Formation of Reducing Sugars from Sucrose by *Erwinia* Species

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INTRODUCTION

Cultures of *Erwinia carotovora* var. *atroseptica* in a sucrose medium accumulate reducing compounds, a property which distinguishes this organism from *E. carotovora* var. *carotovora* (Dye, 1969*a*; Graham, 1971). These reducing compounds are mainly the disaccharides palatinose and 1- α -D-glucosyl-fructose (GF) (Lund & Wyatt, 1973). The formation of reducing compounds in a sucrose medium has been used to characterize other species of *erwinia* (Dye, 1968, 1969*a,b,c*) but the nature of the compounds formed has not been studied.

In this paper the disaccharides formed by *E. carotovora* var. *atroseptica* are shown to account for a substantial proportion of the initial concentration of sucrose. Dye (1968, 1969*a, b, c*) found other strains of *erwinia* which were able to produce reducing compounds from sucrose; a survey is reported of the ability of these strains to form palatinose and GF in the test conditions used by Graham and by Dye.

One of the most useful applications of the test for production of reducing compounds from sucrose, is to distinguish between *E. carotovora* var. *atroseptica* and var. *carotovora*. The possibility has been investigated that failure of *E. carotovora* var. *carotovora* to accumulate these compounds may be associated with transient production of palatinose and GF during the exponential phase of growth and subsequent metabolism of these compounds.

METHODS

Bacteria. The cultures are listed in Table 1 and include representatives of all the species reported by Dye (1968, 1969*a, b, c*) to form reducing compounds in a sucrose medium. *Erwinia carotovora* var. *atroseptica* GI20 and strains of *E. herbicola*, *E. tracheiphila* and *E. rubrifaciens* were originally obtained from Dr D. C. Graham, Department of Agriculture and Fisheries for Scotland, Edinburgh. Twelve additional strains of *E. carotovora* var. *atroseptica* and three strains of *E. carotovora* var. *carotovora* were isolated in this laboratory, and two strains of *E. carotovora* var. *atroseptica* were isolated by Dr M. E. Stanghellini, University of Arizona, Tucson, Arizona, U.S.A. The corn-stalk-rot organisms (Dye, 1969*a*) were obtained from Professor R. S. Dickey, Cornell University, Ithaca, New York, U.S.A., and cultures labelled '*Enterobacter agglomerans*' were from Dr W. H. Ewing, Centre for Disease Control, Atlanta, Georgia, U.S.A.; the remaining organisms were from the National Collection of Plant Pathogenic Bacteria (NCPBB), Harpenden, Hertfordshire.

Culture media. A medium containing peptone (Difco), 1 % (w/v), and beef extract powder (Lab. Lemco), 0.4 % (w/v), was used, without added sugar (basal medium), with 4 % (w/v) sucrose (sucrose medium) or with 4 % (w/v) palatinose (palatinose medium).

Test for the production of reducing compounds from sucrose. Cultures were initially screened

for the ability to accumulate reducing compounds in sucrose medium using the methods of Graham (1971) and Dye (1968). The bacteria were grown in 10 ml medium in 100 ml flasks at 25 °C for 48 h on a shaker (Mark V Orbital Shaker, L.H. Engineering Co., Stoke Poges, Buckinghamshire) with a 1 inch rotary movement, at a speed of 250 rev./min. After incubation, an equal volume of Benedict's solution (qualitative) was added and the culture was heated in a boiling water bath for 10 min. Development of a yellow to brown colour was regarded as a positive reaction.

The identity of reducing sugars formed in the sucrose medium. The bacteria were grown in 100 ml sucrose medium in 1 l flasks at 25 °C on the rotary shaker at 250 rev./min. Samples were removed periodically for measurement of growth in terms of E_{650} and for examination of reducing sugars by chromatography and electrophoresis. For these last analyses the bacteria were removed by centrifugation at 30000 g for 30 min at 3 °C and the culture liquid was stored at -25 °C. In cultures of *E. dissolvens* NCPPB1862 and NCPPB2209 and *Enterobacter agglomerans* biogroup 2, the medium became very viscous; any high molecular weight material was therefore removed by precipitation with 3 volumes of 100 % ethanol. Paper chromatography, paper electrophoresis and spray reagents were as described previously (Lund & Wyatt, 1973), except that the silver nitrate stain used was that of Trevelyan, Proctor & Harrison (1950) which reacted with reducing sugars and, more slowly, with sucrose. Except when they were present in trace amounts, the identities of palatinose and GF were confirmed by their electrophoretic mobility and formation of characteristic colours with an aniline-diphenylamine spray reagent (Lund & Wyatt, 1973).

Assay of palatinose and GF formed by E. carotovora var. atroseptica G120. A culture was grown in 100 ml sucrose medium and harvested after 63 h; the E_{650} was 5.5, equivalent to a dry weight of organisms of about 2.6 g/l. The bacteria were removed by centrifugation and 30 µl samples of the supernatant liquid were analysed by chromatography on Whatman No. 1 paper for 48 h. After drying the paper, reference strips were cut and stained (Trevelyan *et al.* 1950) to locate palatinose and GF. Using these strips, areas containing the disaccharides were located and 5 cm diameter circles containing the sugars were circumscribed with a pencil and compass. The amount of sugar on the discs was estimated directly (Shallenberger & Moores, 1957) by the method of Somogyi (1952). A standard curve was prepared using palatinose, and allowance was made for interference by the paper.

Examination of cultures of E. carotovora var. carotovora. The bacteria were inoculated into basal medium, sucrose medium and palatinose medium (12 or 25 ml, in 150 or 250 ml flasks), and incubated on the rotary shaker. Samples were removed immediately after inoculation, during the exponential phase of growth (at 3, 6, 9 and 12 h), and after 24 and 48 h, for measurement of growth by increase in E_{650} and for chromatography.

RESULTS AND DISCUSSION

Formation of reducing disaccharides by E. carotovora var. atroseptica G120

In shaken-flask cultures in sucrose medium at 25 °C, the exponential phase of growth was completed in about 24 h. When samples of cultures taken after incubation for 24, 42, 48, 65 and 72 h were examined by paper chromatography no residual sucrose was detected and the only reducing sugars found were palatinose and GF.

In preliminary assays, recovery of palatinose after chromatography of five replicate samples was 93 %. Samples of culture liquid from a 63 h culture of strain G120 contained 5 mg palatinose/ml and 9.3 mg GF/ml (5 replicate assays), equivalent to 12.4 and 23 % (w/w), respectively, of the initial sucrose concentration.

Formation of reducing sugars by other species of erwinia

Results of testing 48 h cultures in sucrose medium with Benedict's reagent are shown in Table 1. Cultures which gave a green colour were recorded $\pm$; some of these isolates, as well as those giving a clear positive reaction, were examined to determine the identity of the reducing sugars formed. The exponential phase of growth was usually completed after about 24 h and samples of cultures were removed after 24, 42, 48, 65 and 72 h. Reducing sugars detected by chromatography of culture filtrates are shown in Table 1. In general, where cultures gave a strong reduction in the test with Benedict's reagent, the characteristic reducing sugars had accumulated during the first 24 to 48 h of growth and the concentrations tended to increase during longer incubation. With strains giving an intermediate reaction (green colour) with Benedict's reagent, glucose and fructose were formed during the first 24 to 42 h and the concentrations subsequently decreased. Different results may be obtained when these strains are tested for the production of reducing compounds from sucrose in different laboratories, because the rate of growth and metabolism of remaining glucose and fructose will depend on the efficiency of aeration of shaken-flask cultures.

In Table 1 the species are divided into the four groups distinguished by Dye (1968, 1969*a, b, c*). In group 1 organisms (the *E. amylovora* group) the main reducing sugars formed were glucose and fructose, though unidentified, slow-running compounds, possibly oligosaccharides, were also detected. Two strains of *E. amylovora* formed a compound, with the same R_G as palatinose, as a minor product and the three cultures of *E. salicis* formed traces of compounds with the same R_G values as palatinose and GF. It is possible that alteration of the growth medium would result in an increased formation of these compounds by strains of *E. amylovora* and *E. salicis*. Because of the relatively small amounts formed, these compounds were not isolated, and further tests would be required to confirm their identity.

Of the group 2 organisms, all the 13 strains of *E. carotovora* var. *atroseptica* gave similar results to strain G120. In cultures of *E. rhapontici* the main reducing sugar formed was palatinose, with a relatively smaller concentration of GF than that formed by strain G120; traces of fructose, glucose and slow-running compounds were also detected. Only one strain of *E. chrysanthemi* and one of the corn-stalk-rot organisms gave a clear positive result with Benedict's reagent; these strains accumulated glucose and fructose in the culture liquid.

Of the group 3 cultures initially screened, only one, *E. herbicola* 708, formed palatinose and a small concentration of GF. This was the only strain of *E. herbicola* among 33 studied by Graham which originated from clinical material, and apart from its metabolism of sucrose this strain resembled the remaining 32 in 100 tests (D. C. Graham, personal communication). Many clinical isolates of *E. herbicola* have been studied by Ewing & Fife (1972) who termed them '*Enterobacter agglomerans*'. Strains representative of the 11 biogroups within *Enterobacter agglomerans* were therefore tested, but none resembled *E. herbicola* 708 in forming palatinose and GF.

Of the group 4 organisms, two of the three cultures of *E. dissolvens* formed palatinose and GF as the main reducing sugars, with a lower concentration of GF relative to palatinose than that formed by strain G120. The classification of *E. dissolvens* is in some doubt; Dye (1969*c*) concluded that strains NCPPB1850 and NCPPB1862 resembled the genus *Klebsiella* rather than *Erwinia*, while Graham (1971) concluded that, apart from being non-motile, NCPPB1850 resembled *Enterobacter cloacae*, while NCPPB1862 resembled *Klebsiella aerogenes*. *Klebsiella aerogenes* NCTC418 gave a negative result when tested with Benedict's reagent for the formation of reducing compounds from sucrose, but it would be of interest to know

Table 1. Formation of reducing sugars from sucrose by *Erwinia* species

Culture	Reduction of Benedict's reagent*	Reducing sugars formed†			
		Fru	Glu	Pal	GF
Group 1					
<i>E. amylovora</i> NCPPB683, NCPPB686	+	+	+	TR	—
<i>E. amylovora</i> NCPPB713, NCPPB756	+	+	+	—	—
<i>E. quercina</i> NCPPB1852, NCPPB1853	+	+	+	—	—
<i>E. salicis</i> NCPPB447, NCPPB1466, NCPPB2310	+	+	+	TR	TR
<i>E. tracheiphila</i> 52, Kelman A	+	+	+	—	—
<i>E. rubrifaciens</i> NCPPB2020, G208	+	TR	+	—	—
<i>E. nigrifluens</i> NCPPB564, NCPPB565, NCPPB566	—	NT	NT	NT	NT
Group 2					
<i>E. carotovora</i> var. <i>atroseptica</i> (14 strains)	+	—	—	+	+
<i>E. chrysanthemi</i> NCPPB453	±	TR	TR	—	—
NCPPB516	±	NT	NT	NT	NT
NCPPB1849	+	+	+	—	—
<i>E. rhapontici</i> NCPPB139, NCPPB1578, NCPPB1739	+	TR	TR	+	+
Corn-stalk-rot organisms SR58, SR59, SR80, SR86	±	TR	TR	—	—
SR87	+	+	+	—	—
<i>E. cypripedii</i> NCPPB750, NCPPB751, NCPPB752		NT	NT	NT	NT
Group 3					
<i>E. herbicola</i> NCPPB102	+	+	+	—	—
G142	—	TR	TR	—	—
G708	+	—	—	+	+
<i>E. herbicola</i> var. <i>ananas</i> NCPPB441	+	TR	TR	—	—
NCPPB545, NCPPB1846	±	NT	NT	NT	NT
<i>E. stewartii</i> NCPPB2293, NCPPB2295	—	NT	NT	NT	NT
<i>E. uredoovora</i> NCPPB391	—	TR	TR	—	—
NCPPB800	±	+	+	—	—
NCPPB1416	±	TR	TR	—	—
<i>Enterobacter agglomerans</i> Biogroups 5, 6, 7, G2, G3	—	NT	NT	NT	NT
Biogroups 1, 2, G1, G4	—	TR	TR	—	—
Biogroups 3, 4	±	+	+	—	—
Group 4					
<i>E. dissolvens</i> NCPPB1850	+	+	+	—	—
NCPPB1862, NCPPB2209	+	—	—	+	+
<i>E. nimipressuralis</i> NCPPB440, NCPPB2045	—	NT	NT	NT	NT

±, Intermediate reaction (green colour) with Benedict's reagent; TR, trace; NT, not tested.

* Tested after 48 h incubation at 25 °C in shaken flasks of sucrose medium.

† Detected by chromatography of culture liquid after 24 to 72 h in sucrose medium. Fru, fructose; glu, glucose; pal, palatinose; GF, 1- α -D-glucosyl-fructose.

whether the ability to form palatinose and GF is found widely among different genera of the Enterobacteriaceae.

Examination of cultures of E. carotovora var. carotovora

When three strains of this organism were grown in sucrose medium, neither palatinose nor GF could be detected on chromatograms of culture samples taken after 3, 6, 9, 12, 24 or 48 h incubation. Traces of glucose and fructose were detected in cultures grown for up to 24 h but not after 48 h. When these strains were grown in palatinose medium for up to 48 h no disappearance of palatinose or formation of glucose or fructose could be detected by chromatography. The growth of bacteria in palatinose medium was equivalent to that in the basal medium. Substitution of sucrose for palatinose resulted in an increase of between 3- and 5-fold in growth of the bacteria. The results with these three strains indicate that failure of cultures of *E. carotovora* var. *carotovora* to accumulate palatinose and GF was not due to metabolism of these compounds.

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