Genetic analysis of *Escherichia coli* O9 *rfb*: identification and DNA sequence of phosphomannomutase and GDP-mannose pyrophosphorylase genes

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Subcloning, transposon insertion, and deletion analysis revealed that the *Escherichia coli* O9 *rfb* region is about 12 kb in size. The region encodes at least seven polypeptides of 89, 74, 55, 50, 44, 41 and 39.5 kDa. Southern hybridization analysis of *rfb* regions of *E. coli* O8 and O9, and *Klebsiella* O3 and O5 serotypes (all of these O polysaccharides are mannose homopolymers and the structures of the repeating unit of *E. coli* O9 and *Klebsiella* O3 are identical) showed that a central region specific for *E. coli* O9 and *Klebsiella* O3 is flanked by two regions common to all four. Complementation experiments using strains with known defects and specific tests for the enzymic activity showed that the 50 and 55 kDa polypeptides, encoded by the common region, are phosphomannomutase (PMM) and GDP-mannose pyrophosphorylase (GMP), respectively. Nucleotide sequencing of the region revealed the presence of two genes, *rfbK* and *rfbM*, analogous to the corresponding genes of *Salmonella typhimurium*. In *E. coli* O9, *rfbK* and *rfbM* encode proteins of 460 amino acids (50 809 Da) and 471 amino acids (52 789 Da). The amino acid sequence of GMP was conserved in RfbMs of *E. coli* O7 and *Salmonella* groups B, C1 and C2, CpsB of *S. typhimurium*, AlgA of *Pseudomonas aeruginosa*, and XanB of *Xanthomonas campestris*. The phylogenetic trees of PMM and GMP were different in topology and in the evolutionary distances from ancestors.

Keywords: *Escherichia coli*, *rfb*, phosphomannomutase, GDP-mannose pyrophosphorylase, lipopolysaccharide

INTRODUCTION

Lipopolysaccharide (LPS) is a molecule consisting of three distinct regions; lipid A, an oligosaccharide core, and an O polysaccharide. An *rfb* gene cluster directs the synthesis of O polysaccharide of LPS (for reviews, see Jann & Jann, 1984; Mäkelä & Stocker, 1984; Rick, 1987). Two different pathways of O polysaccharide synthesis are

known. One found in *Salmonella typhimurium* involves both *rfb* and *rfe* genes. The individual repeat units of O polysaccharide are synthesized first and they are polymerized by the *rfe* gene product. Another pathway was found in *Salmonella* groups C1 and L, and *Escherichia coli* groups O8 and O9. In these bacteria, the *rfe* gene is required in addition to *rfb* for O polysaccharide synthesis. The expression of *Klebsiella pneumoniae* O1 polysaccharide has been shown recently to be *rfe*-dependent also (Clarke *et al.*, 1992). Meier-Dieter *et al.* (1992) indicated that Rfe is most probably a N-acetyl glucosamine transferase in enterobacterial common antigen synthesis; however, the role of the *rfe* gene in O polysaccharide synthesis is so far unclear. *E. coli* O8 and O9 synthesize the O polysaccharide by the addition of single sugars to the elongating O polysaccharide. In addition to *E. coli* O8 and O9, *Klebsiella* O3 and O5 are known to have mannose homopolymers as their O polysaccharides. The structures of the O poly-

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Abbreviations: GMP, GDP-mannose pyrophosphorylase; PMM, phosphomannomutase.

The GenBank accession number for the nucleotide sequence reported in this paper is D13231.

Table 1. Bacterial strains, phages and plasmids

Strains, phages and plasmids	Relevant characteristics	Source
Wild-type strains		
F719	<i>E. coli</i> O9:K ⁻ :H ⁻	G. Schmidt, Borstel
CO8	<i>E. coli</i> O8:K?	This laboratory
K49S	<i>K. pneumoniae</i> O3:K49 ⁻ , non-capsulated mutant of the reference strain of <i>Klebsiella</i> K49 (strain 6115)	This laboratory
K61S	<i>K. pneumoniae</i> O5:K61 ⁻ , non-capsulated mutant of the reference strain of <i>Klebsiella</i> K61 (strain 5710/52)	This laboratory
<i>E. coli</i> K12		
HU1190	Δ (<i>sbcB-rfb</i>) <i>hsdR4 recA56 srl::Tn10</i>	S. Hull, Texas Medical Centre
HB101	F ⁻ <i>hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44</i>	This laboratory
MXR	Δ (<i>lac-pro</i>) <i>galE thi recA1</i> (pULB113)	A. Toussaint, Brussels
D21A	<i>his-51 trp-30 proA23 lac-28 ampA1 strA173 tsx-81 recA</i>	This laboratory
JA200	F ⁻ :Tn1000 ⁺ <i>trpE5 recA1 thr leuB6 lacY</i>	T. Sekizaki, Tsukuba
P678-54	<i>thr leuB1 supE lacY tonA gal mal ara mtl min</i>	J. Hacker, Würzburg
<i>S. typhimurium</i>		
SL901	<i>rfc</i>	P. H. Mäkelä, Helsinki
his-660	<i>rfbK M</i>	P. H. Mäkelä, Helsinki
his-642	<i>rfbK</i>	P. H. Mäkelä, Helsinki
Plasmids		
pACYC184	Cloning vector, Tc ^R Cp ^R	This laboratory
pBluescript II SK(+)	Cloning vector, Ap ^R	TOYOBO
pULB113	RP4::miniMu	A. Toussaint, Brussels
pNKB26	Derivative of a cloning vector pACYC184 carrying <i>E. coli</i> O9 <i>rfb</i>	This study
R'-CO8	Derivative of pULB113, carrying <i>his-rfb</i> region of CO8	This study
R'-K49	Derivative of pULB113, carrying <i>his-rfb</i> region of K49S	This study
R'-K61	Derivative of pULB113, carrying <i>his-rfb</i> region of K61S	This study
Phage		
Ω 9	Recognizes strains containing <i>E. coli</i> O9 LPS	G. Schmidt, Borstel

was centrifuged at 20 000 *g* for 30 min at 4 °C. The supernatants were used for enzymic analysis. Protein concentration of cell extracts was determined by the Bio-Rad Protein Assay (Bio-Rad) as instructed by the manufacturer. Bovine serum albumin was used as the protein reference substance.

The method of Sá-Correia *et al.* (1987) was used to determine PMM activity. The 2.0 ml reaction mixture contained 0.1 M Tris/HCl buffer (pH 7.5), 20 μ mol MgCl₂, 0.5 μ mol α -D-glucose-1,6-diphosphate, 1.0 U each of phosphoglucose isomerase, glucose 6-phosphate dehydrogenase and phosphomannose isomerase, and 2 μ mol NADP. The reaction was started by the addition of 200 μ l crude extract and 2 μ mol α -D(+)-mannose-1-phosphate. Increases in OD₃₄₀ were recorded at 37 °C with a UV2200 UV-VIS recording spectrophotometer (Shimadzu). Enzyme activities were calculated after subtraction of endogenous activities which were measured in an assay without α -D(+)-mannose-1-phosphate.

GMP activity was measured by the method of Munch-Petersen (1962). The 1.0 ml reaction mixture contained 0.05 M Tris/HCl buffer (pH 7.5), 10 μ mol MgCl₂, 0.1 μ mol ADP, 2 μ mol pyrophosphate, 5 μ mol sodium fluoride, and 0.5 U each of hexokinase, nucleoside-5'-diphosphate kinase, and glucose 6-phosphate dehydrogenase, 0.8 μ mol glucose and 1 μ mol NADP. The reaction was started by the addition of 50 μ l crude extract and 0.4 μ mol GDP-D-mannose. The reduction of NADP was measured at 340 nm as described above. Enzyme activities were calculated after subtraction of endogenous activities which were measured in an assay without pyrophosphate.

RESULTS AND DISCUSSION

Molecular cloning of the *rfb* region of *E. coli* O9

The *rfb* region (*rfb*_{O9}) of *E. coli* O9 strain F719 was predicted to be in a 17.7 kb *Bgl*II fragment of plasmid

Fig. 1. (a) Restriction enzyme map of pNKB26. The numbers indicate Tn1000 insertion plasmids and show the positions of Tn1000 insertions. Plasmids that still direct the synthesis of O9 polysaccharide are shown above the line of pNKB26 and those which do not are shown below. (b) Physical map of subclones and deletion plasmids used in the study. Solid bars represent the cloning vector pACYC184; hatched bars represent the cloning vector pBluescript II SK(+). Dotted lines represent deleted regions. A series of pXX plasmids with various deletions was prepared using an XhoI site in Tn1000 and an XbaI site of pACYC184, by removing the XbaI–XhoI fragment from various plasmids. Other deletion plasmids were prepared by partial digestion with each enzyme. HindIII A and C fragments were subcloned into a HindIII site of pBluescript II SK(+) termed pBHA6 and pBHC4, respectively. Deletion derivatives of pBHC4 were prepared by digestion with *Apal* and *XhoI*, and deletions originating at the *XhoI* site were introduced by exonuclease III and mung bean nuclease treatment (Deletion kit, Takara Shuzo Co.). Synthesis of O9 polysaccharide was determined by SDS-PAGE and immunoblot analysis and the results are shown on the right. The deduced *E. coli* O9 *rfb* region is shown by the shaded bar.

R'-6 (Kido *et al.*, 1989). The *Bgl*II fragment was cloned into the *Bam*HI site of pACYC184 and the newly constructed plasmid, pNKB26, was introduced into the *rfb* deletion strain HU1190, a *rec*⁻ *hsdR* derivative of *E. coli* K12 SΦ874 (Haraguchi *et al.*, 1991). The resulting strain gave positive agglutination with *E. coli* O9 antiserum and was sensitive to *E. coli* O9-specific phage Ω9, whilst the host strain was not agglutinated and was resistant. The LPS profile from SDS-PAGE analysis and the serological properties of HU1190 harbouring pNKB26 were identical to those seen in an *E. coli* O9 wild-type strain and D21A(R'-6) (Kido *et al.*, 1989; Saeki *et al.*, 1993). The LPS profile showed the presence of a typical ladder of bands corresponding to lipid A plus core oligosaccharide substituted with O polysaccharide. The results showed that pNKB26 carried the whole *rfb* region for the synthesis of O9 polysaccharide and that there was no contribution of host *rfb* genes to the synthesis of O9 polysaccharide in

E. coli K12. We used pNKB26 for further analysis of the *rfb* region.

Isolation and characterization of Tn1000 insertion derivatives of pNKB26

To determine the precise region boundaries of *rfb*_{EO9}, we isolated Tn1000 insertion derivatives of pNKB26. Representative Tn1000 insertion plasmids were selected and their insertion sites are shown in Fig. 1(a). From the results it appears that the left end of *rfb*_{EO9} is located between positions 25 and 247. However, the right end of *rfb* was not clear from the results because some insertion derivatives that had Tn1000 insertions in this region of pNKB26 directed the synthesis of O9 polysaccharide, whilst others with insertions at almost the same positions did not. To clarify the situation, we prepared subclones of pNKB26 for analysis.

Fig. 2. Minicell analysis of pNKB26. Lanes: 1, pACYC184; 2, pNKB26; 3, pNKB26::Tn1000-274 (O9 positive control for Tn1000 inserted in pNKB26); 4, pNKB26::Tn1000-62; 5, pNKB26::Tn1000-195; 6, pNKB26::Tn1000-54; 7, pNKB26::Tn1000-305; 8, pNKB26::Tn1000-131; 9, pNKB26; 10, pNKB26::Tn1000-243; 11, pNKB26::Tn1000-47; 12, pNKB26::Tn1000-48; 13, pXX195; 14, pNKB26-H13. The size of each polypeptide (kDa) is indicated on the right. CAT, chloramphenicol acetyl transferase encoded by the vector.

Subcloning of pNKB26

Deletion derivatives and subclones of pNKB26 are shown in Fig. 1(b). pXX274-6, having a deletion between the vector and Tn1000 insertion position 274, directed the synthesis of O9 polysaccharide. However, plasmids with more extended deletions, pXX195 and pNKB26H-13, did not direct the synthesis of O9 polysaccharide. We constructed some subclones using pBluescript II SK(+), as these are compatible with pACYC184-based deletion derivatives (the former has a ColE1 replicon, whilst the latter has a P15A replicon). When plasmids pBHC4 (Fig. 1) and pXX195 were put into HU1190, the resulting strain synthesized O9 polysaccharide. Plasmids with various lengths of deletions from the left side of the fragment of pBHC4 were prepared and their ability to synthesize O9 polysaccharide was analysed by co-transformation with pXX195. From the results, the left end of *rfb*_{EO9} was determined to be between the left ends of pBHC4-d4 and -d5, almost the same as that of Tn1000 insertion position 25.

Other plasmids with deletions at the right end of pNKB26 were examined. pNKB26-H14, pNKB26-B5 and pNKB26-S1 did not direct the synthesis of O9 polysaccharide. We put plasmid pBHA6 (Fig. 1) together with pNKB26-S1 into strain HU1190. This strain synthesized O9 polysaccharide and, because no deletion plasmids of pBHA6 could substitute for pBHA6, the whole region of the *Hind*III A fragment must be necessary for synthesis of O9 polysaccharide. Thus, we concluded that the minimum region essential for O9 polysaccharide synthesis lies between the Tn1000 insertion position 247 and the right end of the *Hind*III A fragment of pNKB26, and is about 12 kb (Fig. 1b). Expression of the O9 polysaccharide in spite of Tn1000-119 within the 12 kb fragment is

thought to be due to its insertion into a non-essential region. Some clones carrying Tn1000 insertions well outside the *rfb*_{EO9} region, but nevertheless inhibiting O polysaccharide synthesis, were also isolated. The same phenomenon was observed by Kessler *et al.* (1991). These authors cloned the *rfb* region from *Yersinia pseudotuberculosis* serotype IIA and located the *rfb* region by Tn5 mutagenesis. They isolated one clone carrying a transposon insertion outside the region for O polysaccharide synthesis, but inhibiting O polysaccharide production. The question of whether the actual transposon insertion, or a second mutational event within the *rfb* region, caused this effect, was not investigated. Transposon insertion derivatives of pNKB26, pNKB26::Tn1000-57, -45 and -39 carrying transposon insertions outside the minimal region for O9 polysaccharide synthesis blocked O9 polysaccharide synthesis. A subclone carrying a *Sac*I-*Sa*I fragment which covers this region did not confer the ability to synthesize O9 polysaccharide to these strains. Minicell and maxicell analyses revealed that the 50 kDa polypeptide, shown to be PMM in this study, was missing (data not shown). The *rfbK* gene for PMM is not located near these transposon insertions. These results suggested that the second mutational event(s) in *rfbK* may cause the loss of O polysaccharide synthesis in these strains. However, it is not clear why a Tn1000 insertion outside *rfb*_{EO9} inhibits synthesis of PMM distant from the insertion positions.

Analysis of ³⁵S-labelled proteins encoded by pNKB26 in minicells

Minicell analysis was carried out to determine the number and size of polypeptide gene products encoded by *rfb*_{EO9}. pNKB26 encoded nine major polypeptides with mol-

Fig. 3. Physical maps of plasmids used for the determination of PMM and GMP activities. A *SacI*–*HindIII* fragment of pNKB26 was subcloned into pBluescript II SK(+) and named pBHC1-Sc6. pBHC1-*rfbK* and pBHC1-*rfbM* were constructed by deleting a *SacI*–*StuI* and a *SphI*–*SacI* fragment from pBHC1-Sc6, respectively. Numbers in boxes represent the molecular mass of polypeptides and their gene location deduced by minicell analysis. Numbered bars at the top represent DNA fragments used for Southern hybridization analysis.

ecular masses of 89, 74, 55, 50, 44, 41, 39.5, 36 and 27 kDa in addition to the chloramphenicol acetyltransferase (CAT) of the cloning vector (Fig. 2, lanes 1 and 2). The locations of genes encoding these polypeptides were determined by minicell analysis of the *Tn1000* insertion derivatives of pNKB26. Because *Tn1000* has termination codons at both ends, *Tn1000* insertion into coding DNA terminates protein synthesis and causes a change of molecular mass of the gene product (Sancar *et al.*, 1981). Some extra polypeptides derived from *Tn1000* were detected in the minicell preparations of the *Tn1000* insertion plasmid (Fig. 2, lane 3). The locations of genes for six polypeptides of 89, 74, 55, 50, 44 and 27 kDa were ascertained (Fig. 2, lanes 4–8 and 12). The gene for the polypeptide of 27 kDa was located at *Tn1000* insertion position 48 outside of *rfb*_{EO9} (Fig. 1). A polypeptide of 41 or 39.5 kDa was absent in preparations of pNKB26::*Tn1000*-243 and -47 (Fig. 2, lanes 10 and 11). However, it was difficult to determine the molecular mass of the band because the mobility differed slightly in the preparations of O polysaccharide-positive and -negative minicell preparations due to the effect of LPS in the preparations. The location of the gene for the 39.5 kDa polypeptide was determined as follows. The polypeptide was absent from the pNKB26-H13 preparation (Fig. 2,

lane 14) but present in the pXX195 preparation (Fig. 2, lane 13). The gene for the 39.5 kDa polypeptide was therefore located in the deleted fragment. The gene for the polypeptide of 41 kDa might be located at insertion positions 243 and 47. The location of the gene for the polypeptide of 36 kDa was not determined but it is probably between the vector and *Tn1000* insertion 274 because only a faint band was observed in the pXX195 preparation. From these results, at least seven polypeptides of 89, 74, 55, 50, 44, 41 and 39.5 kDa are encoded by *rfb*_{EO9}, implicating them in the synthesis and assembly of the O9 polysaccharide. Since [³⁵S]methionine was used, it is also possible that polypeptides lacking methionine or others produced in very low amounts are also involved in O9 polysaccharide synthesis and have not been detected. The sum of the sizes of the seven polysaccharides accounts for a coding region of about 10.7 kb of DNA, assuming that there are no overlaps and no non-coding regions between structural genes. It may be possible that there are some genes in the remaining 1.3 kb of the approximately 12 kb *rfb*_{EO9} region. The deduced locations of the genes are shown in Fig. 3.

Two polypeptides of 74 and 89 kDa were absent from the pNKB26::*Tn1000*-305 preparation (Fig. 2, lane 7) but

Fig. 4. (a) Ethidium bromide-stained agarose gel. Lanes: 1 and 5, R'-CO8; 2 and 6, R'-K49; 3 and 7, R'-K61; 4, *Hind*III-digested λ DNA. DNA preparations were digested with *Eco*RI (lanes 1–3) or *Pst*I (lanes 5–7). (b, c) Autoradiograms of gels with DNA fragment 3 (b) or 5 (c) (see Fig. 3).

Table 2. Summary of the results of Southern hybridization analysis

Probe DNA*	R'-CO8 (<i>E. coli</i> O8) [†]	R'-K49S (<i>Klebsiella</i> O3) [†]	R'-K61S (<i>Klebsiella</i> O5) [†]
1	+	+	+
2	+	+	+
3	+	+	+
4	—	+	—
5	—	+	—
6	—	+	—
7	+ ^w	+	+ ^w
8	+	+	+
9	+	+	+

* Probes are shown in Fig. 3.

[†] +, Positive; +^w, hybridized weakly; —, negative.

only the 89 kDa preparation was absent from the pNKB26::Tn1000-131 preparation (Fig. 2, lane 8), suggesting that the genes were transcribed from one promoter from the gene for the 74 kDa polypeptide towards that for the 89 kDa polypeptide. Other Tn1000 insertion plasmids lost only one protein band from the preparations, indicating that other genes may have their own promoters. These observations support the idea that the O9 LPS region is organized as a gene cluster, as observed in the O7 LPS region by Marolda *et al.* (1990), rather than a single operon.

Southern hybridization analysis of *rfb* genes

In a number of *E. coli* and *Klebsiella* strains the *rfb* gene cluster has been cloned and shown to be linked to *his*

Table 3. Complementation test of mutations in *E. coli* O9 *rfb* by *rfbK* and *rfbM* genes of the *S. typhimurium* *rfb* cluster

Plasmids*	<i>E. coli</i> O9 expression [†] in:			
	HU1190 (<i>rfbK</i> [—] <i>rfbM</i> [—])	SL901 (<i>rfbK</i> ⁺ <i>rfbM</i> ⁺)	his-660 (<i>rfbK</i> [—] <i>rfbM</i> [—])	his-642 (<i>rfbK</i> [—] <i>rfbM</i> ⁺)
pNKB26	+	+	+	+
pNKB26::Tn1000-62	—	+	—	+
pNKB26::Tn1000-195	—	+	±	±
pXX195	—	+	—	±

* Plasmid maps are shown in Figs 1 and 3.

[†] *E. coli* O9 expression was determined by an agglutination test in anti-*E. coli* O9 serum and was confirmed by the silver-stained SDS-PAGE gel method and immunoblot analysis of LPS preparations. +, Positive; —, negative; ±, trace amount.

(Clarke & Whitfield, 1992; Kido *et al.*, 1989; Valvano & Crosa, 1989). R' plasmids carrying the *his-rfb* regions of *E. coli* O8 (strain CO8), and *Klebsiella* O3 (strain K49S) and O5 (strain K61S) were isolated according to the method reported previously and termed R'-CO8, R'-K49 and R'-K61, respectively (Kido *et al.*, 1989; Saeki *et al.*, 1993). Strain D21A, carrying these plasmids, agglutinated with antisera against the parental strains. DNA homologies among *rfb* genes of these bacteria were investigated by Southern hybridization using DNA fragments of *rfb*_{EO9} as probe. Plasmid preparations were digested with restriction enzymes *Eco*RI and *Pst*I and separated by 0.9% agarose gel electrophoresis (Fig. 4a) and probed with DNA fragments of *rfb*_{EO9}. Represent-

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10      20      30      40      50      60      70      80      90      100     110     120
AAATGCGGAAAGTGTGGTGGCGTTTGGCGGCCAGGTGAGGGAAGAGCATGTTAAAGCAGAGATATGCTGAAGGTATATCTCTGCTTTTAGGTTTTCAGGACACGCGTTCCGCCCGGCTG

130     140     150     160     170     180     190     200     210     220     230     240
TCATTGTAGCCGTGACTGAACGATCTGCCGACAGCCTGAAGCGAAAGAGGGTGGCGTTTAGCCTGGAGCACGGGAGCAGTACGCATTGGGTGGGACAGCCAATGGCGGTAGCGTGGC
-35 ? -10 ?

250     260     270     280     290     300     310     320     330     340     350     360
TGAAAAGGCGCTTCCACCCAGCGGTGATTGATTGATAGTAAATAAGCAATTATTTATTTAAGGATTTTATTGTTACTCCCTGTAATTATGGCTGGTGGTACCGGCAGTCGTCT
SD M L L P V I M A G G T G S R L
start rfbM Kpn I

370     380     390     400     410     420     430     440     450     460     470     480
CTGGCCGATGTCTCGCGAGCTTTACCCGAAACAGTTCCTCCGCTGTTCCGGCAGAATCCATGCTGCAGGAAACCATACCCGACTCTCGGGCTTGAAATCCATGAACCGATGGTCAT
W P M S R E L Y P K O F L R L F G Q N S M L Q E T I T R L S G L E I H E P M V I
Pst I

490     500     510     520     530     540     550     560     570     580     590     600
CTGTAACGAAGACCCGCTTCTGGTGGCGAACAGCTGCGCCAGCTCAACAAGCTGTGGAACAACATTATTCTTGAGCCGGTCCGGCGCAACACCGCCCGGCCATCGCCCTGGCGGC
C N E E H R F L V A E Q L R Q L N K L S N N I I L E P V G R N T A P A I A L A A

610     620     630     640     650     660     670     680     690     700     710     720
CCTTAGGCCACCCGTCACGGCGATGACCCGCTGATGCTGGTCTCGCCCGGACCATATCATCAATAACGAGCCGCTTCCACGACGCCATCCGCGTCCCGGACGATGCGCGATGA
L Q A T R H G D D P L M L V L A A D H I I N N Q P V F H D A I R V A E Q Y A D E

730     740     750     760     770     780     790     800     810     820     830     840
AGGCCATCTGGTCACCTTCGGTATCGTGCCGAACGCCCGGAAACCGGCTACGGCTACATCCAGCGCGGCTGGCCCTCACCGACAGCGCCACACCCCGTACCAGGTGGCCCGCTTCGT
G H L V T F G I V P N A P E T G Y G Y I O R G V A L T D S A H T P Y Q V A R F V

850     860     870     880     890     900     910     920     930     940     950     960
GGAGAAGCCGGACCGCGAGCGCGCGAGGCTACCTCGCCTCCGGGAGTACTACTGGAACAGCGCATGTTTATGTTCCGCGCAAAAAATACCTCTCCGAGCTGGCCAAATTCGCCCC
E K P D R E R A E A Y L A S G E Y Y W N S G M F M F R A K K Y L S E L A K F R P
Stu I

970     980     990     1000    1010    1020    1030    1040    1050    1060    1070    1080
GGATATCTCGAAGCCTGCCAGGCCGCGTCAATGCCGCCGATAACGGCAGCGACTTCATCAGCATCCCGCATGACATTTCTGCGAGTGCCCGGACGAGTCCGTGGACTACGCGGTGAT
D I L E A C Q A A V N A A D N G S D F I S I P H D I F C E C P D E S V D Y A V M

1090    1100    1110    1120    1130    1140    1150    1160    1170    1180    1190    1200
GGAGAAAACCCGCGACGCGGTGGTGGTGGTCTCGATGCCGACTGGAGCGAGCTCGGCTCTGGTCCGCCCTGTGGGAGGTACAGCCGAAAGACGGGACGGGCAACGTCCTCAGCGCGCA
E K T A D A V V V G L D A D W S D V G S W S A L W E V S P K D G Q G N V L S G D
Tn1000-62

1210    1220    1230    1240    1250    1260    1270    1280    1290    1300    1310    1320
CGCGTGGGTGCACAAACGCGAAAACGTACATCAACAGCGACGAGAAGCTGGTGGCGGCCATTGGCGTGAGAACCTGGTGATTGTGAGCACCAGGATGCCGTGCTGGTGATGAACCG
A W V H N S E N C Y I N S D E K L V A A I G V E N L V I V S T K D A V L V M N R

1330    1340    1350    1360    1370    1380    1390    1400    1410    1420    1430    1440
TGAGCGTTCACGAGCGTGAAGAAGCGGTGAGTTCCTCAAGCAGAACCAGCGAGCGAGTACAAGCGCCACCGCGAGATTTACCGTCCCTGGGGCCGCTCGGACGTGGTGGTCCAGAC
E R S Q D V K K A V E F L K O N Q R S E Y K R H R E I Y R P W G R C D V V V Q T

1450    1460    1470    1480    1490    1500    1510    1520    1530    1540    1550    1560
CCCGCGCTTCAACGTCAACCGCATCACCGTGAACCGGGCGGCCCTTCTCGATGCAGATGCACCACCACCGCGCGAGCACTGGGTCAATTCGCGCGCACCGGCCAGGTGACGGTCAA
P R F N V N R I T V K P G G A F S M Q M H H R A E H W V I L A G T G Q V T V N

1570    1580    1590    1600    1610    1620    1630    1640    1650    1660    1670    1680
CGGCAAGCAGTTCTGCTGACCGAGAACCAGTCCACCTTTATTCGATTGGCGCCGAGCACAGCTGGAAAACCCGGGCGCATTCCGCTGGAAGTGCTGGAGATCCAGTCGGGGTCGTA
G K Q F L L T E N Q S T F I P I G A E H S L E N P G R I P L E V L E I Q S G S Y
Sma I

1690    1700    1710    1720    1730    1740    1750    1760    1770    1780    1790    1800
CCTCGCGGAGGACGACATATTGTTATTAAGACCAATATGGTTCGTTGCTAATTTTTCGGGACAAAACGCAGAAATGACACAGTTAATCATGCTTTAAGGCTTATGACATCCGTGGTGAAC
-35 ? -10 ? SD
L G E D D I I R I K D Q Y G R C * M T Q L T C F K A Y D I R G E L
start rfbK

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Fig. 5. For legend see facing page.


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1810 1820 1830 1840 1850 1860 1870 1880 1890 1900 1910 1920
TGGGCGAGGAGCTGAACGAGGACATCGCCTACCGTATCGGCCGCGCCTATGGCGAATTTCTGAAACCCGGGAAGATAGTGGTGGGGGGCGATGTGCGCCTCACCAGCGAGTCGCTGAAGC
G E E L N E D I A Y R I G R A Y G E F L K P G K I V V G G D V R L T S E S L K L
                                     Sma I
1930 1940 1950 1960 1970 1980 1990 2000 2010 2020 2030 2040
TGGCGCTGGCCCGCGGCTGATGGACGCCGCGCACCAGCTGCTGGATATTGGCCTGAGCGGCACGGAAGAGATTACTTCGCCACTTTCACCTCGGGGTGGACGGCGGCATCGAGGTGA
A L A R G L M D A G T D V L D I G L S G T E E I Y F A T F H L G V D G G I E V T

2050 2060 2070 2080 2090 2100 2110 2120 2130 2140 2150 2160
CGGCCAGCCACAACCCGATGAACATAACGGCATGAAGCTGGTGGCGGAGAACCGCAAGCCATCAGCGGGACACCGGCTGCGGGATATCCAGCGCCTGGCGGAGGAGAAATCAGTTCC
A S H N P M N Y N G M K L V R E N A K P I S G D T G L R D I Q R L A E E N Q F P

2170 2180 2190 2200 2210 2220 2230 2240 2250 2260 2270 2280
CGCCGGTGGACCGCGCGCTGCGGGACCTCGCCAGATTTCGGTGTGAAGAGTACGTTGACCACTGATGGGTATGTGGACCTGGCGAACTTACCCTCGCTCGCTGAAGCTGGTGG
P V D P A R R G T L R Q I S V L K E Y V D H L M G Y V D L A N F T R P L K L V V

2290 2300 2310 2320 2330 2340 2350 2360 2370 2380 2390 2400
TGAATCCGGCAACGGGCGCGGGGACGTCGATTGATGAGGTGGAGAACGCTTCGCGCGCGCGGGGCGCGGTGACCTTTATCAAGGTGCATCACCAGCGCGGACGGCCATTTCCGA
N S G N G A A G H V I D E V E K R F A A A G A P V T F I K V H H Q P D G H F P N

2410 2420 2430 2440 2450 2460 2470 2480 2490 2500 2510 2520
ACGGTATCCGGAACCGCTGCTGCCGAGTGCCGCCAGGACACCGCGGACGCGGTGCGTGACATCAGCGGGACATGGGTATCGCCTTTGACGGCGACTTCGACCGCTGCTTCCTGTTG
G I P N P L L P E C R O D T A D A V R A H Q A D M G I A F D G D F D R C F L F D
                                     Tn1000-195
2530 2540 2550 2560 2570 2580 2590 2600 2610 2620 2630 2640
ATGACGAGGCGTCGTTTATCGAGGGTACTACATTGTGCGCCTGCTGGCGGAGCGTTCCTGAGAAAGCAGCGGGGGCGAAATCATTACGACCGCGCTGACGTGGAACACGGTGG
D E A S F I E G Y Y I V G L L A E A F L Q K O P G A K I I H D P R L T W N T V D
                                     Pst I
2650 2660 2670 2680 2690 2700 2710 2720 2730 2740 2750 2760
ACATCGTGACCCGACGCGCGCGGACCGGTGATGTGCAAGACCGGCATCGTTTCATCAAGGAGCGGATGCGCCAGGAAGACGCTATCTACGGCGGGAGATGAGTGCGCACCATTA
I V T R S G G Q P V M S K T G H A F I K E R M R O E D A I Y G G E M S A H H Y F
                                     Sph I
2770 2780 2790 2800 2810 2820 2830 2840 2850 2860 2870 2880
TCCGCGACTTCGCTACTGCGACAGCGGGATGATCCCGTGGCTGCTGGTGGCGGAGCTGCTGCTGAAAGAACAGTTGCTGAAATCGCTGGTGGCGGACCGCCAGGCGCGTTCGCGG
R D F A Y C D S G M I P W L L V A E L L C L K N S S L K S L V A D R Q A A F P A

2890 2900 2910 2920 2930 2940 2950 2960 2970 2980 2990 3000
CGTCGGGGAGATCAACCGAAGCTGGGAAATGCGCGGAGCGGATAGCGCGCATCCGGGCGCAGTATGAGCGCGGCGCGCACACATCGACACAACGACGGATCAGTATTGAATACC
S G E I N R K L G N A A E A I A R I R A Q Y E P A A A H I D T T D G I S I E Y P

3010 3020 3030 3040 3050 3060 3070 3080 3090 3100 3110 3120
CTGAGTGCGCTTTAACCTGCGCACGTCCAACACGGAGCCGGTGGTGGCTGTAACGTTGAGTCCAGAGCGGATAGTGGTTAATGAATGAGAAAACCGCGAGCTGCTCAACCTGTTAA
E W R F N L R T S N T E P V V R L N V E S R A D T A L M N E K T A E L L N L L K

3130
AAGAGGAATCGCTTTGA
E E S L *

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Fig. 5. DNA sequence and the deduced amino acid sequences of *rfbK* and *rfbM* of *E. coli* O9. Possible promoter regions are boxed and the potential Shine–Dalgarno sequences are double underlined.

tative results are shown in Fig. 4(b, c) and all results are summarized in Table 2. From these results *E. coli* O9 *rfb* seems to be composed of two regions: a region common to the four *rfb* clusters (probes 1–3 and 8–9) and a region specific to *E. coli* O9 and *Klebsiella* O3 which have identical O polysaccharides (probes 4–7). The strain-specific region, which seems to determine the polysaccharide structures, is flanked by two common regions, which may include genes encoding either mannose

pathway enzymes or transferases common to the synthesis of these four O polysaccharides.

Complementation of *S. typhimurium rfb*

Well-defined *rfb* mutants of *S. typhimurium* are available which are deleted in either *rfbK* or in both *rfbK* and *rfbM* (Nikaido *et al.*, 1967) and these strains were used in a complementation study with pNKB26 and its derivatives

Table 4. Specific activity of enzymes in the mannose pathway

Strain	Plasmid	Specific activity (mU mg ⁻¹)	
		PMM	GMP
HU1190	—	37.6	0.9
HU1190	pBHC1-Sc6	175.3	13.7
HU1190	pBHC1- <i>rfbK</i>	317.1	0.0
HU1190	pBHC1- <i>rfbM</i>	32.5	23.9

(Table 3). The results indicated that the defects in the genes for polypeptides of 50 and 55 kDa were complemented by *rfbK* and *rfbM* of *S. typhimurium*, respectively. Thus, the genes for polypeptides of 50 and 55 kDa correspond to *rfbK* and *rfbM* of *S. typhimurium* and hereafter we use *rfbK*_{EO9} and *rfbM*_{EO9} for genes of 50 and 55 kDa polypeptides of *E. coli* O9.

DNA sequences of *rfbK*_{EO9} and *rfbM*_{EO9}

The DNA sequence of a 3.1 kb fragment including *rfbK*_{EO9} and *rfbM*_{EO9}, and the predicted amino acid sequences are shown in Fig. 5. Two open reading frames for *rfbK*_{EO9} and *rfbM*_{EO9} were found encoding proteins

of 460 amino acids (50809 Da) and 471 amino acids (52789 Da), respectively. These were in agreement with the minicell analysis. *rfbK*_{EO9} was located between sequence positions 1755 and 3137, and *rfbM*_{EO9} between positions 317 and 1732. The Shine–Dalgarno sequences were observed 11 bp in front of the ATG initiation codon of *rfbK*_{EO9} and 7 bp in front of the initiation codon of *rfbM*_{EO9} (Fig. 5). Sequences homologous to the consensus sequence of *E. coli* σ^{70} -dependent promoters were found 31 bp upstream of *rfbK*_{EO9} and 79 bp upstream of *rfbM*_{EO9}. No inverted repeat sequence was observed downstream of the translational stop codon of *rfbK*_{EO9} and *rfbM*_{EO9}. The G + C content of both genes is 0.62, a value higher than that of *E. coli* (0.5). Insertion positions of pNKB26::Tn1000-62 and -195 are also shown in Fig. 5.

Evidence for an *in vivo* activity of promoters for *rfbK* and *rfbM* was found with plasmid pBHC4 (Fig. 1) and its deletion derivative pBHC4-d10A carrying only *rfbK*. pBHC1-Sc6 (Fig. 3) was used for the detection of enzyme activities of PMM and GMP and found to direct the synthesis of both enzymes *in vivo* (see below). Plasmids pBHC4 and pBHC4-d10A carry their DNA fragment in the opposite direction to pBHC1-Sc6. Both strains, HU1190(pBHC4)(pXX195) and HU1190(pBHC4-d10A)(pNKB26::Tn1000-195), synthesized the O9-specific LPS. This indicated that *rfbK* and *rfbM* are expressed in *E. coli* independently of the *lacZ* promoter. These data suggested that promoters for the expression of the *rfbK* and *rfbM* genes are contained in the fragment.

Table 5. Comparison of amino acid identities of deduced sequences from PMM and GMP genes of various bacteria

By using the ODEN program of the National Institute for Genetics, Mishima, Japan, we calculated amino acid identities between PMM and GMP. Numbers show the percentage identities among the deduced amino acid sequences of PMM and GMP.

PMM							
	RfbK _{EO7}	RfbK _B	RfbK _{C1}	RfbK _{C2}	CpsG	AlgC	XanA
RfbK _{EO9}	73	15	75	15	75	30	57
RfbK _{EO7}		14	84	13	83	29	55
RfbK _B			16	67	17	19	17
RfbK _{C1}				14	96	29	60
RfbK _{C2}					15	17	17
CpsG						29	60
AlgC							31
GMP							
	RfbM _{EO7}	RfbM _B	RfbM _{C1}	RfbM _{C2}	CpsB	AlgA	XanB
RfbM _{EO9}	49	58	59	57	61	52	57
RfbM _{EO7}		47	53	50	52	42	46
RfbM _B			57	77	58	49	54
RfbM _{C1}				57	70	54	58
RfbM _{C2}					58	50	55
CpsB						54	61
AlgA							59

Determination of enzymic activities

To investigate the specific enzyme activities of PMM and GMP directly, we subcloned a *SacI*–*HindIII* fragment of pNKB26 including both *rfbK*_{EO9} and *rfbM*_{EO9} into pBluescript II SK(+) and termed it pBHC1-Sc6 (Fig. 3). Plasmids pBHC1-*rfbK* and pBHC1-*rfbM*, encoding single proteins, were constructed from pBHC1-Sc6 by deleting fragments. For the construction of pBHC1-*rfbK*, the region including the promoter and about one-third of *rfbM*_{EO9} was removed. To construct pBHC1-*rfbM*, the region including about one-third of the C terminus of *rfbK*_{EO9} was removed (Fig. 3). Using these plasmids, we measured PMM and GMP activities. As shown in Table 4, pBHC1-Sc6 and pBHC1-*rfbK* exhibited PMM activity, whereas pBHC1-*rfbM* did not. pBHC1-Sc6 and pBHC1-*rfbM* showed GMP activity. The results confirmed that the 50 kDa polypeptide is PMM and the 55 kDa polypeptide is GMP.

Comparison of PMM and GMP of bacterial GDP-mannose pathways

We compared the nucleotide and deduced amino acid sequences of PMM and GMP genes in *rfb* clusters of *E. coli* O9 (this study) and O7 (Marolda & Valvano, 1993), and *Salmonella* strains of group B (Jiang *et al.*, 1991), C1 (Lee *et al.*, 1992) and C2 (Brown *et al.*, 1992), in the *cps* cluster of *S. typhimurium* directing the synthesis of the mucoid (M) antigen (Stevenson *et al.*, 1991), in the *xan* cluster of the xanthan synthetic pathway of *Xanthomonas campestris* (Köplin *et al.*, 1992) and in the *alg* cluster of the alginate synthetic pathway of *Pseudomonas aeruginosa* (Shinabager *et al.*, 1991; Zielinski *et al.*, 1991). The following results were obtained.

(i) Comparison of amino acid identities of PMM and GMP. Table 5 shows the sequence identities between the enzymes. There was pronounced identity between PMM sequences of RfbK_{EO9}, RfbK_{EO7}, RfbK_{C1} and CpsG. XanA showed relatively high sequence identities with RfbK_{EO9}, RfbK_{EO7}, RfbK_{C1} and CpsG. Identities between AlgC and the other PMM sequences were low. As reported previously (Lee *et al.*, 1992), RfbK_{C1} and CpsG were almost identical and RfbK_B and RfbK_{C2} were similar. However, the degree of sequence identity between GMPs of these bacteria was comparable. No strong divergence was found in GMP sequences.

(ii) Phylogenetic trees of deduced amino acid sequences from the PMM and GMP genes. The phylogenetic trees of PMM and GMP genes were constructed to show their evolutionary relationships. The trees derived from PMM and GMP genes are shown in Fig. 6. The two phylogenetic trees differed in the evolutionary distances from ancestors. Moreover, they differed from each other in topology. In the case of GMP genes, an ancestor of RfbM_{EO9}, RfbM_{EO7}, RfbM_{C1} and CpsB diverged from that of RfbM_B, RfbM_{C2}, XanB and AlgA first and then diverged into each gene, whereas in the case of the PMM gene, AlgC, XanA, RfbK_{EO9}, RfbK_{EO7}, RfbK_{C1} and CpsG are considered to be

Fig. 6. Phylogenetic trees of deduced amino acid sequences from PMM and GMP genes. Branch length is proportional to evolutionary distance.

monophyletic, although their divergences are ancient. Differences in the evolutionary distance from ancestors and in the topology of phylogenetic trees suggest that genes for RfbK and RfbM have evolved independently, though they are both required for the synthesis of GDP-mannose in bacteria.

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