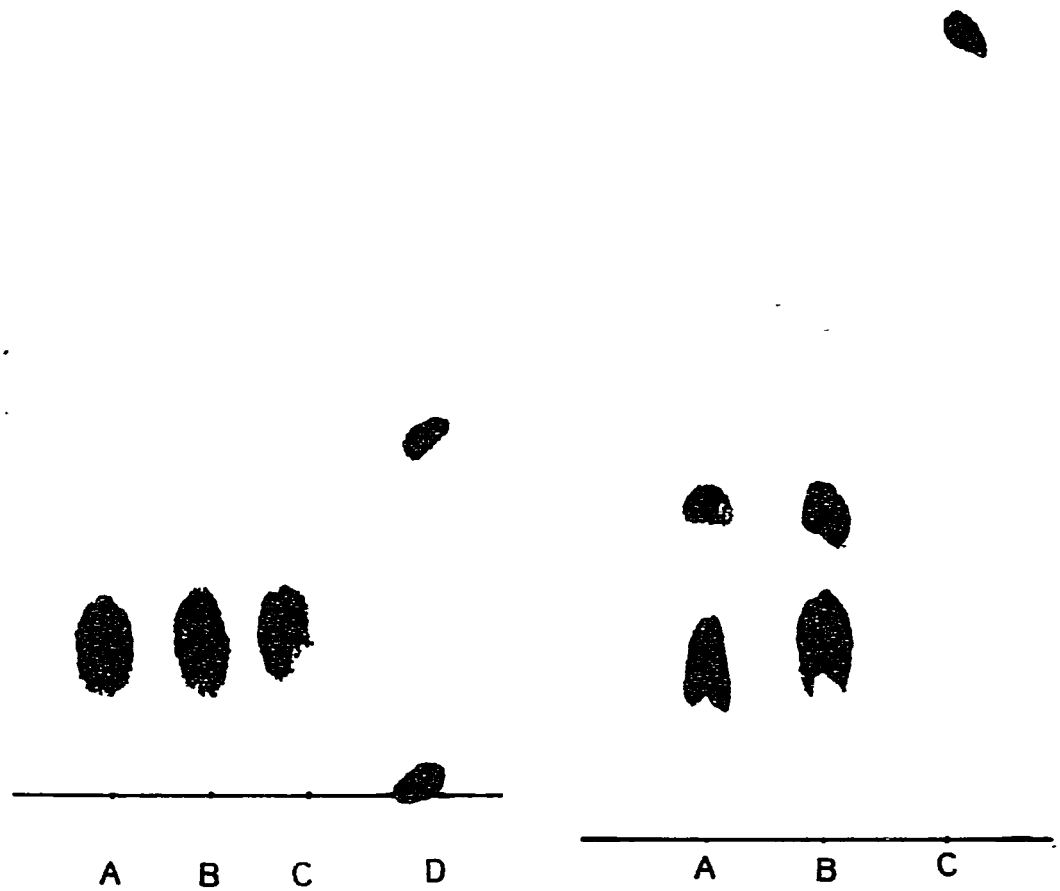

Fig. 1 (left). Chromatograms of oligosaccharides **1** from strains 22 (A) and 22-33 (B), and oligosaccharide **2** (C) by high-voltage paper electrophoresis. Controls, glucose and glucuronic acid (D).

Fig. 2 (right). Chromatograms of hydrolyzates of oligosaccharides **1** (22-33) (A) and **2** (B) by intracellular, specific β-D-glucanase of *Flavobacterium* M64. Control, glucose (C).

charides **1** and **2** by action of the intracellular, specific β-D-glucanase of *Flavobacterium* M64 were also compared (Fig. 2). The hydrolyzates of the two preparations each gave two spots, the slower-moving one being much the stronger. If the oligomer is split at a single glucosidic bond, the two spots should have nearly the same size. Therefore, the slower-moving spot may be composed of two or more principal compounds. Further studies are required on the isolation of pure components and elucidation of their structure, but our present results indicate that oligosaccharide **1** is similar to, or the same as **2**, formed by enzymic hydrolysis of succinoglucan.

Next, we tested whether the oligosaccharide **1** could be formed by hydrolysis with an enzyme formed by strain 22-33. Cells were cultured for 3, 6, and 9 days in a medium containing 0.1% of yeast extract and were then collected by centrifugation; some of the cells were sonicated. The actions of these preparations (culture supernatant, intact cells, and sonication-disrupted cells) on succinoglucan in phosphate buffer, pH 7 and 8, on prolonged incubation were examined. No hydrolysis of succinoglucan was observed. Thus, formation of the oligosaccharide is probably not due to hydrolysis of succinoglucan. Some changes in the cell surface or in the system for biosynthesis of succinoglucan, caused by prolonged incubation in a medium containing yeast extract, or by addition of some reagents, may cause formation and release of the oligosaccharide **1**. It seems likely that **1** is formed from certain intracellular components that are intermediates in the synthesis of succinoglucan.

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REFERENCES

1 T. HARADA, *Arch. Biochem. Biophys.*, 112 (1965) 65–59.
2 T. HARADA, *Process Biochem.*, 9 (1974) 21–25.
3 A. AMEMURA, K. MOORI, AND T. HARADA, *Biochim. Biophys. Acta*, 334 (1974) 398–409.
4 M. HISAMATSU, K. SANO, A. AMEMURA, AND T. HARADA, *Carbohydr. Res.*, 61 (1978) 89–96.
5 T. HARADA, A. AMEMURA, H. SAITO, S. KANAMARU, AND A. MISAKI, *J. Ferment. Technol.*, 46 (1968) 679–684.
6 A. AMEMURA AND T. HARADA, *J. Ferment. Technol.*, 49 (1971) 559–564.
7 A. AMEMURA, M. HISAMATSU, J. ABE, AND T. HARADA, *Abstr. Annu. Meet. Soc. Ferment. Technol. Jpn.*, (1977) p. 87.
8 T. HARADA AND T. YOSHIMURA, *Biochim. Biophys. Acta*, 83 (1964) 374–376.
9 J. G. JOHNSON AND D. B. WILSON, *J. Bacteriol.*, 129 (1977) 225–236.
10 G. SIEWERT AND J. L. STROMINGER, *Proc. Natl. Acad. Sci. U.S.A.*, 57 (1967) 767–773.
11 H. AKAI, K. YOKOBAYASHI, A. MISAKI, AND T. HARADA, *Biochim. Biophys. Acta*, 234 (1971) 422–429.
12 A. MISAKI, H. SAITO, T. ITO, AND T. HARADA, *Biochemistry*, 8 (1969) 4645–4650.