Carbohydrate Polymers **13** (1990) 423–436

Action of *Endo*-(1 → 6)-α-D-Glucanases on the Soluble Dextrans Produced by Three Extracellular α-D-Glucosyltransferases of *Streptococcus sobrinus*

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(Received 6 April 1989; revised version received 4 November 1989; accepted 24 November 1989)

ABSTRACT

Four different α-D-glucosyltransferases (GTF) have been obtained from culture filtrates of Streptococcus sobrinus *strains grown in the chemostat at pH 6·5 in complex medium supplemented with Tween 80. Three of the enzymes, GTF-S1, GTF-S3 and GTF-S4, converted sucrose into soluble glucans. Their limit of hydrolysis with endodextranase, the proportion of linear to branched oligosaccharides among the end products of enzymic degradation, and methylation analysis, all supported the view that the glucans were dextrans. The S1-dextrans were highly branched (32% of α-(1 → 3)-branch points), S3-dextrans were linear, and the branching of S4-dextrans was intermediate in value (9%). The enzymes that catalyze the synthesis of three such diverse dextrans were thus proved to be three different GTF, each with a characteristic specificity. Conditions of growth in the chemostat could be varied to provide maximum yields of either GTF-S1, -S3 or -S4.*

INTRODUCTION

Four α-D-glucosyltransferases (GTF) have been separated from culture filtrates of *Streptococcus sobrinus* grown in the chemostat (Walker *et al.*, 1990). The activity and distribution of these enzymes depends on the growth rate, the pH of growth, the limiting nutrient and on the presence

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Carbohydrate Polymers 0144-8617/90/$03.50 —

of Tween 80. The structure of the extracellular α-D-glucans synthesized from sucrose by the streptococci is thus closely related to the growth conditions of the microorganisms. Therefore, when the structure of *S. sobrinus* α-D-glucans is reported, it is imperative to specify all the conditions under which the GTF were released.

In the absence of Tween 80 the two major enzymes are: GTF-S1, that catalyzes the synthesis of highly branched soluble dextran, and GTF-I, that catalyzes the synthesis of α-(1→3)-D-glucan. Growth in medium supplemented with Tween 80 results in the release of significant amounts of two more enzymes of the GTF-S type, i.e. the product is soluble. Under some conditions, the production of the new enzymes GTF-S3 and GTF-S4 exceeds that of GTF-S1 (Walker *et al.*, 1990).

The soluble dextrans synthesized by GTF-S1, -S3 and -S4 have now been hydrolyzed with specific endodextranases. The rate of hydrolysis and the limit of enzymic degradation indicated that the three dextrans have different structures. Separation of the oligosaccharide end products of *Penicillium funiculosum* endodextranase action on the three dextrans supported this conclusion, and further proof was provided by methylation analysis.

METHODS AND MATERIALS

Enzymes and substrates

Streptococcus sobrinus strains were grown in the chemostat, and the extracellular GTF were separated as described previously (Walker *et al.*, 1990). For maximum production of GTF-S3 and GTF-S4, the medium pump was set to give a dilution rate (D) of 0·45 h^{-1}, and the medium was supplemented with D-glucose (0·5%) and Tween 80 (0·5%), with the pH being maintained at 6·5. Maximum production of GTF-S1 was obtained at D 0·05 h^{-1} and pH 6·5 in glucose-limited medium, with Tween 80 having no significant effect on yield. Soluble glucans designated S1-, S3- and S4- dextrans were prepared with HTP-column fractions (4 ml) that contained peak activities of GTF-S1, -S3 and -S4, respectively. The fractions (usually 3) were pooled, and incubated with sucrose (4 g) and 15 mM citrate buffer (pH 6) in 50 ml. *Streptococcus sobrinus* S1-dextran (150 μg) was added as primer to the incubation mixtures containing GTF-S1. After incubation at 35°C for 2 days under toluene, S1- and S3-dextrans were precipitated and washed with 66% ethanol, while S4-dextrans were treated similarly with 50% ethanol. The dextrans were then dissolved in water (the S3- and S4- dextran suspensions being

briefly sonicated), then they were reprecipitated and washed with methanol, dissolved or suspended in water and freeze-dried. Native dextran from *Leuconostoc mesenteroides* strain NRRL B-512(F) was kindly provided by Dr Allene Jeanes. The dextrans were methylated and analyzed with the aid of capillary gas–liquid chromatography-mass spectrometry (Slodki *et al.*, 1986).

Penicillium funiculosum endodextranase (EC 3.2.1.11) was prepared as described previously (Walker, 1972), *Chaetomium gracile* endodextranase (Dextranase DL-2) was provided by Miles Laboratories Australia Pty Ltd and *Bacillus coagulans* endodextranase was purchased from Calbiochem, Los Angeles. *Streptococcus mitis* exodextranase, dextranglucosidase (Walker & Pulkownik, 1974) was prepared as described previously. Endoglucanase activity was assayed by the increase in reducing power of dextran (Sigma 2000) expressed as apparent conversion into isomaltose, while *exo*-dextranase activity was measured by the release of D-glucose from isomaltose.

Dextran hydrolysis and analysis of products

Each dextran (10 mg) was incubated for up to 7 days at 35°C in digests (1 ml) that contained sodium citrate buffer (10 mM, pH 6·0) and endodextranase (0·26 U). Samples were withdrawn at intervals to determine the concentration of reducing sugars (Dygert *et al.*, 1965) and soluble carbohydrates (van Handel, 1968), and thus obtain the limit of hydrolysis. The initial rate of hydrolysis by the endodextranases was determined in similar digests but with 14 mU of enzyme, and samples were taken at 30 min intervals.

The end products of the hydrolysis of *S. sobrinus* dextrans by *P. funiculosum* dextranase were separated with a Varian model 5000 liquid chromatograph equipped with a manual loop injector. The column consisted of a Dextropak cartridge (0·8 × 10 cm) fitted with a precolumn insert and held in a radial-compression separating system (Z-module and RCSS C_{18} Guard PAK, Waters Associates). The solvent, water, was filtered through a Norganic purification system (Millipore Corporation) comprising a scavenger resin and a membrane filter (HA, pore size 0·45 μm). The samples were deionized with Amberlite MB-3 resin, and then prepared for HPLC by centrifuging through a microfilter (MF-1, Bioanalytical Systems Inc.) fitted with a regenerated cellulose membrane (RC 58, pore size 0·2 μm). Separation of the oligosaccharides was monitored with a differential refractometer (R 401, Waters Associates) and peak areas were measured with a chromatography data system (CDS 111, Varian). The products of dextranase action on *S.*

sobrinus S3-dextran and on *L. mesenteroides* B-512(F) dextran were compared by chromatography on Whatman No. 3MM paper as previously described (Hare *et al.*, 1978); detection was effected with alkaline silver nitrate (Trevelyan *et al.*, 1950).

RESULTS AND DISCUSSION

The rate of hydrolysis and limit of hydrolysis of *S. sobrinus* S1-dextran, S3-dextran and S4-dextran were compared with those of *L. mesenteroides* B-512(F) dextran (Tables 1 and 2). The dextranases displayed characteristic differences between their relative rates of hydrolysis of the four dextrans, the *Chaetomium gracile* enzyme hydrolyzing *S. sobrinus* dextrans more rapidly than the other dextranases. The final limits of hydrolysis of a particular dextran, however, did not alter, the three dextranases giving a remarkably similar result for the apparent conversion into isomaltose. S1-dextran was hydrolyzed more slowly, and

TABLE 1
Rate of Hydrolysis of *S. sobrinus* Strain K1-R Dextrans with Endodextranases

Source of dextranase	*Rate of hydrolysis*[a]		
	S1-dextran	*S3-dextran*	*S4-dextran*
Penicillium funiculosum	43	72	74
Chaetomium gracile	81	171	117
Bacillus coagulans	49	141	63

[a]Relative to B-512(F) dextran = 100.

TABLE 2
Limit of Hydrolysis of *S. sobrinus* Strain K1-R Dextrans with Endodextranases

Source of dextranase	*Limit of hydrolysis (%)*			
	S1-dextran	*S3-dextran*	*S4-dextran*	*B-512(F) dextran*
Penicillium funiculosum	45	123	85	125
Chaetomium gracile	44	—	74	112
Bacillus coagulans	49	—	86	134

reached a lower limit of hydrolysis than the other dextrans, and these results were those expected for a highly branched dextran. By contrast, S3-dextran was hydrolyzed even more rapidly than B-512(F) dextran by *C. gracile* and *B. coagulans* dextranase, and the limit of hydrolysis by *P. funiculosum* dextranase was the same as that of B-512(F) dextran. The values exceeded 120% hydrolysis, and were indicative of dextran with a very low degree of branching. S4-dextrans were different from both S1- and S3-dextrans, as shown by the intermediate value for their relative rate and limit of hydrolysis.

Hydrolysis of 6715-13-201 dextrans was similar to the results shown for K1-R dextrans (Table 2), except for the hydrolysis of S1-dextran, which was limited to 28%.

Separation and identification of the products of hydrolysis of several dextrans with *P. funiculosum* endodextranase have provided insights into their specific structural features (Taylor *et al.*, 1985). The distribution of products from S1-, S3- and S4-dextrans from strains K1-R and 6715-13-201 was therefore investigated in order to establish the essential differences between each type. The endodextranase gives glucose and isomaltose as the end products from linear dextran. Any product with a higher degree of polymerization will contain a linkage other than α-(1→6)-, usually an α-(1→3)-branch linkage. Several α-(1→6)-glucosidic linkages in the vicinity of a branch point in dextrans are resistant to hydrolysis by endodextranase (Bourne *et al.*, 1963; Walker & Pulkownik, 1974) and branched dextrans yield a lower proportion of glucose and isomaltose together with a higher proportion of branched oligosaccharides. Almost identical profiles were obtained from HPLC separations of oligosaccharide products from dextrans synthesized by two strains of *S. sobrinus*, and only the results obtained with the three dextrans from strain 6715-13-201 are depicted in Fig. 1, where the products are compared with those from B-512(F) dextran. The values for retention times (RT) and peak areas of oligosaccharides derived from strain K1-R and B-512(F) dextrans are given in Table 3. The two homologous series of branched oligosaccharides, Bn-1 and Bn-2, that result from the hydrolysis of B-512(F) dextran, have previously been identified as 3^3-α-isomaltosyl- and 3^3-α-glucosylisomaltosaccharides, respectively (Taylor *et al.*, 1985). These arise from regions in the backbone bearing 2-unit and 1-unit side chains, respectively. A single series of branched oligosaccharides, Bn-2, was obtained from branched dextrans containing 1-unit side chains exclusively.

Streptococcus sobrinus S1-dextrans were distinguished by the low proportion of products from the linear regions, and by the high proportion of B_4 (3^3-α-glucosylisomaltotriose) which, despite the absence of a

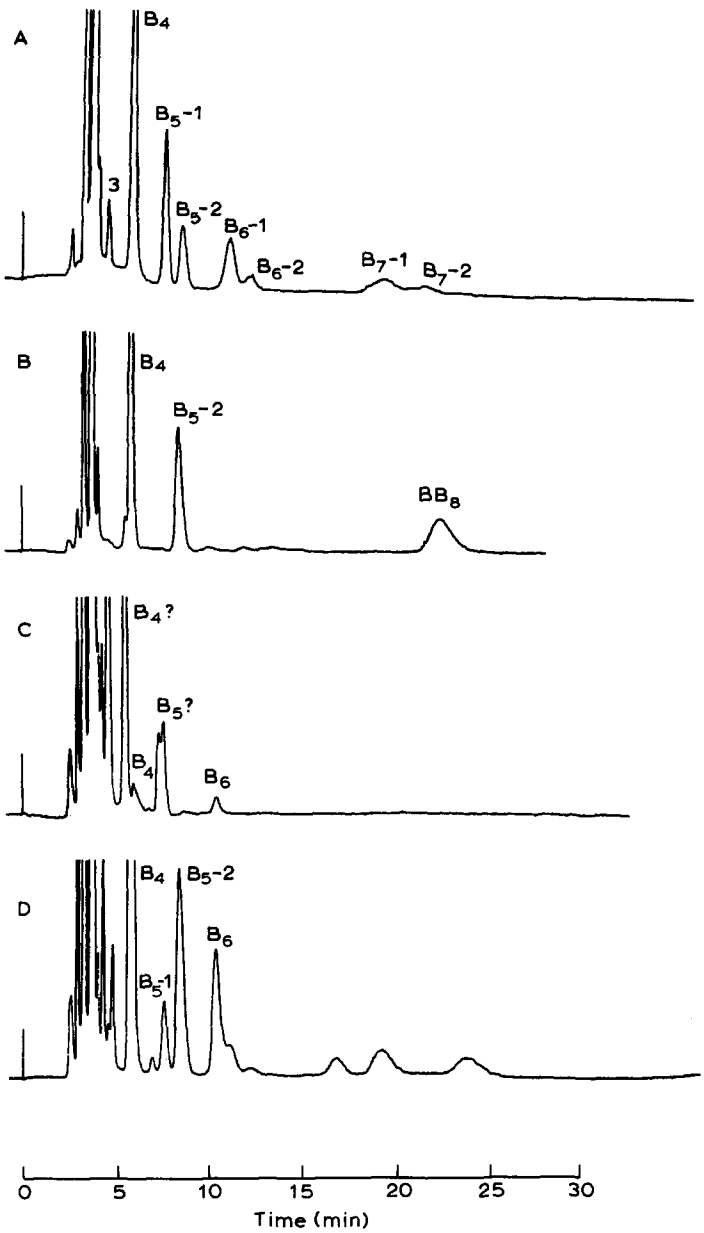

Fig. 1. Separation by HPLC of low molecular weight linear and branched oligosaccharide products of *P. funiculosum* endodextranase on (A) B-512(F) dextran; (B–D) *S. sobrinus* 6715-13-201 S1-, S3- and S4-dextrans respectively. The flow rate increased linearly from 0·5 to 3·0 ml per min during the first 10 min of the operating programme. Symbols are as in Table 3 and in Taylor *et al.*, 1985.

TABLE 3
HPLC Separation of Oligosaccharide Products of Hydrolysis of *S. sobrinus* K1-R Dextrans with *P. funiculosum* Endodextranase

Oligosaccharides			*Products separated from dextran hydrolyzates*							
Stds[a]	*Others*[b]	*RT (min)*[c]	*B-512 dextran*		*S1-dextran*		*S3-dextran*		*S4-dextran*	
			RT	*Area (%)*	*RT*	*Area (%)*	*RT*	*Area (%)*	*RT*	*Area (%)*
Glc		1·7	1·7	18·6	1·7	8·6	1·7	12·4	1·7	10·9
IM_2		1·9	2·0	55·8	2·0	41·2	2·0	65·1	2·0	38·7
IM_3		2·8	2·8	0·7	2·7	1·3	2·7	13·3	2·7	1·7
	B_4[d]	3·4					3·4	4·8		
B_4-2		4·0	4·0	9·3	3·9	16·0	4·0	0·5	3·9	17·8
IM_4		4·2								
	B_5	5·2							5·2	0·3
B_5-1		6·1	6·1	4·5			5·9	4·0	6·0	2·1
B_5-2		7·3	7·3	2·6	7·0	7·4			7·0	7·0
IM_5		7·5								
	B_6	10·1					10·1	0·7	10·1	7·2
B_6-1		11·0	11·0	3·4						
B_6-2		12·5	12·5	1·0					12·3	0·6
IM_6		14·2								
	B_7	18·6							18·6	2·3
B_7-1		23·0	23·0	2·2					22·1	3·5
B_7-2		26·1	26·1	1·9					25·6	0·8
IM_7		26·8								
	BB_8	28·0			27·0	11·4			29·0	3·5
	B_8-2	46·9							46·5	2·4
	BB_9	62·3			62·3	14·0			66·0	0·9

[a]See Taylor *et al.* (1985). IM_n refers to oligosaccharides of the isomaltose series. B_n oligosaccharides also contain a single α-(1 → 3)-glucosidic linkage, and BB_n signifies two α-(1 → 3)-branch linkages.
[b]Tentative description.
[c]Flow rate 2 ml/min.
[d]This was not 3^3-α-glucosylisomaltotriose.

true branch linkage, is the first member of the Bn-2 series (Table 3). This was the result expected for a highly branched dextran. Among the products of higher degrees of polymerization (d.p.) the retention times for B_5 and B_6 correspond to those of the Bn-2 series, indicating that the side chains in S1-dextrans consisted of single glucosyl residues. Two more products separated from K1-R S1-dextran hydrolyzate had retention times higher than isomaltoheptaose (IM_7), but they belonged to

neither the Bn-1 nor the Bn-2 series. Members of these series are susceptible to hydrolysis by dextranglucosidase (Walker & Pulkownik, 1974) when $n \geqslant 6$, glucose being released from the non-reducing terminals leaving B_5-2 as the final product. Because of the strong probability that higher oligosaccharides derived from S1-dextrans were doubly branched, these products have been designated BB_8 and BB_9 (Table 3). BB_9 was hydrolyzed by dextranglucosidase to give BB_8 and glucose. Tentative structures proposed for BB_8 and BB_9, that are consistent with the specificity of dextranglucosidase and *P. funiculosum* endodextranase, are $3^{3,5}$-α-diglucosylisomaltohexaose and $3^{3,5}$-α-diglucosylisomaltoheptaose, respectively. BB_9 was not detected among the products from 6715-13-201 S1-dextran.

The highly branched structure of a K1-R S1-dextran was confirmed by methylation analysis, the molar proportion of 2,4-di-o-methyl ether being 32%, a result that was in complete agreement with the analysis previously published (Hare *et al.*, 1978) for *S. sobrinus* OMZ176 S1-dextran.

The products from the enzymic degradation of S3-dextrans were those expected from virtually linear dextrans. The proportion of the products (glucose, IM_2 and IM_3) released from the linear regions of S3-dextrans was higher than 90% (Table 3), and this is close to the value published (Reese & Parrish, 1966) for the products of the enzymic degradation of synthetic, linear α-1,6-dextran. It is calculated that over 98% of the residues in S3-dextrans and linear dextran are joined by α-(1→6)-glucosidic linkages. No branched oligosaccharides were detected by paper chromatography under conditions where spots corresponding to B_5-, B_6- and B_7-fractions from B-512(F) dextran were clearly visible on the chromatogram. Furthermore, no dimethyl ethers were found among the products of methylation analysis of S3-dextrans, and the proportion of 2,3,4,6-tetra- to 2,3,4-trimethyl ether indicated a d.p. of less than 25.

Separation of the enzymic hydrolyzates by HPLC revealed *c.* 0·5% of B_4-2, together with 4·8% of a new product with a retention time (3·4 min) between that of IM_3 and B_4-2. An oligosaccharide eluted at this time could be designated a B_4. Analysis of this material, and the apparent B_5-1 found among the products, would help to complete the structural details of S3-dextrans. A partially purified glucosyltransferase from a *S. sobrinus* strain was recently found to synthesize a low molecular weight water-soluble dextran (Hanada & Takehara, 1987). Gel filtration, methylation analysis and NMR analysis (Cheetham *et al.*, submitted) all supported the conclusion that *S. sobrinus* S3-dextrans have a low molecular weight (d.p. 20–50). If some of the chains have retained a fruc-

tofuranosyl end group, then their hydrolysis with endoxtranase would yield significant amounts of 6^G-α-glucosyl and/or 6^G-α-isomaltosylsucrose. Such products would not be detected as reducing sugars on paper chromatograms, but could account for the peaks of apparent B_4 and B_5 recorded in Fig. 1.

The S4-dextrans were hydrolyzed to give the products expected from a branched dextran. Although the yield of B_4-2 was high, the degree of branching was shown by methylation analysis to be 8–10%, a value that was intermediate between the highly branched S1-dextrans and the linear S3-dextrans. Methylation analysis, supported by enzymic hydrolysis with *Cladosporium resinae* α-(1→3)-endoglucanase (Walker & Hare, 1977), revealed that S4-dextrans also contained *c.* 16% α-(1→3)-linked glucosidic sequences (Pearce *et al.*, 1990). Since these could be products arising from small amounts of GTF-I present in the preparation of GTF-S4, another S4-dextran was prepared with GTF-S4 from strain 6715-13-27, a mutant that released little or no GTF-I. Methylation analysis of this S4-dextran indicated the molar proportion of 2,4,6-tri-o-methyl ether was 6% and the result was in good agreement with the linkage analysis of dextran synthesized by the single extra glucosyltransferase (Shimamura *et al.*, 1983) found when *S. sobrinus* 6715 was grown in Tween 80 medium.

CONCLUSIONS

The variation in results obtained in different laboratories for the structure of *S. sobrinus* dextrans is explained by the effect of growth conditions on the nature and relative proportion of the different GTF that can be released. These results have proved that three different GTF of the GTF-S type can be separated by column chromatography on hydroxyapatite.

In early work with *S. sobrinus* several fractions of GTF were separated from strain OMZ176, and all the fractions catalyzed the synthesis of water-insoluble α-D-glucan. Most investigators have since found GTF-I together with a primer-dependent GTF (GTF-S1) that synthesized a soluble dextran. It has long been known (Hare *et al.*, 1978) that the S1-dextran is highly branched, with the molar proportion of 2,4-dimethyl sugar obtained by methylation analysis being in the range of 30–32%. Nevertheless, when *S. sobrinus* strains were grown in Tween 80-medium the organisms released a primer-independent GTF that catalyzed the synthesis of a soluble dextran having no more than 5–7% of branch points (Inoue *et al.*, 1982; Shimamura *et al.*, 1983). The

characteristics of a GTF-S isolated from *S. sobrinus* strains B-13 (Namiki *et al.*, 1985) and OMZ176 (Inoue *et al.*, 1982) were not discussed in relation to the presence of Tween 80 in the medium, nor was this surfactant specified by name in the publications. Furthermore, the linkage analysis of the new soluble dextran synthesized by the GTF-S was not contrasted with that published earlier (Hare *et al.*, 1978) for strain OMZ176. Thus, no indication was given for this strain that two very different dextrans had been synthesized by two very different enzymes that had been released as the major GTF-S under two different conditions of growth. Such confusion could be avoided if, when the structure of bacterial polysaccharides is under consideration, the conditions of growth are always described in detail, and the particular synthetases released are also specified. The new type of GTF-S released during growth in batch cultures containing Tween 80 most likely corresponds to GTF-S4, and analysis of S4-dextrans from several strains (Cheetham *et al.*, submitted; Pearce *et al.*, 1990) has indicated that the GTF has a similar specificity to the GTF-S4 released by *S. sobrinus* 6715-13-27 in the absence of Tween 80. It appears that the chromatofocusing and isoelectric focusing procedures adopted previously for the fractionation of GTF succeeded in separating GTF-S4 from GTF-S1, but generally failed to resolve GTF-S3. Isoelectric focusing is not a method of choice because GTF-S3 and GTF-S4 can change to give isozymes with both higher and lower pI values, and the two enzymes can overlap.

It is likely that the enzyme designated GTF-S3 is involved in the synthesis of the soluble dextrans with molecular weight less than 10 kDa, obtained by fractionation of the glucans synthesized by culture filtrates from the mutans group of streptococci (Inoue & Koga, 1979; Sund *et al.*, 1987). A high limit of hydrolysis by dextranglucosidase indicated that, as in S3-dextrans, branch linkages were virtually absent from the low molecular weight dextran fractions.

The redistribution of GTF that occurs in Tween 80-medium has provided good yields (Walker *et al.*, 1990) of two primer-independent enzymes, GTF-S3 and GTF-S4. Because these enzymes synthesize more — linear dextrans, their products can stimulate the activity of primer-dependent GTF-I more effectively than the highly branched S1-dextran (McCabe, 1985; Walker & Schuerch, 1986). Even at the low growth rates that are normal in dental plaque, the greatly increased release of GTF by *S. sobrinus* growing in the presence of Tween 80 includes the correct, high, relative proportion of GTF-I necessary for the interaction with GTF-S types to produce an abundance of adhesive, water-insoluble glucan from sucrose. Tween 80 is listed among the surfactants that can safely be used in food processing, and it is commonly included as an

emulsifier in bread, fruit drinks and in foods (Benson, 1967) such as chocolate, cake, ice-cream, jellies and desserts with cream toppings that are eaten at the end of a meal. Since both detergents and nutrients tend to accumulate at interfaces, the ingestion of Tween-supplemented 'take-away' sweet desserts is likely to stimulate the synthesis on the tooth surface of those adhesive, water-insoluble glucans that are implicated in the colonization of *S. sobrinus* and in the induction of dental caries.

The mechanism by which surfactants stimulate the increased synthesis and/or secretion of GTF is as yet unknown. Tween 80 increases the octadecenoic acid residues in the fatty acids of the bacterial membrane (Umesaki *et al.*, 1977; Jacques *et al.*, 1985; Pitty & Jacques, 1987), leading to altered fluidity. Protein secretion can be dramatically affected by the physical state of the membrane lipids, and a change in membrane fluidity may have diverse effects including altered activity of membrane-bound components of the inducer system (Reese, 1972) and the secretory apparatus (Briggs & Gierasch, 1986).

ACKNOWLEDGEMENTS

This investigation was supported by grants from the National Health and Medical Research Council of Australia. The authors are grateful to Miss Barbara Pearce and Mrs Eva Fiala-Beer for their assistance with the continuous cultures.

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