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ON THE EFFECT OF rfe MUTATION ON THE BIOSYNTHESIS OF THE 08 AND 09 ANTIGENS OF E.COLI

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SUMMARY: From phosphomannose isomerase-less mutants of E.coli strains 08 and 09, rfe derivatives were constructed by recombination with a Salmonella rfe donor. In contrast to membranes from the parent E.coli strains, those from the rfe recombinants did not synthesize the 08 or 09 mannan from GDP mannose in vitro. They could, however, be restored to biosynthetic activity with butanol extracts from the E.coli rfe bacteria. This indicated that the rfe mutation affects the synthesis of a hydrophobic acceptor.

The 08 and 09 antigens of Escherichia coli are lipopolysaccharides that have α-mannans as O-specific polysaccharides. Both polysaccharides contain only α-1,2 and α-1,3 linkages in different ratios. The repeating unit of the 08 mannan is a trisaccharide (1), and that of the 09 mannan a pentasaccharide (2). We have previously shown (3-5) that both polysaccharides are synthesized by a mechanism that differs from that described for the O antigens of Salmonella of O groups B or E (6,7). The polymerization of the 08 and 09 mannans takes place by sequential mannosyl transfer reactions at the nonreducing end. Both mannans when isolated from the cytoplasmic membrane (as the biosynthetic intermediate hapten, ref. 7,8), have glucose at the reducing end (4,5). A hydrophobic mannose acceptor, presumably a glucolipid, can be extracted from biosynthetically active membranes of E.coli 09 and used to restore the mannan-synthesizing capacity of inactive membranes (Kanegasaki & Jann, Eur. J. Biochem., submitted). Reciprocal membrane reconstiution experiments showed this intermediate to be common to E.coli 08 and 09 (Goldemann et al., manuscript in preparation).

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The biosynthesis of the E.coli O8 and O9 antigens depends on the function of the rfe gene(s) (9) that were first shown to be necessary for the synthesis of the O antigens of Salmonella O groups C and L but not B (10), and also for the synthesis of the enterobacterial common antigen ECA (11). The properties of rfe mutants suggested that the product of the rfe gene(s) may participate in the biosynthesis or modification of a lipid intermediate used in the biosynthesis of ECA and certain but not all O-antigenic polysaccharides. However, a direct demonstration of this gene product or its function has been lacking.

In this communication we show that the effect of the rfe-mutation in E.coli O8 and O9 can be overcome in vitro by reconstitution of rfe^- membranes (as enzyme source) with the hydrophobic mannose acceptor isolated from rfe^+ bacteria.

MATERIALS AND METHODS

Bacteria and cultivation: The pmi mutants F945 and F860 of E.coli O8 and O9, respectively, have been described before (3,4). The rfe-derivatives EH737 and EH743 of these were obtained by introduction of the rfe-4274 mutant allele from SH5454, a rfe mutant of Salmonella typhimurium Hfr K1-2 as described before (9). The bacteria were grown to the late logarithmic phase in L-broth (12) with the addition of 0.5% glucose.

Analytical procedures ; Preparation of EDTA-bacteria, membranes and butanol extracts; Assay of mannose incorporation; Characterization of products: These were all described previously (3,4,13, Kanegasaki & Jann, Eur.J.Biochemistry, submitted).

RESULTS

Membrane reconstitutions: Membranes from the rfe^- mutants of E.coli O8 and O9 incorporated very little mannose (Table 1). Their reconstitution with butanol extracts from the corresponding rfe^+ strains resulted in active incorporation. The butanol extracts were active not only in the homologous system, but also in heterologous combination. The O8 system seems to be somewhat less efficient, since the incubations had to be prolonged from 10 minutes (standard incubation time - Kanegasaki & Jann, Eur.J.Biochem., submitted) to 30 minutes, to ob-

Table I. Biosynthetic activities of membranes from the *rfe*$^-$ mutants with and without reconstitution with butanol extracts from the *rfe*$^+$ bacteria.

Membranes from	Butanol extract from	Incubation time (min)	Mannose incorporation (pMole/mg protein)
EH737 (08 *rfe*$^-$)	none	10	2.0
		30	2.6
	F945 (08 *rfe*$^+$)	10	58.8
		30	237.8
	F860 (09 *rfe*$^+$)	10	61.3
		30	234.5
EH743 (09 *rfe*$^-$)	none	10	3.0
		30	3.5
	F860 (09 *rfe*$^+$)	5	1 072
		10	1 350
	F945 (08 *rfe*$^+$)	5	1 027
		10	1 370

The incubation mixtures contained membranes (200 µg protein, final), TRIS-HCl (100 mM final, pH 7.5), butanol extractable material from 2.5 x 10^9 bacteria (in 30 µl water), (or 30 µl water) and GDP (^{14}C) mannose (5 µM, final; 25000 cpm) in a total volume of 100 µl. The reconstitution technique is described in ref. 13. After incubation at 35°C, the membranes were precipitated with acetic acid, filtered, washed and counted in a Packard liquid scintillation counter.

tain significant incorporation.With the O9 system (EH 743 membrane) the incubation time could be reduced to 5 minutes without an appreciable change in the mannose incorporation. No mannose incorporation was observed in these experiments when butanol extracts of the rfe$^-$ mutants were used (data not shown). These results give direct support to the hypothesis that the rfe mutation affects the mannose acceptor lipid rather than mannosyl transferase.

Characterization of the products of incorporation: To analyze the products, the incubation mixtures were scaled up 10 times. Incubations were stopped by cooling and the membranes were collected by centrifugation, washed with buffer, the pellet was then desintegrated with sodium dodecyl sulfate and subjected to polyacrylamide gel electrophoresis in the presence of dodecyl sulfate (3). Figure 1 shows the radioactivity profiles obtained with the products of reconstitutions in the O8 and O9 systems. The radioactive bands obtained after 30 minutes have the same electrophoretic mobility as the intermediate which was previously identified as the mannan bound to the acceptor lipid (3). It is interesting to note that the product of the O8 system seems to be smaller after 10 minutes of incubation than after an incubation period of 30 minutes. This is in accord with the incorporation shown in Table 1 and further attests to the lesser efficacy of the O8 system in these conditions.

When the products were liberated from the reconstituted membrane by mild acid hydrolysis (0.01 N HCl at 100°C for 20 minutes) they were excluded from Sephadex G50 and included in Sephadex G100. After total acid hydrolysis (1 N sulfuric acid at 100°C for 3 hours) more than 90% of the radioactivity had the same paper chromatographic mobility as mannose. The products of the incubations in the reconstituted system therefore behave as the biosynthetic products of the corresponding rfe$^+$ bacteria (3-5) which were shown to possess the authentic structure of the O8 and O9-specific mannans, respectively.

DISCUSSION

From the results described in this and in earlier communications (3-5), one can postulate that the biosynthesis of the

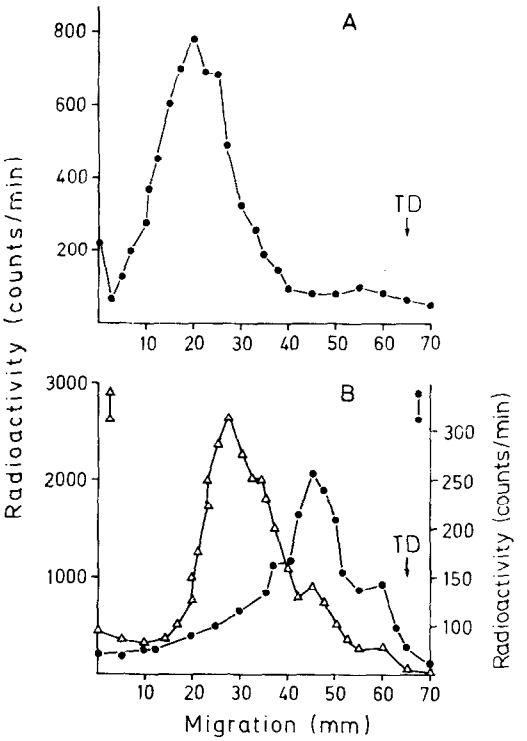

Figure 1
SDS polyacrylamide gel electrophoresis of the radioactive products from incubations with GDP (^{14}C)mannose of reconstituted rfe$^-$ membranes.
A: Electrophoretic pattern of the product obtained with reconstituted EH743 (O9rfe$^-$) membranes after 10 minutes incubation;
B: Electrophoretic pattern of the product obtained with reconstituted EH737 (O8rfe$^-$) membranes after 10 minutes (- ● -) and 30 minutes (- △ -) incubation.
Butanol extracts from E.coli O9rfe$^+$ were used in all experiments. The incubation mictures were tenfold scale ups of that described in the legend to Table 1. After incubations the membranes were washed with TRIS-HCl (100 mM, pH 7.5) by ultracentrifugation, desintegrated and aliquots (equal in 1B) were electrophoresed on 5.6% gels as described in ref. 3. Tracking dye (TD) was pyronin G. Gel slices (2 mm) were hydrolyzed in HCl and the radioactivity was counted in a Packard scintillation counter.

O8 and O9 antigens proceeds in the following stages: First a mannose acceptor is synthesized by the glucosylation of a yet unknown lipid. This is then substituted at the glucose by the growing mannan chain which is elongated at the non reducing end. Finally, the mannan with glucose at the reducing end is

transferred from the lipid to the core-lipid A complex with the formation of the complete O8- or O9-antigenic lipopolysaccharide.

The biosynthesis of the O8 and O9 antigens may be blocked at various stages by mutation of relevant genes. One of these genes (or gene clusters) is the rfe locus, which is required for the biosynthesis of several polsaccharide antigens: the O antigens of Salmonella strains belonging to O groups C1 and L, the Salmonella T1 antigen, the enterobacterial common antigen ECA and the O8, O9, O20 and O100 antigens of E.coli (9,10,11,12). Although the rfe function is not known, it has been speculated that it may determine the synthesis or modification of a lipid intermediate in the biosynthesis of these polysaccharides. Since only the biosynthesis of the E.coli O8 and O9 antigens has been studied in sufficient detail to analyze the biochemical consequences of rfe mutation, we have constructed rfe mutants from both coli strains and subjected them to membrane reconstitution experiments.

The results show clearly, that in both mutants the mannosyl transferase system is intact whereas the synthesis of the mannose acceptor lipid is defective. We cannot yet say whether the defect is in the synthesis of the lipid or in its glucosylation. This is especially difficult since the nature of the lipid is not yet known. However, it is fair to assume that the reaction in question is also limiting in other rfe mutants of Salmonella and E.coli. If the rfe gene(s) govern the glucosylation of the acceptor lipid, then the mechanism of polymerization of the rfe-dependent Salmonella and E.coli polysaccharides must be the same as with the E.coli O8 and O9 mannans, and the respective haptens should also have glucose at the reducing end. Experiments to differentiate between the two possible rfe-dependent reactions are being carried out.

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