Change in Ability of *Agrobacterium* to Produce Water-soluble and Water-insoluble β -Glucans

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Four of nine stock cultures of *Agrobacterium* tested formed mixtures of two types of colonies, which were distinguished on plates of aniline blue medium because of the difference in their production of two exocellular polysaccharides. The ability of organisms from the white colonies of strain IFO12665 to produce large amounts of water-soluble polysaccharide was unstable; when kept on nutrient agar slants or in inorganic salt solution the bacteria mutated spontaneously to yield blue colonies producing large amounts of curdlan-type polysaccharide instead.

INTRODUCTION

Curdlan is a β -glucan which is composed entirely of (1 $\rightarrow$ 3)- β -glucosidic linkages and it forms a firm, resilient gel when heated in aqueous suspension (Maeda *et al.*, 1967; Harada, Misaki & Saito, 1968*a*; Saito, Misaki & Harada, 1968). This polymer was first found to be formed in high yield by a mutant strain (10C3K) (Harada *et al.*, 1966) of *Alcaligenes faecalis* var. *myxogenes* 10C3 which produces a water-soluble, acidic polysaccharide (succinoglucan) (Harada, 1965). Recently, we have investigated the distribution of curdlan-type polysaccharide in micro-organisms by using the aniline blue method in which organisms forming curdlan-type polysaccharide are detected as blue colonies on aniline blue plates. In this way, some strains of *Agrobacterium* were found to produce curdlan-type polysaccharide with water-soluble polysaccharide (Nakanishi *et al.*, 1976). In addition, some stock cultures of *Agrobacterium* formed mixtures of blue and white colonies on aniline blue plates; these variant forms developed spontaneously under normal conditions for maintenance of bacteria on agar slants. The present paper describes studies on the instability of polysaccharide production in *Agrobacterium*.

METHODS

Strains. Seven strains of *Agrobacterium radiobacter*, one strain of *Agrobacterium rhizogenes* and one strain of *Agrobacterium tumefaciens* (see Table 1) were obtained from the Institute of Fermentation, Osaka.

Media. The stock culture medium (PY medium) contained (% w/v): peptone (Takeda), 1; yeast extract, 0.5; NaCl, 0.5; pH 7.2. PY agar contained, in addition, 2% agar. AB medium [containing (% w/v): glucose, 1; yeast extract, 0.5; aniline blue, 0.005; agar, 2; pH 7.2] was used to detect colonies forming curdlan, because aniline blue combines specifically with curdlan (Nakanishi *et al.*, 1974). The synthetic medium (S

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Table 1. *Formation of water-soluble and water-insoluble polysaccharides by strains isolated from various stock cultures of Agrobacterium*

Strain	Appearance on AB plates	Exocellular polysaccharide produced [mg (100 ml) ⁻¹]		Wt of organisms formed [mg dry wt (100 ml) ⁻¹]
		Water-soluble	Water-insoluble	
<i>A. radiobacter</i> IFO12607	Blue	70	500	170
IFO12664	White	270	0	155
IFO12665	Blue and white	670	15	100
IFO13127	Blue and white	400	80	120
IFO13256	Blue (two types)	130	120	150
IFO13532	White	300	0	100
IFO13533	White	1200	0	80
<i>A. rhizogenes</i> IFO13259	Blue (two types)	120	480	160
<i>A. tumefaciens</i> IFO3058	White	180	0	60

medium) described previously (Amemura & Harada, 1971) was used to examine polysaccharide production by the organisms.

Culture conditions for polysaccharide production. S medium (95 ml) in 500 ml conical flasks was inoculated with a seed culture (5 ml) which had been grown in the same medium. The culture was shaken reciprocally at 120 strokes min⁻¹ at 30 °C for 5 days.

Determination of water-soluble and water-insoluble polysaccharides. Amounts of water-soluble and water-insoluble polysaccharides were assayed by the method of Harada *et al.* (1968*b*).

RESULTS AND DISCUSSION

Coexistence of different types of organisms in stock cultures of Agrobacterium

The amounts of water-soluble and water-insoluble polysaccharides produced by nine strains of *Agrobacterium* were measured (Table 1). Organisms from white colonies of strains 12665 and 13127 and those obtained from slimy blue colonies of strains 13256 and 13259 were selected for examination, because white colonies or slimy blue colonies may be the parent strains, as shown for *Alcaligenes faecalis* var. *myxogenes* (Hisamatsu *et al.*, 1976). Stock cultures of the other five strains were used directly. Organisms giving only blue colonies produced considerable amounts of water-insoluble polysaccharide; those giving only white colonies produced only water-soluble polysaccharide. Organisms giving white colonies, selected from cultures growing as a mixture of white and blue colonies, produced considerable amounts of water-soluble polysaccharide, whereas organisms giving slimy blue colonies, selected from cultures in which two types of blue colonies were present, produced both water-soluble and water-insoluble polysaccharides. These results confirm that water-insoluble polysaccharide (curdlan-type polysaccharide) forms a complex with aniline blue, as previously reported (Nakanishi *et al.*, 1974).

Agrobacterium radiobacter IFO12665 was selected for the following experiments, because it formed very distinct blue and white colonies. Blue and white colonies were picked at random from a plate of the organism and the pure cultures derived from them were spread on AB medium plates (Fig. 1*a*, *c* and *d*). The strain 12665W obtained from a white colony was unstable yielding blue colonies (Fig. 1*b*), whereas strains 12665B1 and 12665B2 obtained from blue colonies were stable for up to 60 days on PY agar slants. The organism giving white colonies may be the parent strain and it may mutate to organisms giving blue colonies. The production of polysaccharide by these organisms was measured. Strain 12665W produced large amounts (6.7 mg ml⁻¹) of water-soluble polysaccharide but only small amounts of water-insoluble polysaccharide (0.15 mg ml⁻¹), while strains 12665B1 and 12665B2 produced large amounts of water-insoluble polysaccharide (9.9 and 5.6 mg ml⁻¹,

Fig. 1. Colonies of *Agrobacterium radiobacter* after growth at 30 °C for 5 days on AB medium: (a) strain 12665W (fresh pure culture); (b) strain 12665W (culture incubated for 60 days after purification); (c) strain 12665B1 (fresh pure culture); (d) strain 12665B2 (fresh pure culture).

respectively) with small amounts (0.87 and 0.89 mg ml⁻¹) of water-soluble polysaccharide. The colonies of strains 12665B1 and 12665B2 were slightly different in shape.

Spontaneous mutation of ability to produce polysaccharide

Colonies producing curdlan-type polysaccharide are stained well with aniline blue, whereas those producing only succinoglucan are not (Nakanishi *et al.*, 1976). The insoluble polysaccharide of *A. radiobacter* is a curdlan-type polysaccharide (Nakanishi *et al.*, 1974) and the soluble polysaccharide was similar to succinoglucan, as described below.

Strain 12665W was maintained on PY agar slants for 60 days. Spontaneous mutant strains 12665wb1, 2, 3 and 4, which were stained blue with aniline blue, were then isolated and tested for production of water-soluble and water-insoluble polysaccharides (Table 2).

Table 2. *Formation of water-soluble and water-insoluble polysaccharides by strains derived spontaneously from A. radiobacter strain 12665W*

Mutant strain	Exocellular polysaccharide produced [mg (100 ml) ⁻¹]	
	Water-soluble	Water-insoluble
12665wb1	10	1100
12665wb2	84	600
12665wb3	72	330
12665wb4	0	190

Table 3. *Effect of incubation time in sodium chloride solution or phosphate buffer on the appearance of blue colonies on aniline blue plates*

Agrobacterium radiobacter strain 12665W was incubated at 30 °C in sodium chloride solution or phosphate buffer. Periodically samples were removed and 0.1 ml portions of appropriately diluted suspension were spread on aniline blue plates. Numbers of blue and white colonies were counted after 5 days at 30 °C. Values are the averages from two experiments.

	Type of colony	Dilution	No. of colonies per plate after incubation for (days):							
			0	1	2	3	4	6	8	10
0.9 % NaCl solution	Blue	1	0	0	1	1	14	80	102	9
	White	10 ⁵							8	1
		10 ⁶	132	101	93	90	62	21		
0.02 M-phosphate buffer pH 6.0	Blue	1	0	1	28	31	105			
		10					9	212	94	0
	White	10 ⁵							297	2
		10 ⁶	127	123	105	94	90	82	31	0

The ability of these strains to produce the polysaccharides was stable during 60 days incubation on PY agar slants. The production of the two polysaccharides by strains 12665B1 and 12665B2 (described above), and by strains 12665wb2 and 12665wb3 was similar. Thus, strains 12665B1 and 12665B2 in the stock culture of IF012665 seem to have mutated from a strain giving white colonies.

The mutation occurred even when a suspension of strain 12665W was kept in saline or 0.02 M-phosphate buffer pH 6.0 (Table 3). Before experiments, the culture was repurified on an AB medium plate and a freshly grown colony was transferred to a PY agar slant. After 2 days, the organisms were inoculated into PY medium. Exponentially growing cultures were centrifuged and the cells were washed with 0.9 % (w/v) sodium chloride solution and resuspended in the same solution. Portions of this suspension (1 ml, containing about 1.3×10^{11} bacteria) were inoculated into 100 ml of 0.9 % sodium chloride solution or 0.02 M-phosphate buffer pH 6.0 in 500 ml conical flasks and incubated at 30 °C with shaking. Samples were removed periodically, and 0.1 ml portions of appropriately diluted suspension were spread on AB medium plates. After 5 days at 30 °C, the numbers of blue and white colonies which had developed were counted. Strain 12665W formed white colonies which were stained slightly with the dye. It was difficult to count the blue colonies when the parental white colonies predominated on the plate, but good reproducibility of determinations was achieved when the ratio of blue to white colonies was higher than 1 in 5×10^8 . The pure fresh culture of strain 12665W used gave a ratio of less than 1 in 5×10^8 . When incubated in saline, the number of blue colonies increased significantly on day 4 and continued to increase until day 8, but decreased on day 10. In contrast, the number of white colonies decreased steadily throughout the incubation, probably due to autolysis. On day 8 of incubation, about 0.6 % of the bacteria were viable, and the ratio of blue colonies to white colonies was about 1 in 8×10^8 . When incubated in phosphate

buffer, the number of blue colonies was at its maximum on day 6 of incubation, the ratio of blue colonies to white colonies being about 1 in 4×10^4 . On day 10, when most of the bacteria had died, the ratio was about 1 in 2×10^4 . This change is similar to the change seen in the production of succinoglucan and curdlan by *Alcaligenes faecalis* var. *myxogenes* (Hisamatsu *et al.*, 1976). The mechanism of spontaneous mutation is unknown.

Recently, we showed (Hisamatsu *et al.*, 1977) that all the water-soluble polysaccharides from *Agrobacterium* have succinic acid as an acidic component and β -glucan as a moiety of the polysaccharide which is hydrolysed to a polymer with a degree of polymerization of about 12 (Hisamatsu *et al.*, 1977) by a specific β -glucanase, succinoglucan depolymerase from *Flavobacterium* m64 (Amemura, Moori & Harada, 1974). They are similar to the succinoglucan of *Alcaligenes faecalis* var. *myxogenes* 10C3 (Harada, 1965; Misaki *et al.*, 1969; Saito, Misaki & Harada, 1970). It is interesting that spontaneous mutation in the ability to produce water-soluble β -glucan (acidic β -glucan containing succinic acid) and water-insoluble β -glucan (curdlan-type polysaccharide) occurs frequently in both genera. It has been generally thought that isolates from different sources from gall-type growths, or non-infective variants of *Agrobacterium*, are readily confused with members of the genus *Alcaligenes* (Allen & Holding, 1974). There seem to be no definitive characteristics for classification of organisms in the genera *Agrobacterium* and *Alcaligenes* other than their abilities to form galls. Thus, the spontaneous mutation described here may be useful in studies on strains of *Alcaligenes* and *Agrobacterium*.

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