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Variation in Composition and Yield of Exopolysaccharides Produced by *Klebsiella* sp. Strain K32 and *Acinetobacter calcoaceticus* BD4

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The expolysaccharides produced by *Klebsiella* sp. strain K32 and *Acinetobacter calcoaceticus* BD4 under different growth conditions have been analyzed for sugar composition. The first use of ion chromatography for the quantitative determination of microbial exopolysaccharide composition is reported. *Klebsiella* sp. strain K32 produced a polymer composed of rhamnose, galactose, and mannose early in its fermentation. The composition of the polymer varied markedly depending on the growth stage of the organism. *Klebsiella* sp. strain K32 grown in a fermentor produced a polymer which was rich in mannose during early exponential growth in a complex medium, but in the late stationary phase it did not contain detectable levels of mannose. The rhamnose present in the polymer increased from 12 to 55% over the course of growth, whereas galactose decreased from 63 to 45%. *A. calcoaceticus* BD4 produced a polymer containing rhamnose, glucose, mannose throughout its growth and stationary phase. *Klebsiella* sp. strain K32 and *A. calcoaceticus* BD4 were grown on various carbon sources in shake flasks. The polymer yield and composition from both organisms were found to vary with the carbon source. The exopolysaccharide with the highest mannose composition was obtained by using rhamnose as a carbon source for both organisms. These and other data suggest that regulatory changes caused by growth on different substrates result in either the production of a different distribution of polymers or a change in exopolysaccharide structure.

The growth of bacteria is often accompanied by the production of polysaccharides which are found outside the cell wall. These exopolysaccharides may be found as a capsule attached to the bacteria or they may be released to the environment as slime or both (14). A polysaccharide may be important to the bacterium (i.e., adhesion, infection, and protection), but it may have commercial value as well. Some polysaccharides are known to have gelling properties (i.e., agar and gelrite) (3, 9), others have emulsifying properties (8, 13), and still others may represent a source of certain important monosaccharides. A polysaccharide has potential commercial value depending on its characteristic composition, the quantity produced by a culture, and the ease of harvest and processing of the polysaccharide.

Although the composition and amount of microbial exopolysaccharide produced are genetically determined, it is possible to influence both by altering culture conditions (14). Exopolysaccharide formation is generally favored by the presence of excess nutrient carbohydrate and low temperature (6, 14, 17). Both exopolysaccharide yield and composition may be influenced by limiting nitrogen, carbon, phosphorus, and sulfur nutrients (7). Exopolysaccharide composition has been reported to be independent of carbon source in *Klebsiella* sp. strain 54 and *Serratia marcescens* when grown on a variety of sugars (1, 16).

Klebsiella sp. strain K32 and *Acinetobacter calcoaceticus* BD4 both produce exopolysaccharides with a high rhamnose content (8, 15). We report the variation in rhamnose content of these exopolysaccharides as a function of both carbon source and growth stage. Our results indicate that researchers studying exopolysaccharide composition should carefully examine the growth conditions which are being used. Finally, ion chromatography was used for the first time in the determination of microbial exopolysaccharide composition.

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MATERIALS AND METHODS

Microorganisms. *Klebsiella* sp. strain K32 was the gift of G. G. S. Dutton of the University of British Columbia. *A. calcoaceticus* BD4 was obtained from the American Type Culture Collection (ATCC 33304). Cultures were maintained on nutrient agar plates at 4°C and transferred monthly.

The complex medium used in shake flasks (medium 1) contained (per liter) 100 g of glucose, 0.5 g of $MgSO_4 \cdot 7H_2O$, 10 g of soy peptone (Sigma Chemical Co.), 0.5 g of yeast extract (Difco Laboratories), 17.4 g of K_2HPO_4, and 13.6 g of KH_2PO_4. The defined medium used in shake flasks (medium 2) contained (per liter) 1.0 g of $(NH_4)_2SO_4$, 13.6 g of KH_2PO_4, 17.4 g of K_2HPO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 2.8 mg of $FeSO_4 \cdot 7H_2O$, 2.9 mg of $ZnSO_4 \cdot 7H_2O$, 2.4 mg of $Ca(NO_3)_2 \cdot 4H_2O$, 2.4 mg of $CoCl_2 \cdot 6H_2O$, 2.5 mg of $CuSO_4 \cdot 5H_2O$, 2.4 mg of $Na_2MoO_4 \cdot 2H_2O$, and 1.7 mg of $MnSO_4 \cdot H_2O$ supplemented with 1% glucose, mannose, rhamnose, ethanol, succinate, or glutamate. The defined medium used in the fermentor (medium 3) contained (per liter) 20 g of sodium succinate, 1.69 g of $(NH_4)_2SO_4$, 17.4 g of K_2HPO_4, 13.6 g of KH_2PO_4, 1.5 g of $MgSO_4 \cdot 7H_2O$, 28 mg of $FeSO_4 \cdot 7H_2O$, 28.8 mg of $ZnSO_4 \cdot 7H_2O$, 23.6 mg of $Ca(NO_3)_2 \cdot 4H_2O$, 23.8 mg of $CoCl_2 \cdot 6H_2O$, 25 mg of $CuSO_4 \cdot 5H_2O$, 24.2 mg of $Na_2MoO_4 \cdot 2H_2O$, and 16.9 mg of $MnSO_4 \cdot H_2O$. The complex medium used in the fermentor (medium 4) contained (per liter) 100 g of glucose, 0.5 g of $MgSO_4 \cdot 7H_2O$, 10 g of soy peptone (Sigma), 0.5 g of yeast extract (Difco), 0.75 g of K_2HPO_4, and 0.75 g of NaH_2PO_4. All media were adjusted to pH 7.0 before inoculation.

Cultivation conditions. Organisms were grown overnight in 1 ml of nutrient broth (Difco) and transferred directly to 100 ml of medium 1 or medium 2 in 500-ml baffled shake flasks

TABLE 1. Relative sugar composition of exopolysaccharide produced on a defined medium during 7 days of incubation in shake flasks at 26°C[a]

Organism	Carbon source	Exopolysaccharide		Relative sugar composition (%)			
		g liter^{-1}	g per g of cells	Rhamnose[b]	Galactose[c]	Glucose[d]	Mannose[e]
Klebsiella sp strain K32	Glucose	0.38	0.10	65	35	ND[f]	ND
	Mannose	0.28	0.05	66	34	ND	ND
	Rhamnose	0.20	0.10	64	35	ND	1
	Succinate	0.08	0.05	47	53	ND	ND
	Glutamate	0.07	0.04	28	72	ND	ND
	Ethanol	0.02	0.04	33	67	ND	ND
	Glucose[g]	1.11	0.19	57	43	ND	ND
A. calcoaceticus BD4	Glucose	0.01	0.06	41	ND	50	9
	Mannose	0.01	0.01	48	ND	45	7
	Rhamnose	0.01	0.02	29	ND	25	46
	Succinate	0.17	0.05	57	ND	29	14
	Glutamate	0.26	0.09	62	ND	32	6
	Ethanol	0.23	0.15	62	ND	32	6
	Glucose[g]	0.61	1.50	22	ND	54	24

[a] The pH at harvest was 7.0 ± 0.9.
[b] Maximum deviation between duplicate samples was 3%.
[c] Maximum deviation between duplicate samples was 0.5%.
[d] Maximum deviation between duplicate samples was 3%.
[e] Maximum deviation between duplicate samples was 4%.
[f] ND, Not detectable (<0.1%).
[g] Complex medium plus glucose.

(Bellco). When used for experiments with various carbon sources, shake flasks were incubated on a rotary shaker at 200 rpm at 25°C. When used to inoculate a fermentor, cultures in shake flasks were incubated overnight and transferred to a 3-liter Applicon fermentor containing 1.9 liters of medium 3 or medium 4. The fermentor had a working volume of 2 liters and was equipped with automatic pH and foam controls. The pH was continuously adjusted to 7.0 with 5 N sodium hydroxide when *Klebsiella* sp. strain K32 was grown in medium 4 and with 4 N sulfuric acid when *A. calcoaceticus* BD4 was grown in medium 3. A 10% solution of Antifoam A (Sigma) was used in control foaming.

Isolation of exopolysaccharide. Cells were removed by centrifugation at 10,000 × *g* for 20 min, and the pellet was frozen and lyophilized. The supernatant (1 volume containing 0.1% [wt/wt] added sodium chloride) was poured into 4 volumes of acetone with stirring and allowed to sit at 4°C overnight. The precipitated solids were suspended in distilled water and sodium azide (0.01% [wt/wt]) was added to prevent bacterial growth. The slurry was dialyzed at room temperature for 24 h against running deionized water, frozen, and lyophilized.

Determination of exopolysaccharide molecular weight. The exopolysaccharide was dissolved in 1 ml of 0.1 M sodium chloride and passed through a 0.22-μm Millex-GS filter (Millipore Corp.). The filtered sample was loaded onto a 2.5- by 100-cm Fractogel TSK HW-75(F) column (Pierce Chemical Co.) and eluted at 1.5 ml min^{-1} with 0.1 M sodium chloride (pH 7) at 25°C. Dextran standards (1 mg ml^{-1} in 0.1 M sodium chloride) having molecular weights ranging from 100,000 to 5,000,000 (Sigma) were used to estimate exopolysaccharide molecular weight. Fractions were collected, and the elution profiles were measured by a total sugar assay (4) after hydrolysis with trifluoroacetic acid.

Hydrolysis of exopolysaccharide. Polysaccharide sample (4 mg in 1 ml of distilled water) was placed in a 100- by 15-mm screw-top glass tube, 8 N trifluoroacetic acid (1 ml) was added, and the tube was tightly capped (Teflon-lined cap) and heated for 2 h at 100°C. The hydrolyzed sample was then cooled to 25°C and uncapped, and 0.5-ml samples were dried under reduced pressure for 48 h until only a residue remained (10).

Thin-layer chromatography. Thin-layer chromatography plates (0.25 mm) were prepared with silica gel G (Merck & Co.) impregnated with 0.3 M monobasic potassium phosphate. Plates were developed in ethyl acetate-isopropanol-water (65:23.5:11.5) (5), and sugars were visualized by spraying with *p*-anisaldehyde reagent (60 ml of glacial acetic acid, 0.5 ml of concentrated sulfuric acid, 0.5 ml of *p*-anisaldehyde [Aldrich Chemical Co.]) followed by spraying with aqueous sulfuric acid (50%, vol/vol) and heating (ca. 3 min) with a hot-air gun until the colored spots appeared. Standard sugars (1 μg) (Sigma) were used for identification.

Ion chromatography. Ion chromatography (12) was performed on a Dionex QIC ion chromatography system equipped with a 50 μl sample loop and at an eluent flow rate of 1.0 ml min^{-1}. The detector was a Dionex IonChrom/Pulsed Amperometric Detector. A gold working electrode, a silver-silver chloride reference electrode, and a glassy carbon counter electrode were used. The potentiostat applied three potentials (E_1 = +0.20 V, E_2 = +0.60 V, E_3 = −0.80 V), and pulsed durations were t_1 = 60 ms, t_2 = 60 ms, and t_3 = 240 ms. An HPIC-AS6 anion-exchange column (27.5 cm by 0.75-cm outer diameter) equipped with an HPIC-AG6 guard column (7.5 cm by 0.75-cm outer diameter) was used. The 0.15 N sodium hydroxide eluent was protected from carbon dioxide by an Ascarite trap. Samples were prepared for analysis as follows: 0.5 ml of trifluoroacetic acid hydrolysate was evaporated to dryness under vacuum, the dried sample was dissolved in 5 ml of glass-distilled water, and the pH was adjusted to 7. Samples (1 ml) of the aqueous solution were analyzed for monosaccharides by ion chromatography. Standard sugars (0.02 to 1.0 μg) were used to determine retention times.

RESULTS

Effects of carbon source on exopolysaccharide composition. To determine the effect of various carbon sources on the composition and yield of the exopolysaccharide produced, *Klebsiella* sp. strain K32 and *A. calcoaceticus* BD4 were

grown in baffled shake flasks on medium 1 or medium 2 supplemented with glucose, rhamnose, mannose, succinate, glutamate, or ethanol. Cultures were grown in duplicate and harvested after 4 and 7 days of incubation on a rotary shaker (Table 1).

The gross composition of the exopolysaccharides produced by *Klebsiella* sp. strain K32 or *A. calcoaceticus* BD4 were similar. *Klebsiella* sp. strain K32 produced a rhamnose-galactose exopolysaccharide (with mannose a minor component when cells were grown on rhamnose); *A. calcoaceticus* BD4 produced a rhamnose-glucose-mannose exopolysaccharide. The relative amounts of the sugars comprising the exopolysaccharide varied with the carbon source (Table 1).

The polymer produced by *Klebsiella* sp. strain K32 grown on ethanol had a rhamnose/galactose molar ratio of 1:2. When this strain was grown on defined medium containing glucose, mannose, or rhamnose, however, the rhamnose/galactose ratio was quite different, almost 2:1. When this strain was grown in a complex medium the rhamnose/galactose molar ratio was approximately 1:1.

The sugar composition of the polymer produced by *A. calcoaceticus* BD4 varied dramatically depending on carbon source. The molar ratio of exopolysaccharide produced on rhamnose (rhamnose/glucose/mannose, approximately 1:1:2) was quite different from that produced on succinate (rhamnose/glucose/mannose, 4:2:1) or on glutamate or ethanol (rhamnose/glucose/mannose, 10:5:1)

The exopolysacharide samples harvested at 4 days had sugar compositions similar to those of samples harvested at 7 days. The analysis performed with ion chromatography gave a standard deviation of 0.4% between four determinations of standard sugars, and the maximum average difference between duplicate hydrolyzed exopolysaccharide samples ranged from 0.5 to 4% (depending on the sugar analyzed).

Growth and exopolysaccharide production. The kinetics of *A. calcoaceticus* BD4 growth and exopolysaccharide production was determined in a fermentor (Fig. 1). *A. calcoaceticus* BD4 had a doubling time of 4 h on medium 3 (defined medium plus succinate) at 27°C and reached the stationary phase after 31 h of incubation. The culture produced base during the fermentation, but the pH was maintained at 7.0 by the addition of 4 N sulfuric acid. Exopolysaccharide was produced at a constant rate of 4 mg h^{-1} per g of dry cell weight. Total production of exopolysaccharide at harvest was 6.4 g $liter^{-1}$.

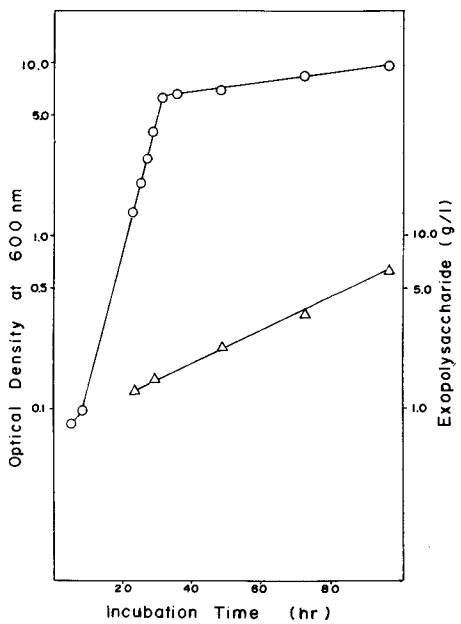

FIG. 1. Growth and exopolysaccharide production of *A. calcoaceticus* BD4 on a defined medium plus succinate in a fermentor at 26°C at pH 7.0. Symbols: (○) optical density at 600 nm, (△) exopolysaccharide.

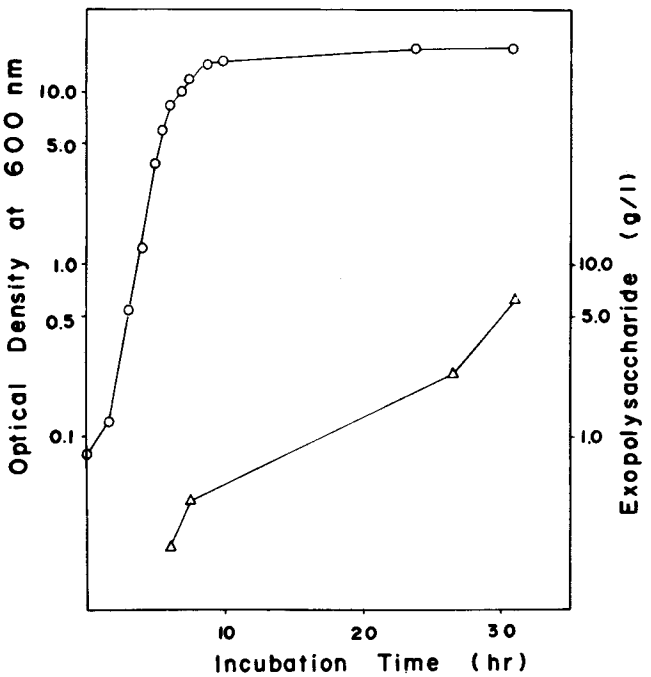

FIG. 2. Growth and exopolysaccharide production of *Klebsiella* sp. strain K32 on a complex medium plus glucose in a fermentor at 37°C at pH 7.0. Symbols: (○) optical density at 600 nm, (△) exopolysaccharide.

The kinetics of *Klebsiella* sp. strain K32 growth and exopolysaccharide production was determined in a fermentor (Fig. 2). *Klebsiella* sp. strain K32 had a doubling time of 45 min on medium 4 (complex medium plus glucose) at 37°C and reached the stationary phase after 8 h of incubation. Acid was produced during the fermentation, but the pH was maintained at 7.0 by the addition of 5 N sodium hydroxide. Exopolysaccharide was produced at an approximate rate of 8 mg h^{-1} per g of dry cell weight. Total production of exopolysaccharide at harvest was 6.4 g $liter^{-1}$.

Molecular weight of exopolysaccharide. The exopolysaccharide from *Klebsiella* sp. strain K32 grown on glucose was analyzed by gel-permeation chromatography. Throughout the entire fermentation an exopolysaccharide of 1×10^6 molecular weight observed. Late in the stationary phase of fermentation, higher-molecular-weight exopolysaccharides (3×10^6 to 20×10^6) also appeared. However, we cannot rule out the possibility that these were aggregrates.

Sugar composition of exopolysaccharide produced during fermentation. The polysaccharide samples were hydrolyzed; the resulting monosaccharides were identified by thin-layer chromatography, and their relative concentrations were determined by ion chromatography. The monosaccharides comprising the polymer were qualitatively the same regardless of where in the growth curve the sample was taken.

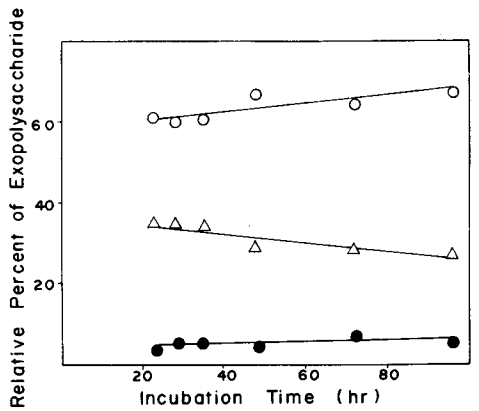

FIG. 3. Relative sugar composition of the exopolysaccharide produced by *A. calcoaceticus* BD4 in a fermentor on defined medium plus succinate at 26°C at pH 7.0. Symbols: (○) percentage of exopolysaccharide determined to be rhamnose, (●) percentage of exopolysaccharide determined to be mannose, (△) percentage of exopolysaccharide determined to be glucose.

Sugars identified by thin-layer chromatography in the exopolysaccharide produced by *Klebsiella* sp. strain K32 were rhamnose, galactose, and, until the late stationary phase, mannose. Those identified in the exopolysaccharide produced by *A. calcoaceticus* BD4 were rhamnose, glucose, and mannose.

The relative proportions of sugars comprising the exopolysaccharide samples isolated at various times during the growth period of *A. calcoaceticus* BD4 and *Klebsiella* sp. strain K32 were determined by ion chromatography (Fig. 3 and 4, respectively). The exopolysaccharide produced by *A. calcoaceticus* BD4 had nearly constant relative proportions of monosaccharides over the growth period. In contrast, the composition of the exopolysaccharide samples taken over the growth period of *Klebsiella* sp. strain K32 varied in the relative proportions of monosaccharides present. The rhamnose portion increased, galactose decreased, and mannose decreased to undetectable levels late in the fermentation.

DISCUSSION

The total yield of exopolysaccharide produced by *Aerobacter* (*Klebsiella*) *aerogenes*, *Aerobacter cloacae*, *Escherichia coli* (6, 16, 17), and *Xanthomonas juglandis* (7) depends on the composition of the medium and on the incubation conditions. Polysaccharide composition has generally been thought to be independent of the carbon source utilized for growth (1, 16), although only sugars were tested as substrates. The results presented in Table 1 show that both yields and composition of exopolysaccharides produced by both *Klebsiella* sp. strain K32 and *A. calcoaceticus* BD4 depend on carbon source utilized; however, in most cases different sugar substrates caused little variation in polysaccharide composition. The growth conditions used were similar for each test substrate (temperature, pH, rotations per minute, flask size, incubation time, etc.). No variation in exopolysaccharide composition was observed when a culture was grown on a given substrate harvested in the stationary phase at days 4 and 7.

Klebsiella sp. strain K32 grew well on all of the carbon sources tested. The yields of exopolysaccharides and cell material were highest on sugars. We have also established that *Klebsiella* sp. strain K32 grew poorly when given its own exopolysaccharide as a substrate. *A. calcoaceticus* BD4 grew very poorly on rhamnose and mannose, and no growth was observed on glucose. Exopolysaccharide, however, was produced even when little or no growth occurred. It is interesting that *A. calcoaceticus* BD4 produced a polymer containing rhamnose, glucose, and mannose, but could not grow well on the individual sugars comprising this polymer. *A. calcoaceticus* BD4 was also unable to grow when given its own exopolysaccharide as a substrate. The polymer produced by *A. calcoaceticus* BD4 is reportedly a bioemulsifier (8, 13), and perhaps its only natural function is to make other carbon sources such as hydrocarbons more available for growth.

Cell growth and exopolysaccharide production by *Klebsiella* sp. strain K32 and *A. calcoaceticus* BD4 followed expected kinetic patterns (Fig. 1 and 2). Exopolysaccharide was produced at a nearly constant rate by both organisms, 4 and 8 mg h^{-1} per g of dry cell weight for *A. calcoaceticus* BD4 and *Klebsiella* sp. strain K32, respectively. No variation in sugar composition was seen in samples of exopolysaccharide taken over the growth period of *A. calcoaceticus* BD4. The exopolysaccharide produced late in the stationary phase appeared to be nearly identical with that produced during exponential growth (Fig. 3). The sugar composition of exopolysaccharide produced by *Klebsiella* sp. strain K32 was variable (Fig. 4). The polymer produced in early exponential growth did not have the same sugar composition as the polymer taken from the medium during the stationary phase. Rhamnose content increased from 12% in early exponential growth to 55% in the final sample. When *Klebsiella* sp. strain K32 was grown in a fermentor on medium 3 containing glucose instead of succinate (in the absence of yeast extract and soy protein) at 37°C and pH 7.0, the exopolysaccharide composition was similar. The relative sugar composition of the exopolysaccharide varied from 3%

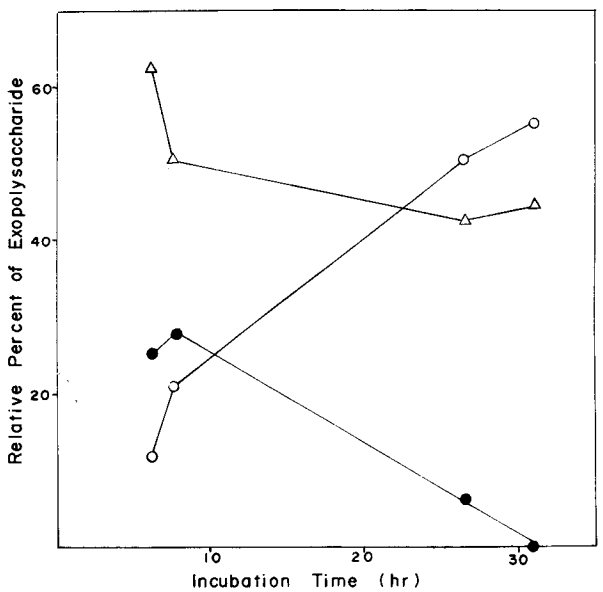

FIG. 4. Relative sugar composition of the exopolysaccharide produced by *Klebsiella* sp. strain K32 in a fermentor on a complex medium plus glucose at 37°C at pH 7.0. Symbols: (○) percentage of exopolysaccharide determined to be rhamnose, (●) percentage of exopolysaccharide determined to be mannose, (△) percentage of exopolysaccharide determined to be galactose. A zero time precipitation showed that the yeast extract contributed less than 3% of the precipitable polysaccharide.

rhamnose in the early exponential phase to 63% rhamnose in the late stationary phase. A similar variation in the glucose content of an exopolysaccharide from *Serratia marcescens* during growth has been reported (1).

Variation in exopolysaccharide composition during growth requires that the organism be able to produce more than one type of polymer or to modify the polymer extracellularly. The structure of an exopolysaccharide from *Klebsiella* sp. strain K32 has been studied (2), and there is no evidence that the polysaccharide is branched. The structure was reported as →3)-α-D-galactose-(1→2)-α-L-rhamnose-(1→3)-β-L-rhamnose-(1→4)-α-L-rhamnose-(1→, with pyruvate linked to the 3,4-hydroxyl groups of the first rhamnose residue. We speculate that the exopolysaccharide produced by *Klebsiella* sp. strain K32 is not modified after release, but rather that the variation in its composition is the result of the production of a spectrum of different polymers, similar to the reported production of a spectrum of polymers produced by *S. marcescens* (1) and by *Rhodopseudomonas capsulata* (11). There are no structural data available for the polysaccharide produced by *A. calcoaceticus* BD4. Without evidence that extracellular modification occurs after production of its exopolysaccharide, we speculate that it, too, can produce a spectrum of different polysaccharides.

The variation in exopolysaccharide composition which we measure can be explained by two possible mechanisms. The first is the production of similar polymers, each with a defined (but different) primary structure or repeating unit. The distribution of individual polymers within this spectrum might vary depending on the growth stage or carbon source. Polymer composition is measured on the gross mixture of exopolysaccharide, and thus the observed variation could be explained on this basis. The second possibility is that the primary structure (repeating unit) of the individual polymers may change. For example, a mannose-rich polymer might be produced when the level of mannose (or activated mannose) is high in the cell, whereas a rhamnose-rich polymer might result when rhamnose (or activated rhamnose) level is elevated in the cell. We cannot differentiate between these mechanisms on the basis of the data presented here. Work on this question is being vigorously pursued.

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