BUTANOL-SOLUBLE GLYCOSYL TRANSFERASES IN *KLEBSIELLA AEROGENES*

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1. Introduction

The bacterial cell membrane is the site of synthesis of several polymers which are composed, at least in part, of oligosaccharide repeating units. In capsulate Gram negative bacteria such as *Klebsiella aerogenes* these products are mucopeptide, lipopolysaccharide and exopolysaccharide (capsule and slime). In biosynthesis of each such polymer, formation of the oligosaccharides involves transfer of monosaccharides from nucleoside diphosphate sugars to a carrier lipid identified as a phosphorylated C_{55}-isoprenoid alcohol [1–3]. Exopolysaccharide synthesis in *K. aerogenes* follows this pattern [3], the first two reactions in the biosynthesis of the capsule of type 8 strains being [4]:

Lipid–P + UDP–glucose → Lipid–P–P–glucose + UMP
Lipid–P–P–glucose + UDP–galactose → Lipid–P–P–glucose–galactose + UDP

The enzymes responsible for these reactions form part of the membrane complex and earlier attempts to obtain soluble transferases were unsuccessful [4]. They are probably highly hydrophobic molecules resistant to extraction with aqueous solvents except under drastic conditions under which enzyme activity is destroyed.

A possible technique for the extraction and purification of hydrophobic proteins from bacterial cell membranes was provided by the acid–butanol method of Strominger and others [5]. Use of this extraction procedure on cell membranes from *Staphylococcus aureus* solubilised a number of proteins from which an isoprenoid alcohol kinase was purified by low temperature precipitation from butanol followed by chromatography on DEAE-cellulose [6]. Preliminary experiments in our laboratory with *K. aerogenes* indicated that 4–5% of the membrane protein could be extracted with acid butanol (H.A. McArthur, unpublished results). We now report the extraction of two glycosyl transferases from *K. aerogenes* type 8 and the reconstruction of an active enzyme system.

2. Materials and methods

The bacterial strain used was a non-mucoid derivative of *K. aerogenes* A4 (type 8) known to be active in glucose transfer to lipid and in galactose transferases I and II [7]. It was used in preference to the wild type cells when extracellular polysaccharide was absent. It was grown overnight in 30 ℓ of trypticase soy broth (Baltimore Biologicals Laboratories, Baltimore, Md., USA) containing 1% (w/v) glucose in 15 ℓ-fermenters (L.H. Engineering Ltd., Stoke Poges, England), harvested by centrifugation at 10 000 *g* for 20 min and washed in saline (pH 7.5). The bacteria were broken by passage through a French pressure cell (Aminco-American Instrument Co., Inc., Silver Spring, Md., USA) suspended in Tris buffer (600 ml, pH 7.5) and nucleic acids were destroyed by the addition of RNAase and DNAase and gentle shaking for 30 min at 30°C. 'Cell membrane' material was recovered by centrifugation at 50 000 *g* for 1 hr and extracted with acid butanol as described [6].

Ficaprenol for use as glycosyl acceptor was prepared from the leaves of *Ficus elasticus* [8]. Reverse-phase chromatography on silica gel revealed a mixture of C_{50}-, C_{55}- and C_{60}-isoprenoid alcohols. Crude isoprenoid alcohol kinase was prepared from a culture of *Staphylococcus* [6].

Incubation mixtures contained: 100 μg ficaprenol, 0.6% Triton X-100, 0.1 mM dimethyl sulphoxide,

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10 mM $CaCl_2$, 10 mM $MgSO_4$, 50 mM Tris buffer (pH 8.0), 5 mM ATP and UDP–[^{14}C]glucose (50 nmoles, 0.2 μCi) in a total volume of 450 μl. They were prepared by adding the prenol dissolved in light petroleum to each tube and evaporating the solvent under reduced pressure. This was repeated after addition of the butanol solution of protein. Triton, dimethylsulphoxide and buffer were then added and thoroughly mixed with a fine rod. All the other components except the nucleoside diphosphate sugars were added and thoroughly mixed on a gyratory mixer. Immediately after the addition of the UDP–glucose, and at intervals thereafter, samples (50 μl) were withdrawn and pipetted into 4 ml of chloroform/methanol (2:1, v/v). Extraction of lipid-bound monosaccharide and scintillation counting were performed as described previously except that Whatman 1PS papers were used to separate the organic phase from aqueous material. To detect galactose transferase activity, radioactive glucose was omitted from the incubation mixtures and UDP–[^{14}]galactose (50 nmoles, 0.2 μCi) was added.

3. Results and discussion

3.1. *Protein extraction*

The initial extraction of the bacterial membrane material yielded 4.1–4.8% of the membrane protein in several experiments. Much of this protein precipitated along with other material when the soluble extract was held at 0°C for 36 hr. It was removed by centrifugation. Cooling of the resultant supernatant fluid to −20°C for 16–24 hr yielded a small amount of brownish precipitate which was recovered by rapid centrifugation at −20°C and 8000 *g*. The yield was approx. 0.4% of the membrane protein in the best preparation. Lower yields were obtained when cell breakage was sub-optimal, suggesting that the acid butanol solution only extracts significant amounts of protein from bacterial membranes and not from whole bacteria. The coloured precipitates were redissolved in butanol and kept at 2–4°C. A small amount of material precipitated from the butanol solution after 1–2 weeks at this temperature. The mixture was normally homogenised gently before use.

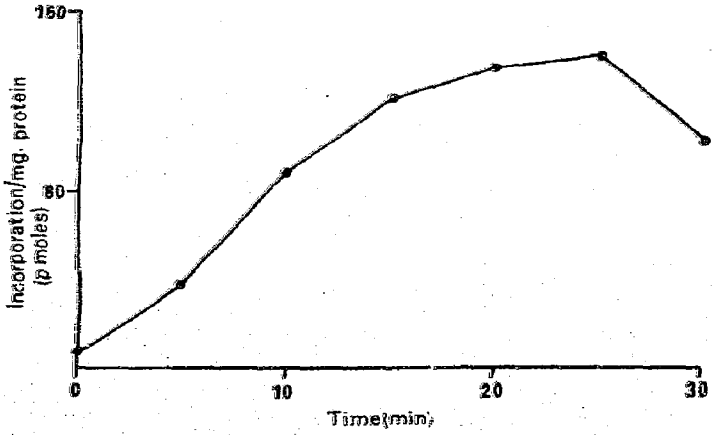

Fig. 1. Transfer of [^{14}C]glucose to lipid by butanol soluble enzyme. Ordinate: Incorporation/mg protein (pmoles). Abscissa: Time (min). Incubation mixtures prepared as described were held for 30 min at room temp. after addition of ATP. UDP-glucose was added and samples (50 μl) withdrawn at 5 min intervals to measure incorporation into chloroform extractable material.

Table 1
Transfer of [^{14}C]glucose from UDP–[^{14}C]glucose to lipid.

Incubation mixture		Additions	(pmoles glucose transferred/ 30 min)
Complete		–	429
	–ATP	–	0
	–Ficaprenol	–	0
Complete		*Staphylococcus* BSE*	1363
Complete	–ATP	CTP	87
	–ATP	GTP	36
	–ATP	TTP	43
	–ATP	UTP	21

BSE* – butanol soluble enzyme.

The incubation mixtures were prepared as described in the Methods and incubated for 30 min at room temp. after addition of the nucleoside triphosphate. Thereafter the UDP–glucose was added and samples extracted with chloroform/methanol 0, 15 and 30 min after incubation at room temp. (18°C). Each reaction mixture contained 4 mg butanol soluble protein.

3.2. *Enzymatic activity*

The ability of the bacterial extracts to transfer glucose and galactose from the UDP-monosaccharides to lipid was tested at all stages of the preparation. The initial activity of the cell lysates from the pressure cell was confirmed for both glucose and galactose transfer. Activity was also found in the butanol extracts even though organic solvent was present. On the basis of

activity per mg protein, there was a considerable loss of enzyme function at this stage, transfer being approx. 55–60% less than in the original cell lysates. This may, in part, be due to the presence of butanol in the aqueous incubation mixtures. The procedures used, in which some protein is precipitated and the active protein can only with difficulty be resuspended in aqueous solutions, also prevent accurate comparisons.

The expected reaction is the transfer of glucose-1-phosphate to prenol-phosphate. Transfer to free prenol was not expected and no glucose was found in the organic phase following incubation of UDP–glucose with ficaprenol and the butanol soluble protein. The addition of ATP permitted transfer of glucose to chloroform-soluble material. Consequently, incubation mixtures were kept for 30 min at room temp. to allow phosphorylation of the lipid prior to addition of the UDP–monosaccharides. Although other nucleoside triphosphates could replace ATP, none were as effective as judged by subsequent glucosyl transfer (table 1). These observations indicate that the butanol extracts contain an isoprenoid alcohol kinase resembling that reported by Higashi et al. [5]. The addition of a crude preparation of this enzyme from *Staphylococcus* [6] stimulated the incorporation of glucose but was unable to replace the *Klebsiella* preparation as a source of glucosyl transferase.

Incorporation of glucose from the nucleoside diphosphate monosaccharide into lipid increased with time to a maximum after 25 min (fig. 1). Thereafter a decrease occurred. The reason for this is not known although similar results have been obtained with membrane preparations prior to butanol extraction. The glucosylated lipid may be unstable under the experimental conditions, or other enzymes present in the butanol extract may affect this product. Galactose incorporation resembled that of glucose and in a typical experiment, replicate tubes in which labelled substrates were UDP–[^{14}C]glucose and UDP–[^{14}C]galactose respectively, incorporated 107 pmoles of glucose and 87 pmoles of galactose after 10 min. We have not yet obtained the ratio glucose:galactose 1:2 found in membrane preparations of type *8* strains [4, 7]. The galactosyl transferase II [7] may therefore be absent from the soluble proteins. The reproducibility of hexose incorporation has varied considerably between experiments and this can almost certainly be ascribed to failure to achieve uniform suspension of the hydrophobic protein and lipid.

Recovery of glucosylated lipid from the incubation mixtures by the standard chloroform methanol extraction procedure was followed by chromatography on paper and on silica gel (TLC). On paper, after descending chromatography in ethanol/1 M ammonium acetate, pH 7.3 (7:3, v/v) 1 cm strips of the paper were counted in scintillation fluid. Most of the radioactivity moved with $R_{galactose}$ 1.21 but a small amount was also detected with R_{gal} 0.4 and 0.1. The major peak corresponds well to the position of glucosylated lipid from normal incubation mixtures with membrane material [4]. On TLC in diisobutyl ketone/acetic acid/water (20:15:2) most of the radioactivity had R_f 0.2 with a smaller amount having R_f 0.47. Preparations of mannosyl lipid showed a similar mobility to the major product [9].

Further attempts to separate and purify the glycosyl transferases from *K. aerogenes*, which are involved in the synthesis of the exopolysaccharides of this genus, are continuing. It is of interest that in earlier studies, using a different strain of *K. aerogenes*, extraction of membranes with *neutral* butanol at –25°C left the glycosyl transferases of this strain in the residual particulate material [3].

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