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A New Locus for Exopolysaccharide Overproduction in *Escherichia coli* K-12

Karen Zinkewich-Péotti

A Thesis

in

The Department

of

Chemistry

Presented in Partial Fulfillment of the Requirements
for the Degree of Master of Science at
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ABSTRACT

A New Locus for Exopolysaccharide Overproduction in *Escherichia coli* K-12

Karen Zinkewich-Peotti

A new locus for exopolysaccharide overproduction in *Escherichia coli* K-12 was mapped using insertion mutagenesis. Sixty-two percent linkage to *serA*, which is located at 62 minutes on the linkage map, was shown by P1 transduction. The polysaccharide produced by the mutant was purified and its chemical structure shown to be similar to colanic acid. While mutations at various map positions cause overproduction of colanic acid by *Escherichia coli* K-12, none of the previously recognized mutations map near this new locus, nor are their phenotypes with respect to growth media and incubation temperature identical with that of the insertion mutant. The insertion site maps near the *kps* locus responsible for K antigen synthesis by some *Escherichia coli* strains. Although *E. coli* K-12 is not known to elaborate a K antigen, the site of insertion might suggest that a K-type antigen is being synthesized.

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INTRODUCTION

This study is concerned with a new locus for the overproduction of an exopolysaccharide by *Escherichia coli* K-12. This exopolysaccharide is physically similar to colanic acid (M antigen) but the gene affecting its overproduction maps near a gene cluster for K-antigen biosynthesis. Since studies on polysaccharide formation in *E. coli* K-12 have focused mainly on the overproduction of M-antigen, the structure and biosynthesis of colanic acid will be reviewed, and the genes implicated in control of colanic acid synthesis [*capR* (*lon*), *capS*, *capT*, *rcsA*, *rcsB* and *rcsC*] discussed. The influence of growth conditions, the effect of an amino acid analogue, p-fluorophenylalanine and the correlation between antigen formation and the presence of certain outer membrane proteins will be examined.

E. coli K-12 is not known to make K-antigens; however, the results of our genetic studies suggest that the overproduced exopolysaccharide may be a K-type antigen, so the chemistry and genetics of K-antigens will be described.

Insertion mutagenesis was used to obtain the mutants studied in this project; therefore, this introduction will conclude with a discussion of the Mu-d(Ap *lac*) insertion element.

E. coli K-12 is surrounded by a complex structure composed of an outer membrane, a cell wall, and an inner

membrane enclosing the cytoplasm. The inner membrane is the site of lipid biosynthesis, active transport and ATPase activity. Concentric to this is the peptidoglycan cell wall. The outer membrane structure is composed of major and minor proteins, lipopolysaccharides and lipids. Few enzymatic functions have been associated with the outer membrane (17), though its receptors have been implicated in interactions with other cells, phages, and macromolecules.

The exopolysaccharides of E. coli are attached to the outer membrane in an unknown manner. They may be tightly associated with the cell surface forming a capsule as in the case of the K-antigens; or they may be more loosely joined to the outer membrane, giving rise to a slime layer. Capsular polysaccharides are not essential for cell growth, but they do strongly influence the manner in which bacterial cells interact with their environment. This interaction can occur at various levels such as cell-host, cell-virus and cell-cell (52).

Exopolysaccharides from Enterobacter aerogenes, Streptococcus pneumoniae and E. coli have been well studied. Analysis of these polymers has uncovered a wide variety of chemical components and structures. This small group of bacteria are able to produce a large number of different polysaccharides, most of which consist of repeating sugar units and are termed heteropolysaccharides (50). The types of complex carbohydrates which E. coli can synthesize include lipopolysaccharides, O-antigens, K-antigens, and M-

antigen. The way in which the cell regulates the production of such complex carbohydrates has only partially been elucidated and the problem is an exciting one.

I Colanic acid

The structure of colanic acid is shown in Figure 1. This polysaccharide was first isolated in 1927 from a mucoid strain of *E. coli* by Dorothea Smith (36). Kauffman subsequently designated a slime substance from *Salmonella paratyphi B* " M antigen" (36). This same polysaccharide was named "colanic acid" by Goebel when he isolated it from *E. coli* K235 in 1963 (14). It was later shown that serologically and chemically identical products were elaborated by many *E. coli* strains, *Enterobacter cloacae*, and *Salmonella paratyphi B* and other *Salmonella* strains (36).

The repeating unit of colanic acid contains six monosaccharides: D-glucose, D-galactose, L-fucose and D-glucuronic acid, in a ratio of 1:2:2:1. In addition, there are acetyl and pyruvyl groups. Sutherland determined that acetyl groups are primarily attached to glucose whereas pyruvyl groups are located on galactose (48). Garegg et al.(11) further demonstrated that alkyl groups may be linked at various positions on the terminal D-galactose residue of the repeating hexasaccharide unit.

The biosynthetic pathway for colanic acid is shown in Figure 2 . Thirteen enzymes are involved in the formation of the four monosaccharides. The polymerase(s) responsible

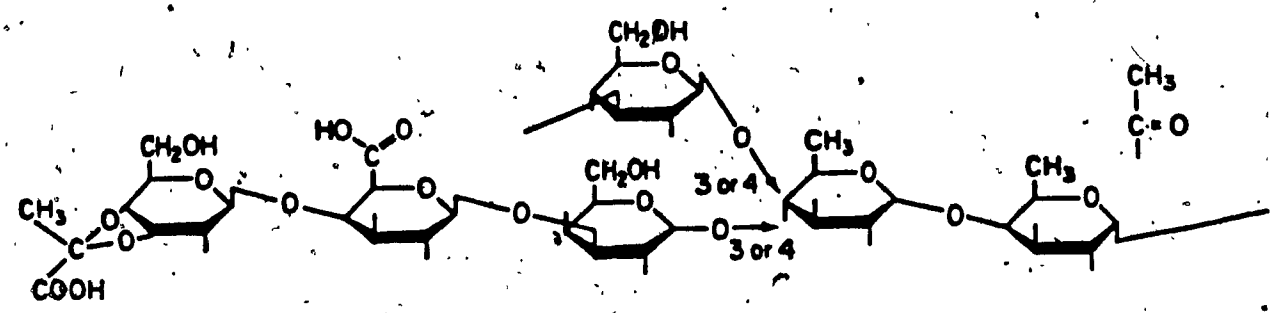


Figure 1 The proposed structure of colanic acid from E. coli K-12 after Lawson et al., Garegg et al., and Markovitz (36).

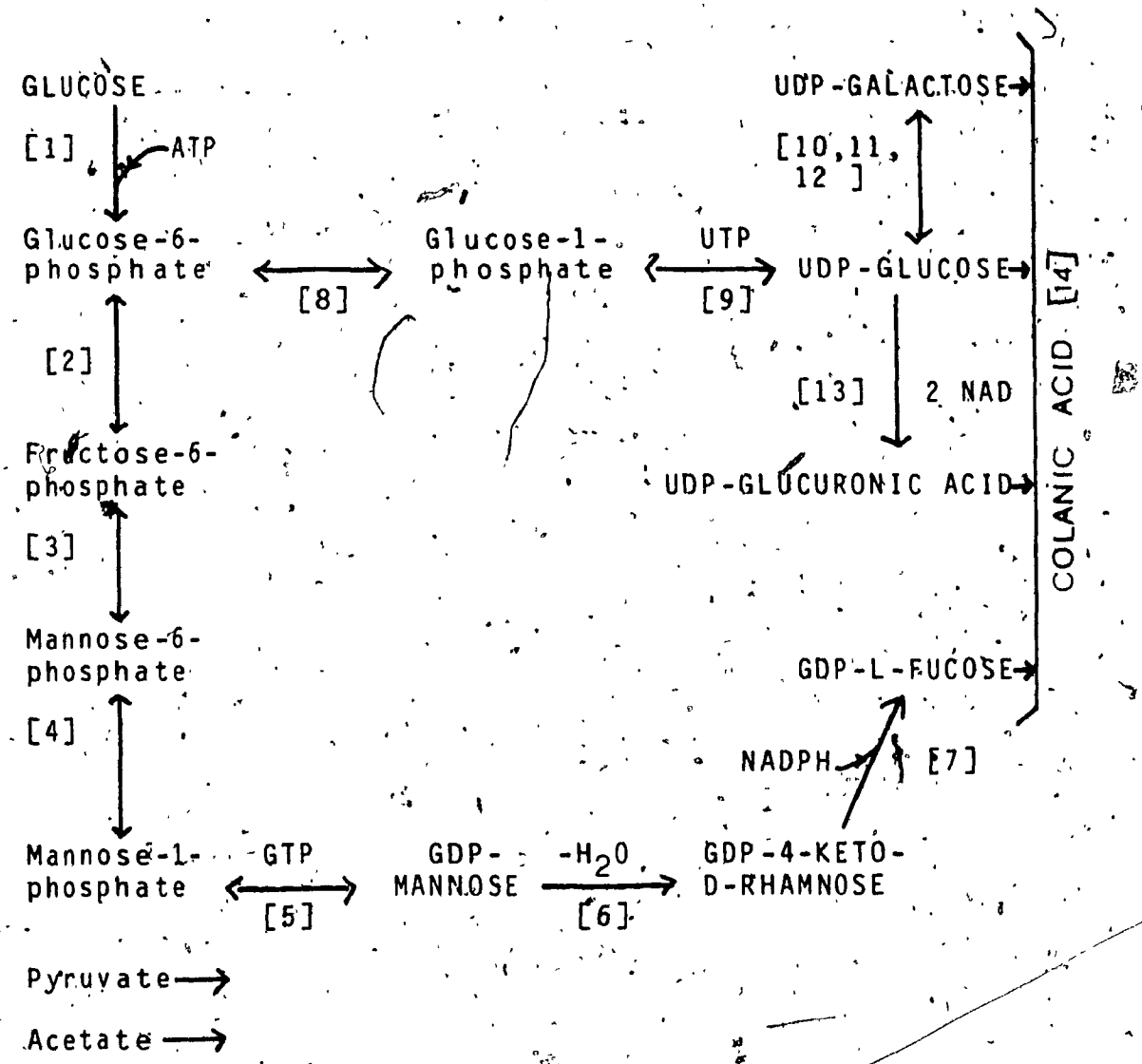


Figure 2: Postulated pathway for the biosynthesis of colanic acid by E. coli K-12. After Markovitz et al. (36).

ENZYMES:

[1] hexokinase; [2] phosphoglucose isomerase;

[3] phosphomannose isomerase; [4] phosphomannose mutase;

[5] GDP-D-mannose pyrophosphorylase;

[6] GDP-D-mannose hydrolyase; [7] GDP-L-fucose synthetase;

[8] phosphoglucomutase; [9] UDP-D-glucose pyrophosphorylase;

[10] UDP-D-galactose-4-epimerase;

[11] UTP-D-galactose-1-phosphate uridyl transferase;

[12] galactokinase; [13] UDP-D-glucose dehydrogenase;

[14] Colanic acid polymerase(s).

for the assembly of these building blocks have not been identified.

Wild type E. coli K-12 produces almost no colanic acid, whereas the mucoid mutants overproduce it. The structural genes for colanic acid biosynthesis are located in at least five separate positions on the chromosome (51). The enzymes of the colanic acid pathway are not all derepressed in mucoid mutants. Indeed as shown in Table 1, the pattern of derepression varies with the mutation.

There is some disagreement about the levels of the gal enzymes in the lon mutant. The genes making up the gal operon, galE, galT and galK (coding for UDP-D-galactose-4-epimerase, UTP-D-galactose-1-phosphate uridyl transferase and galactokinase respectively), are under the regulation of the galR gene. According to Markovitz (36) the gal operon enzymes are all derepressed in capR (lon) and capT mutants but not in the capS mutants. Moreover, Mackie and Wilson (33) found elevated levels of gal mRNA in capR6 strains, and suggested that the lon mutation causes derepression of gal enzymes at the level of transcription. On the other hand, Gottesman (51), using lac operon fusions to the genes necessary for colanic acid synthesis, found that the gal operon was not derepressed in the capR background. In any case, if there is derepression, it is not a phenomenon mediated through galR since Hua and Markovitz (19) found these enzymes to be derepressed in the capR strain even when the strain carried a deletion in the

Table 1. Levels of colanic acid biosynthetic enzymes in various mucoid mutants of *E. coli* K-12.

Enzyme	Wild type	capR6	capR9	capS	capT
Phosphomannose isomerase	1	4.7	5.0	1	NA
Phosphomanno-mutase	1	NA	2.2	5.3	5.0
GDP-D-mannose pyrophosphorylase	1	5.5	22	11	4
GDP-D-mannose hydrolyase	1	+	5.0*	5.5	NA
GDP-L-fucose synthetase	1	+	4 *	9	NA
Phosphoglucose isomerase	1	1	1	NA	NA
Phosphogluco-mutase	1	NA	1.8	1.8	1.2
UDP-D-glucose pyrophosphorylase	1	7/2*	12/8*	3/7*	3/7*
UDP-D-galactose-4-epimerase	1	2.5	4.3	1.3	2.3
UTP-D-galactose-1-uridyl transferase	1	NA	6.2	1.6	6.4
Galactokinase	1	NA	6.8	1	4.2
UDP-D-glucose	1	NA	17	9	9

The specific activities of enzymes in the wild type strain are assigned a value of 1. Unless otherwise indicated, cells were grown at 23-25° C.

NA = not available * bacteria grown at 35-37° C.

+ = higher than in the wild type. Data from Markovitz (36).

galR cistron.

Mutations in the structural genes of any of the following enzymes are known to render mucoid strains non-mucoid: phosphomannose isomerase (man), UDP-D-glucose pyrophosphorylase (galU), UDP-D-galactose-4-epimerase (galE), UDP-D-glucosedehydrogenase and GDP-D-mannose pyrophosphorylase (36).

II Sugar nucleotides and lipid intermediates

Nucleotide diphosphate monosaccharides are strongly implicated as intermediates in the formation of both lipopolysaccharides and colanic acid in E. coli. These molecules are the precursors of the repeating hexasaccharide units. Some nucleotide diphosphate monosaccharides (e.g. UDP-fucose), are uniquely precursors of exopolysaccharides, while others (e.g. UDP-galactose) are destined for cell wall building as well (50).

That sugar nucleotides are intermediates in colanic acid synthesis was suggested by Lieberman et al. (30), who noted that UDP-D-glucose dehydrogenase (UDPG dehydrogenase) is derepressed in the mucoid capR9 strain and repressed in the non-mucoid wild type. A non-mucoid mutant of capR9, in which UDPG dehydrogenase was still derepressed, accumulated large quantities of nucleotides. Those identified were UDP-glucuronic acid, guanosine triphosphate and adenosine diphosphate sugars. Since UDPG dehydrogenase was derepressed but colanic acid synthesis was blocked at some other site,

the sugar nucleotide intermediates accumulated (30).

The specific activity of UDP-glucose pyrophosphorylase in the wild type strain is much higher than those of the enzymes involved in the synthesis of GDP-L-fucose. This may be accounted for by the requirement for UDPG pyrophosphate for both cell wall and capsule biosynthesis, while the L-fucose is required only for capsular polysaccharide synthesis (29).

A lipid intermediate of the kind found in lipopolysaccharide biosynthesis has not been convincingly demonstrated in colanic acid synthesis. Johnson and Wilson (26) identified an isoprenylpyrophosphate sugar which they believed to be part of the colanic acid pathway, and tentatively identified a second lipid carrier. They suggested that such a complex carbohydrate structure as colanic acid might require more than one lipid linking intermediate.

III The lon mutation

Mutation in the capR gene, also known as lon is the best characterized of the mutations responsible for colanic acid overproduction. It maps at 10 minutes and is linked to proC as determined by P1 transduction (35). Mutations at capR are exceedingly pleiotropic: besides exopolysaccharide overproduction, they render the bacterium hypersensitive to UV light and ionizing radiation, increase its resistance to some antibiotics and to λ and P1 phage infections, and

cause filament formation as well as defective cell division. The capR mutants also show reduced degradation of nonsense and missense as well as normal proteins (6).

The lon gene product has been cloned and identified as an ATP-dependent protease, MW 94,000, with a particular affinity for nucleic acids (6). The activity of this protease is temperature dependent, such that it degrades proteins more rapidly at high temperatures (42°C) than at low ones (32°C) (16).

To determine the relationship between various phenotypic traits of the lon mutants, lon mutants displaying suppression of select phenotypic traits were studied. It was shown that the overproduction of colanic acid is not a determinant for expression of other traits. Mutation in sulA, a cell division inhibitor, eliminates the UV sensitivity and filament formation of lon mutants, but does not preclude mucoidy. Likewise, mutation in galE, (UDP-D-galactose-4-epimerase) necessary for colanic acid biosynthesis, renders the bacterium non-mucoid but does not affect the rest of the phenotype (16).

The current model for defective cell division in lon mutants proposes that the sulA gene product has a longer half-life in the mutant (19 minutes) than in the wild type (1.2 minutes) (51). Following DNA damage, the recA protein is activated and cleaves the lexA protein. Expression of genes controlled by lexA is increased, among them sulA. The sulA gene product is thought to temporarily inhibit

cell division in wild type E. coli; in the lon mutant, however, the sulA gene product is degraded much more slowly, and its inhibitory effect leads to cell death (45). (A similar model for the overproduction of colanic acid will be proposed later).

IV Mutation at capS and capT

Mutations at capS and capT are less well defined than is capR. The capS mutation maps near trp at 22.5 minutes (36). The map position of capT has not been determined. It has been shown that it is not linked to trp contrary to caps, nor is it linked to proC contrary to lon(36). The capT phenotype might actually be the result of two mutations (A. Markovitz, personal communication). Insertion of the capR+ gene on a plasmid, does not alter the mucoid phenotype of capS mutants (3), confirming that these two mutations are distinct.

While capT and capR cause derepression of the gal operon genes, capS has no such effect (18). A double mutant, capRcapT shows no more derepression of these enzymes than do the single mutants, so it is probable that their effect is mediated via a common pathway.

Neither capS nor capT display the UV hypersensitivity of lon. The behaviour of these three mucoid mutants vis-à-vis media and growth temperature is varied (Table 2).

Table 2. Effect of growth media and temperature on colanic acid mutants

	MMNG 30°C	MMNG 37°C	Complex media 30°C	Complex media 37°C
capR	mucoid	mucoid	mucoid	non-mucoid
capS	mucoid	mucoid	mucoid	non-mucoid
capT	mucoid	mucoid	mucoid	mucoid

From Markovitz (36).

V Capsular polysaccharide regulatory genes: rcsA,B and C

Using the Mu d(Ap lac) insertion technique of Casadaban and Cohen (see section XI of this Introduction), Gottesman et al. (51) prepared lac operon and lac protein fusions to genes necessary for capsule synthesis. Non-mucoid insertion mutants were obtained from lon mutant strains. A non-mucoid phenotype indicated that insertion had occurred in a gene required for colanic acid synthesis. Five genes were found to share a common pattern of regulation with respect to lon. Low levels of β-galactosidase were found in lon strains, while 4-to-80 fold higher levels were present in strains carrying the lon mutation. The expression of β-galactosidase is under the control of the promoter of the gene into which Mu d(Ap lac) has inserted. Fluctuations in β-galactosidase activity reflect regulation of the promoter.

The five lon regulated genes are located in a cluster close to the udk gene at 45 minutes on the E.coli K-12 map. They were named cpsA,B,C,D and E (51). A sixth gene, cpsF at 90 minutes on the E. coli K-12 linkage map, was regulated in the same fashion but is not required for colanic acid synthesis. Contrary to a report by Markovitz (18), the gal and manA operons are not regulated by the lon system, at least under the conditions used by Gottesman et al. (51).

A mutation termed " non " (non-mucoid), had previously been located in the udk region. Non shows 31% linkage to h+s. The O antigen cluster in Salmonella typhimurium maps at

this position. This locus, termed rfb, codes for at least eight enzymes, including two which are found in the colanic acid pathway: GDP-mannose pyrophosphorylase and phosphomannose mutase. While E. coli K-12 does not produce O antigen, three rfb genes have been mapped and their location corresponds to that of the non mutation (51).

On the basis of what is known about the physiological activity of the lon protease, it is suggested that the overproduction of colanic acid in lon mutants could be due to retarded degradation of a positive regulator of exopolysaccharide biosynthesis. According to this hypothesis, this positive regulator in the wild-type strain would be degraded rapidly so only a small amount of M-antigen would be synthesized. In the lon mutant, the positive regulator of capsule formation (like the sulA protein), would have a greatly increased half life, leading to overproduction of colanic acid.

The same lac operon fusions engineered by Gottesman and coworkers for their lon mutant studies were used to search for a positive regulator which might mediate the effects of lon on the colanic acid system. In a lon background, β-galactosidase levels of these fusion strains are very low; consequently growth on lactose is slow. Spontaneous and nitrosoguanidine induced mutants with elevated levels of β-galactosidase were selected on plates containing lactose as sole carbon source. Introduction of the wild-type cps region induced mucoidy in a number of these lac mutants.

Lon mutants were identified by their sensitivity to methyl methanesulphenate (MMS). Mutants which were both MMS-resistant and mucoid were presumed to have mutations in regulators of capsular polysaccharide synthesis, whereas mutations rendering lac$^+$lon$^+$ mucoid strains non-mucoid, indicated the presence of a negative regulator.

These isolation procedures revealed three new regulatory genes: rcsA, rcsB and rcsC (15) which regulate genes cpsA to F as does lon. RcsA and rcsB are positive regulators, located at 43 and 47 minutes respectively, on the E. coli K-12 linkage map; rcsC maps at 47 minutes and is a negative regulator. None of these three regulatory genes are linked to the structural genes (cpsA-F) or to lon. Mutations in rcsA-C genes are recessive to the wild type. As with lon, the rcs regulators have no effect on the gal operon; only the mucoid trait of the lon phenotype is affected by these genes suggesting that lon regulates these genes and not vice versa (15).

Further proof that rcsA is a positive regulator was obtained when it was cloned into a multicopy plasmid. In agreement with the proposed model, the multicopy rcsA containing plasmid caused mucoidy in lon+ cells (Torres, Cabassa and Gottesman in preparation, as reported in (15)).

VI Outer membrane proteins

A plasmid carrying genes for the major outer membrane

proteins M2 (also known as "a" or "3b") M5, and M7 repressed colanic acid synthesis in a lon strain. The other pleiotropic effects associated with the lon mutation were not affected. It is thought that protein M2 negatively correlates with polysaccharide production. Indeed, at low temperatures, when polysaccharide formation is generally more abundant, less protein M2 is produced (12). The mechanism by which protein M2 turns off capsular polysaccharide synthesis is unknown.

Another outer membrane protein, found by Paakkanen et al. (42) is present only in E. coli strains which are encapsulated. A function for this protein has yet to be demonstrated.

VII Growth conditions and limiting factors

As mentioned previously, some mutants of E. coli K-12 show the mucoidy phenotype on minimal media plates, but not on complex media. In addition, incubation temperature has a strong influence on mucoid production. Generally, E.coli cells are less mucoid at 37° C than at 30° C. Markovitz has reported that wild type E.coli K-12 produces excess polysaccharide when grown at 15° C and that this polysaccharide is identical to colanic acid (35).

Polysaccharide production by E. coli K-12 increases as the carbohydrate:nitrogen ratio in the medium is increased. Production reaches a maximum beyond which further raising sugar:nitrogen ratio is without effect (53).

Limiting growth with respect to nitrogen, sulphur or phosphorous also stimulates exopolysaccharide production in both wild type strains and mucoid mutants (36). This has been explained (49) in terms of a role for a lipid intermediate in colanic acid production. This intermediate is postulated to be the lipid carrier involved in cell wall synthesis. According to this explanation, there is a competition between cell wall synthesis and exopolysaccharide synthesis for the limited number of lipid carriers. Under "normal" growth conditions, competition for lipid carrier favours cell wall biosynthesis at the expense of colanic acid biosynthesis. Under limiting conditions as well as at low incubation temperatures, however, growth is slower and the demand for lipid carrier for cell wall synthesis is reduced, thus liberating a greater proportion of the lipid intermediates for use in colanic acid production (49).

VIII Induction of mucoidy by p-fluorophenylalanine

An *E.coli* K-12 strain was induced to form mucoid colonies at 37°C by inclusion of 5×10^{-6} M p-fluorophenylalanine (Fphe) in plates, or at 30° C by incorporation of 8×10^{-5} M Fphe in liquid cultures. When a number of the enzymes of the colanic acid pathway were assayed, some were found to be derepressed. The specific activity of phosphomannose isomerase was 3.3 times higher than in cells grown in the absence of Fphe; UDPG-epimerase

was 2.5 fold higher and guanosine diphosphate-L-fucose synthetase was increased (27). There was a positive correlation between the amount of incorporation of [3- ^{14}C]Fphe and the induction of mucoidy. Kang and Markovitz proposed that incorporation of Fphe into a repressor protein altered its efficiency.(the lon gene product had not been identified at the time of this publication). It is equally plausible that the lon protease is deactivated by Fphe incorporation. Unlike the lon system, no derepression of GDP-D-mannose hydrolase, G-6-P dehydrogenase or phosphoglucose isomerase was observed. However, incorporation of Fphe into these enzymes, might have affected their activity.

IX The K-antigens

The K antigens are a group of acidic polysaccharides elaborated by strains of E.coli with a serological K-specificity. K -antigens tend to form a bacterial capsule and have been implicated in the virulence of some E. coli strains. The sugar sequence and the linkage between monosaccharides are recognized by the mammalian immune system and antibodies to a given K-antigen will react with the isolated polysaccharide as well as with the whole cell (22).

The K antigens, which have been immunologically classifed into some one hundred different types, have recently been subdivided into two groups. The first class

have a high molecular weight and low electrophoretic mobility, whereas the second display a low molecular weight and high electrophoretic mobility (23). The high-molecular-weight species are found as thick, copious capsules and consist mainly of glucuronic acids. The low-molecular-weight species usually contain N-acetylneuraminic acid and 2-keto-3-deoxy-D-manno-octanoic acid; they form thin, patchy capsules (24). Two very unusual K-antigens, K88 and K99 are proteins rather than polysaccharides (41); the genes responsible for their elaboration are plasmid-borne (47).

The K antigens are chemically distinct from colanic acid, though some immunological cross reactivity is occasionally observed. Thus, antibodies against K30 antigen of E.coli which is composed of glucuronic acid, galactose and mannose, cross react with M-antigen (20). Antibodies against antigen K29 of E.coli and antigens K8,11, 13,21, and 35 of Klebsiella likewise cross-react with the M-antigen (41). Antigen K27 of E.coli is composed of the same sugars as colanic acid i.e. D-glucuronic acid, D-glucose, D-galactose and L-fucose, but in different ratios (25).

K- and M-antigens can be separated according to Orskov and Orskov (40), by fractional precipitation with Cetavlon (hexadecyltrimethylammonium bromide) (see methods). This procedure has been used by Orskov and Orskov to separate the M- and the K-antigens being produced by the same strain. Depending on the growth conditions, the E.coli strain which they studied produced both K- and M-antigens or only K-

antigen (40).

X Genetics of the K antigens

Certain K-antigens are only associated with cells also bearing specific O antigens. These O antigens include types 08, 09 and 020. The genes controlling the biosynthesis of these particular K- antigens are linked to his and are near the rfb locus responsible for production of the O antigens. In these cases, there is linkage between the O- and K-antigen genes (41). E. coli K-12 is an rfb mutant with a complete lipopolysaccharide core but no O-antigen (Figure 3) nor has E.coli K-12 been shown to elaborate any K-antigen.

Genetic mapping was done on two E.coli strains carrying antigens K10 and K54 (39), by conjugating them with strains carrying other serotypes. It was determined that the genes for antigens K10 and K54 were linked to serA, and that the K-antigen genes of the recipient strain had been replaced by the K-antigen genes of the donor strain. The locus at which the K-antigen genes mapped, kpsA, could also be transferred and expressed in E.coli strains which had never in nature been found to produce K antigens (39). Linkage to serA was shown here as well.

An E. coli K10 ser+ mutant which did not produce a capsule could nevertheless transfer the genetic determinants for K10 antigen into a K54 serA strain, suggesting that there are two loci involved in capsule production. Seventy-

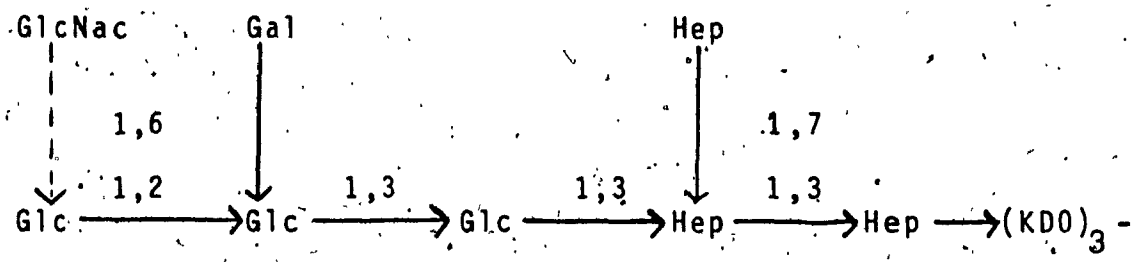


Figure 3. Structure of the core oligosaccharide of *E. coli* K-12, after Orskov et al. (41).

Glc = glucose, Gal = galactose

Hep = L-glycero-D-mannoheptose

GlcNac = N-acetylglucosamine

KDO = 2-keto-3-deoxyoctanoic acid.

two percent of the ser+ conjugants in this mating were also K10 (41). The K-antigen determinants were linked 80% to cys (39).

The genes required for K1 antigen synthesis were analysed by cloning and deletion techniques. The genes for K1 production map near serA; a minimum of twelve polypeptides were found to play a role in K1 biosynthesis. The antigen is an N-acetylneuraminic acid polymer termed colominic acid. Three coordinately regulated gene clusters were uncovered. Silver et al.(46) proposed that these clusters make up the kps regulon. Cluster A is the NANA operon and apparently regulates K1 synthesis. Cluster B codes for five proteins required for translocation of the polysaccharide from its site of synthesis to its position on the cell membrane. Cluster C codes for four necessary proteins of undetermined functions. Deletion mutations produced cells which could make the antigen but could not transport it outside the cell; in these mutants, antigen could only be detected following lysis of the bacteria by sonication or EDTA (46).

Despite the variety of K-antigens only two map locations have been found, one near his and the other near serA. Jann and Jann (23) propose that the genes for the high-molecular-weight antigens are linked to his and that the genes of the low-molecular-weight species are linked to serA, though genetical analysis of many more K-antigen types need to be done before this is shown to be the rule.

XI The Mu d(Ap lac) insertion

Insertion mutagenesis was the technique used in our laboratory to obtain mucoid mutants. Casadaban (5) developed a specialized transducing phage by incorporating the lactose structural genes without the lactose promoter into a derivative of Mu containing the gene for ampicillin resistance. This insertion integrates randomly into sites on the E.coli chromosome. The integration of one insertion does not preclude integration of a second.

The insertion is about 37 kb in length (38), equivalent to approximately 0.8 minutes of the E.coli chromosome.

Upon insertion into the bacterial chromosome, the lactose structural genes fall under the control of the promoter of the gene into which they have integrated. The insertion must be in the same orientation as the transcription of the gene in order for expression to occur. Fluctuations in the levels of β-galactosidase, the product of the lacZ gene, are easily assayed and reflect the activity of the promoter.

A number of considerations need be taken into account when interpreting the data obtained from β-galactosidase assays of Mu d(Ap lac) insertions. While regulation of transcription of the gene into which insertion has occurred should now be reflected in expression of the fused lactose genes, this is true only if the transcription control sites have not been damaged. Fusions to the same promoter may result in different levels of expression of β-galactosidase

activity. Some cells remain lac- and show very low β-galactosidase activities, following Mu d(Ap/lac) insertion; this may be due to the insertion being in an orientation different from that of the promoter, or the insertion having damaged the promoter (51).

Most transposable elements can exert strong polar effects on operons which are some distance from the site of insertion, as well as on adjacent genes (28).

METHODS AND MATERIALS

All enzymes and co-factors were obtained from Sigma and Boehringer Mannheim. Bacto-tryptone, Bacto yeast extract, Bacto casamino acids and Bacto-agar were from Difco. All other chemicals were reagent grade.

A. List of strains

Strain	Relevant genotype	Source
CU1008 Δlac	Δ(lacZ) ilvA	E.B. Newman
M6	thi-1 lon-10	B. Bachmann
ZP10	CU1008 Δlac / Mu d(Ap lac)	this work
MH420	lysA24	M. Herrington
6350	serA12	B. Bachmann
4294	Hfr thi-1	B. Bachmann
4242	Hfr thi-1	B. Bachmann
4316	Hfr thi-1	B. Bachmann
4312	Hfr metB1	B. Bachmann

B. Growth of bacteria

Bacterial strains with the exception of *E. coli* K-12 mutant ZP10, were maintained on Luria broth (LB)(37) plates. ZP10 was maintained on minimal medium (37) containing 0.2 % glucose, 2 mg% isoleucine, 2 mg% valine and 2.5 mg% ampicillin. Bacteria were sometimes cultivated on minimal medium containing 0.2 % glucose and 1% casamino acids. Plates were incubated at 30°C in a Model 6 Precision Scientific Incubator.

Sterilizations were done at 120° C and 20 psi. Antibiotics were filter-sterilized and added to cooled (45° C) media.

C. Preparation of phage lysate : Mu d(Ap lac) from strain MAL103

Duplicate cultures of MAL103 were incubated overnight at 30° C in Super Broth (37). Then 3.0 mL of MAL103 was transferred into tubes containing 5.0 mL of LB and incubated in a 30° C water bath. The tubes were gently aerated through cotton plugged-pipettes connected to an air outlet. After two hours, the temperature of the bath was rapidly raised to 42° C, held at that temperature for 30 to 40 minutes then rapidly lowered to 30° C using ice. The temperature was maintained for one to two hours by which time the culture medium had cleared, indicative of cell lysis.

The medium was transferred to a sterile tube containing 1 mL of chloroform. The tube was shaken, then centrifuged for 5 minutes at speed 7 in an IEC International Clinical Centrifuge Model C7. The supernate was decanted into a second fresh tube containing 1 mL of chloroform and the above procedure was repeated. The supernate thus obtained was mixed with chloroform and stored at 4° C.(37).

D. General lysate preparation

The bacterial strain on which phage were to be grown, was inoculated into 5 mL of LB containing 1 drop of 1 M $CaCl_2$ (LB Ca) and incubated overnight at 37° C. Subculturing was done if required to obtain a logarithmic phase culture. (0.1 mL) of different dilutions (0, 10^{-1} to 10^{-5} using LBCa) of wild type phage were placed in sterile tubes and 4 drops of log-phase culture were added to each. The tubes were placed in a 37°C water bath for 20 minutes, then 3 mL of R top was added, and the phage suspensions poured onto lysate agar plates. R top and lysate agar were prepared as per Miller (37).

E. Transduction using Mu d(Ap lac) or P1

Recipient strains were grown at 37° C overnight in LB. Culture was centrifuged for 5 minutes in a clinical centrifuge and the cell pellet was suspended in 5 mL of MC buffer (37). The resuspension was incubated with occasional shaking in a 30° C water bath for 10 minutes. Aliquots (0.1 mL) of the resuspension were distributed into several sterile tubes, each containing a different dilution of phage lysate in MC buffer: control (no phage), 0 , 10^{-1} ,10^{-2}, 10^{-3} dilutions. These were then incubated at 30° C for at least 30 minutes, after which time 0.5 mL of LB was added to each tube, and the incubation period repeated. 3 mL of F Top agar (37) at 45° C was then

added to each tube, and the mixture was poured onto appropriate plates and incubated at 30°C, usually for two days. When using Mu d(Ap lac), selection was usually done on LB plates containing 2.5 mg% ampicillin (LB Amp). Procedure from Miller (37).

F. Assay for β-galactosidase

The Mu d(Ap lac) insertion element carries the gene for β-galactosidase. This enzyme was assayed according to Miller (37).

Cells were grown in minimal medium containg 0.2% of glucose or other carbon sources. Amino acids were added as needed. Cells were incubated at 30° or 37° C in a New Brunswick Scientific gyrotory water bath shaker (model G76) at speed 5 until their OD 600 was about 0.2. (the actual OD 600 was recorded).

Tubes containing 0.9 mL of Z buffer (37) were set up in triplicate, and 0.1 mL of culture added to each; a control tube containing 1.0 mL Z buffer was prepared. One drop of 0.1% sodium dodecyl sulfate and two drops of chloroform were added to the tubes, which were vortexed for 10 seconds, and then incubated for 5 minutes at 28°C. ONPG (o-nitrophenyl-β-D-galactopyranoside) 200 μl of a 4 mg/mL solution, was then added to each tube with mixing. The exact time of substrate addition was noted. The tubes were returned to the 28°C water bath until a faint yellow colour appeared, at which time (precisely recorded) the reaction

was stopped by the addition of 0.5 mL of 1 M Na_2CO_3. In cases where no colour developed by 1.5 hours, the tubes were left overnight and the reaction terminated the next morning. The optical densities of the assay mixtures were determined at 420 nm and 550 nm using a Perkin-Elmer λ-3 spectrophotometer.

Units of β-galactosidase (U) were determined using the formula of Miller:

$$U = \frac{1000[OD\ 420 - (1.75)\ (OD\ 550)]}{(0.1)(t)(OD\ 600)}$$

t = assay time (minutes)

G. X-gal plates

To determine the presence of β-galactosidase activity or for use as a selective medium, X-gal plates were prepared as follows:

100 mL LB agar or minimal medium with nitrogen and glucose agar, cooled to 45°C.

0.3 mL 20 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactoside in N,N-dimethylformamide.

H. Sugar assays

i) Total Hexose

Total hexose was assayed in duplicate by the phenol-H_2SO_4 method (13). To standards (0-100 μg dextrose) and

samples in a final aqueous volume of 1 mL was added 1 mL of 5% aqueous phenol. Tubes were vortexed, then 5 mL of concentrated H_2SO_4 was added to each tube, which were immediately vortexed. The reaction tubes were incubated at room temperature for 10 minutes and at 25°C for 15 minutes.

Optical densities were read at 488 nm on a Perkin-Elmer λ-3.

ii) Methylpentose assay

Methylpentose was assayed in duplicate by the H_2SO_4 - cystein method (after Dische and Shettles (10)). Standards (0-40 μg fucose) and samples in a final aqueous volume of 1 mL were placed on ice and 4.5 mL of a 1:6 water:sulfuric acid solution was added. The tubes were removed from the ice and left at room temperature for a few minutes, then transferred to a boiling water bath for exactly 10 minutes. The tubes were then cooled under running water and 0.1 mL of 3% aqueous cystein hydrochloride added to each with vortexing.

Optical densities were read at 396 nm and 430 nm. The methylpentose content was calculated from the difference (OD 396 - OD 430).

iii) L-Fucose dehydrogenase assay (after Maler (34)).

Samples (0-80 μl) were hydrolyzed by boiling with equal volumes of 0.2 N HCl in capped microfuge tubes, for 1 hour. Hydrolysates were centrifuged in a Brinkman Eppendorf

centrifuge 5414, for 10 minutes and the supernates were analyzed in duplicate for free L-fucose. Standards containing 0-20 nmoles of L-fucose were similarly analysed (in duplicate).

Each reaction mixture contained 2mM NAD+ (β-Nicotinamide adenine dinucleotide), 0.1 mg α-L-fucose dehydrogenase (E.C. 1.1.1.122 ,1.1 unit/mg protein) and buffer. 100mM Tris-HCl buffer, pH 8.0 was used to bring each reaction mixture to 0.5 mL.

Tubes were mixed gently and incubated for 90 minutes at 37°C and the increase in optical density at 340 nm due to the formation of NADH , was determined.

This method was used to assay the fucose content:

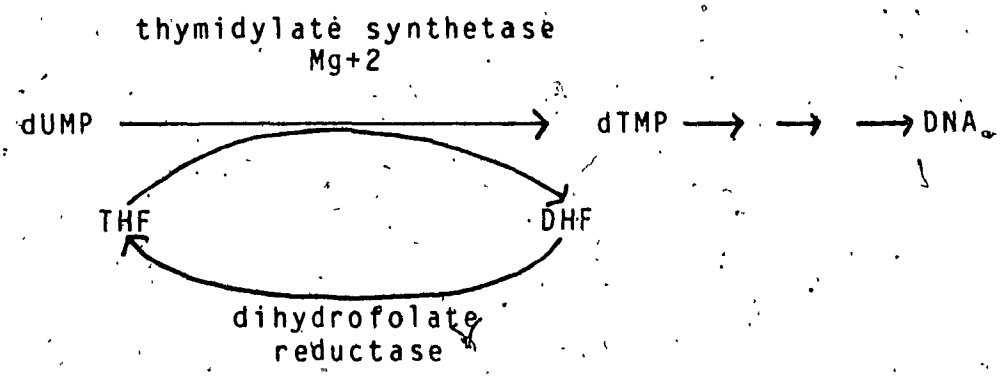
a) in dialyzed supernates of centrifuged liquid cultures.

b) in crude polysaccharide extracts. The latter were prepared by pelleting 20 mL of culture in the IEC at 10,000 rpm for 10 minutes. The supernate was removed and the pellet (in the case of CU) or pellet and slime layer (for ZP10 and M6) were shaken with 45% aqueous phenol at 65° C for 10 minutes. The extract was centrifuged as above and the upper aqueous layer hydrolysed and analysed for fucose.

I. Isolation of thy mutants

Trimethoprim is a potent inhibitor of dihydrofolate (DHF) reductase. If thymidylate synthetase is active in the presence of trimethoprim, the supply of trihydrofolate (THF)

is rapidly depleted and the cells cease to grow. Mutants for thymidylate synthetase supplied with thymidine can grow in the presence of trimethoprim because DHF ⟶ THF is not completely inhibited and the requirement for THF for the formation of dTMP from dUMP has been bypassed.



Aliquots (0.1 mL) of an overnight culture of a bacterial strain were spread on minimal medium containing nitrogen and glucose plates, supplemented with 5 μg/mL of trimethoprim and 50 μg/mL of thymidine (4). Plates were incubated for two days at 37° C and single colonies were purified and maintained on the same type of plates.

J. Isolation of antibiotic resistant strains

Bacterial strains were grown overnight in tubes of LB at 37° C. Aliquots (0.1 mL) were plated onto LB plates containing antibiotic (ampicillin 2.5 mg%, nalidixic acid 2.0 mg% or streptomycin 12 mg%). The plates were incubated at 37°C for about two days and resistant colonies were selected and purified.

K. Conjugations

Donor and recipient strains were grown to mid-exponential-phase in LB.

Aliquots (0.5 mL) of the donor organism were mixed with 10 mL of the recipient strain in 250 mL Erlenmeyer flasks. Flasks were set in a 37°C water bath and gently shaken for five minutes. 0.1 mL of each conjugation mixture was added to 50 mL of warm LB in a 500 mL Erlenmeyer flask. At zero time a 0.5 mL sample was withdrawn and diluted with 2 mL of 0.9% NaCl, and vortexed at high speed for 10 seconds. 0.2 mL of this dilution was added to 3 mL of molten F top agar (45°C), which was then poured onto selective plates. Aliquots were subsequently removed from conjugation mixtures at specified intervals, diluted and plated as described. (37).

L. UV sensitivity

Bacterial strains were streaked on LB plates and exposed for one minute to UV light (UV lamp: Gelman Instrument 253.7 nm); unirradiated control plates were also streaked. Plates were incubated overnight at 37°C and compared.

M. Preparation of Cell Extracts for Enzyme Assays

Cells were grown at 30°C with shaking to late-log phase in minimal medium containing nitrogen and glucose

supplemented with antibiotics or amino acids as required.

Cultures were centrifuged for 10 minutes at 12,000 x g in an IEC refrigerated centrifuge, model B-20, washed with 50 mM Na_2HPO_4 - KH_2PO_4 pH 7.0 and resuspended in this same buffer. Cells were then chilled in an ice bath and disrupted with an Artex Systems Corporation Sonic 300 Dismembrator, (intermediate tip, 60% capacity) 5 x 20 second bursts with intervening 1 minute cooling periods. The sonicate was centrifuged at 34,000 x g for 30 minutes and the crude extract assayed for phosphomannose and phosphoglucose isomerase. The protein content was determined by the Lowry et al. assay (32), using bovine serum albumen as a protein standard.

N. Phosphoglucose isomerase assay

Assay mixture:

50 μl 0.001 M Fructose-6-phosphate

83 μl 1 mg/mL NADP (β-nicotinamide adenine dinucleotide phosphate)

50 μl 0.1 M MgCl

20 μl glucose-6-phosphate dehydrogenase, 12 units/mL (from Bakers yeast type VII)

0-100 μl crude extract

197-297 μl 0.08 M Tris-HCl, pH 7.6

Final assay volume was 500 μl. The production of NADPH was measured spectrophotometrically at 340 nm with the Perkin-Elmer λ-3. (27).

O. Phosphomannose isomerase assay

Assay mixture:

20 µl 0.01 M mannose-6-phosphate

83 µl 1 mg/mL NADP

50 µl 0.1 M $MgCl_2$

20 µl glucose-6-phosphate dehydrogenase 12 units/mL

20 µl phosphoglucose isomerase 16.25 units/mL (Bakers Yeast Type III)

0-100 µl crude extract

207-307 µl 0.08 M Tris-HCl, pH 7.6

Final assay volume was 500 µl. NADPH was monitored at 340 nm. (27).

P. Polysaccharide purification

Exopolysaccharide adherent to the bacterial cell wall was isolated as follows (after Jann and Jann (21) and Orskov and Orskov (40) :

Cells were grown in 3 litre fermentation tanks (New Brunswick Scientific Company Fermentor FS-305) at 30°C in minimal medium with nitrogen and 0.6% glucose. Amino acids were added as required; ampicillin was also included in the media for ZP10. The tanks were aerated and agitated at 100 rpm. Fermentations were terminated when an OD 600 of 0.9 - 1.1 was achieved.

The cultures were harvested by centrifugation (14,000 x g, 20 minutes) in an IEC refrigerated centrifuge. Pellets

were resuspended in a solution of 0.9% NaCl and 2% phenol, precipitated with 3 volumes of cold ethanol and collected by centrifugation (10 minutes, 12,000 x g). Pellets were washed with acetone, recentrifuged, thoroughly dried, and weighed.

Acetone dried bacteria (10.0 g) were suspended in 350 mL of distilled water and warmed to 65°C. To this suspension was added 350 mL of 90% aqueous phenol, (previously warmed to 65°C) and the resulting mixture was shaken for 10 minutes at 65°C.

The extraction mixture was left to separate overnight in a 1000 mL separatory funnel. Three layers were obtained: a phenol layer on the bottom, cell debris in the middle and an upper aqueous layer. The phenol layer was discarded and the two upper layers were centrifuged at 8,000 rpm in the IEC. The aqueous layer was set aside and the cell debris mixed with some phenol, was re-extracted with water. The supernatant thus obtained was added to the initial aqueous layer. The pooled aqueous extracts were dialyzed against distilled water containing a small amount of sodium azide for 48 hours with numerous water changes.

The dialysate was freeze-dried and a 1% solution of the lyophilisate in distilled water was prepared. This solution was centrifuged at 37,000 rpm for 4 hours in a Beckman L5-50B ultracentrifuge. The lipopolysaccharide pellet (Fraction L) was frozen for future analysis.

The supernate was diluted 1:1 in water and the OD 260

measured. The diluted supernatant was incubated overnight at 30°C in the presence of 5 mg% ribonuclease-A and 0.5 mg% deoxyribonuclease I. The solution was dialyzed then for 48 hours against four changes of distilled water and the OD 260 reverified.

The solution was made 0.25 M with respect to NaCl, and 0.5 volumes of a solution of 0.25 M NaCl and 4% Cetavlon (hexadecyltrimethylammonium bromide), added with gentle stirring. Any undigested nucleic acid precipitated (Fraction PI) upon addition of NaCl-Cetavlon and was removed by centrifugation (15 minutes, 14,000 rpm).

Distilled water was slowly added with gentle stirring to the supernate, until a second precipitate (Fraction PII) was obtained and removed by centrifugation in the IEC (15 minutes, 9,000 rpm). More water was added to assure that precipitation of polysaccharide species had been complete.

Fraction PII, a polysaccharide-Cetavlon complex, was dissolved in a small amount of 1 M NaCl, precipitated with 3 volumes of ethanol, and centrifuged (10 minutes; 9,000 rpm). The pellet obtained (Fraction PIII) contained the sodium salt of the acidic polysaccharide; it was redissolved in 1 M NaCl, re-precipitated with ethanol, and lyophilized.

Q. <u>Assay for 2-keto-3-deoxyoctanoic acid</u>

Reagents:

acidic periodate: 0.025 [illegible]O_4 in 0.125 N H_2SO_4

0.04 N sulfuric acid

0.2 N sodium arsenite in 0.5 N HCl

0.3 % aqueous thiobarbituric acid, pH 2.0

Fraction L was dissolved in 5 mL of water and 0.1 mL diluted with 0.1 mL of 0.04 N H_2SO_4 in a microfuge tube. The tube was capped and heated in a boiling water bath for 20 minutes to release KDO.

Acidic periodate (0.25 mL) was added with mixing, and the solution left to stand for 20 minutes at room temperature. Then 0.5 mL of the arsenite solution was added, followed two minutes later by 2 mL of thiobarbituric acid reagent. The reaction mixture was vortexed and heated at 100°C for 20 minutes. Assay tubes were cooled and the optical density at 548 nm measured. An optical density of 1.0 in a 1 cm pathway corresponds to 52.6 nmoles of KDO. (13).

R. Infrared (IR) spectroscopy

IR spectra of purified polysaccharide (KBr pellets) were obtained using a Perkin-Elmer 599 B infrared spectrophotometer.

S. Proton magnetic resonance (PMR) spectroscopy

Purified polysaccharide was repeatedly suspended in 99.96% deuterium oxide and lyophilized. 2.0 mg of deuteriated polysaccharide was dissolved in 0.5 mL of d_6-

dimethyl sulfoxide. Pmr spectra were obtained at 400 MHz at the NMR laboratory at l'Université de Montréal.

RESULTS

The overproduction of exopolysaccharide was studied using the *E. coli* K-12 mucoid mutant ZP10. The results section which follows is divided into four major parts. First the isolation of ZP10 is described; then the phenotype of ZP10 in comparison to the parent CU and to the *lon* mutant M6 is discussed. The quantification and purification of the exopolysaccharide produced by ZP10 are reviewed, followed by an analysis of the genetics of the mutation leading to polysaccharide overproduction and the mapping of the insertion.

I ZP10 - a mucoid mutant

A. Obtaining ZP10

Mu d(Ap *lac*) was transduced into CU1008 Δlac, a non-mucoid isoleucine/valine auxotroph, using P1 phage. Transductants were incubated on LB plates containing 2.5 mg% ampicillin (LB Amp) and the ampicillin resistant colonies which appeared after two days were purified.

The goal of the transduction was to obtain insertion mutants which were visibly mucoid. Due to the insertion, these mutants would be ampicillin resistant and some would have β-galactosidase activity, in addition to their mucoid phenotype. The parent strain having a deletion in the *lacZ* gene does not express β-galactosidase, therefore any β-

galactosidase activity would be due to the insertion.

It was difficult to identify the mucoid transductants on complex medium (LB), so the colonies were transferred onto minimal media containing nitrogen, glucose, isoleucine, valine and ampicillin (MMNG iv amp). Twenty mucoid mutants were distinguished and further purified. None of the colonies grew in the absence of isoleucine and valine, indicating that they were CU1008 Δlac progeny.

The β-galactosidase activities of these mutants covered a wide range, from 3 to 700 units as defined by Miller (37).

After successive purifications, only five mutants were obviously mucoid; these were retained for further work. All five isolates were non-mucoid on LB but mucoid on MMNG iv amp at both 30° C and 37° C. One of these, mutant ZP10, consistently expressed its mucoidy, so despite low β-galactosidase values (4 U), it was chosen for subsequent work.

B. Back-cross

To ensure that only one insertion had integrated into CU1008 Δlac to produce ZP10, and that we were dealing with one mutation, a back cross was carried out. Phage were grown on ZP10 and used to infect CU1008 Δlac. All 45 transductants obtained on LB Amp plates, presented the same phenotype as ZP10. It is therefore highly unlikely that ZP10 carries more than one insertion ie. its phenotype is

due to one mutation. If multiple insertions were responsible for the ZP10 phenotype, then each transductant would have had to have been infected by that number of phage in order to obtain the ZP10 phenotype, an unlikely event.

II Characterization of ZP10

The parent strain CU1008 Δlac (henceforth referred to as CU) and the lon mutant M6 (35) were always used as controls in experiments done with ZP10. Strain M6 is known to produce colanic acid due to the lon mutation, and it has been studied with respect to a large number of parameters (J.Fraser, personal communication). It was important to have a colanic acid-overproducing E. coli K-12 mutant for comparative purposes, and M6 was suited to our needs. Attempts to acquire capS and capT mutants from other labs were unsuccessful.

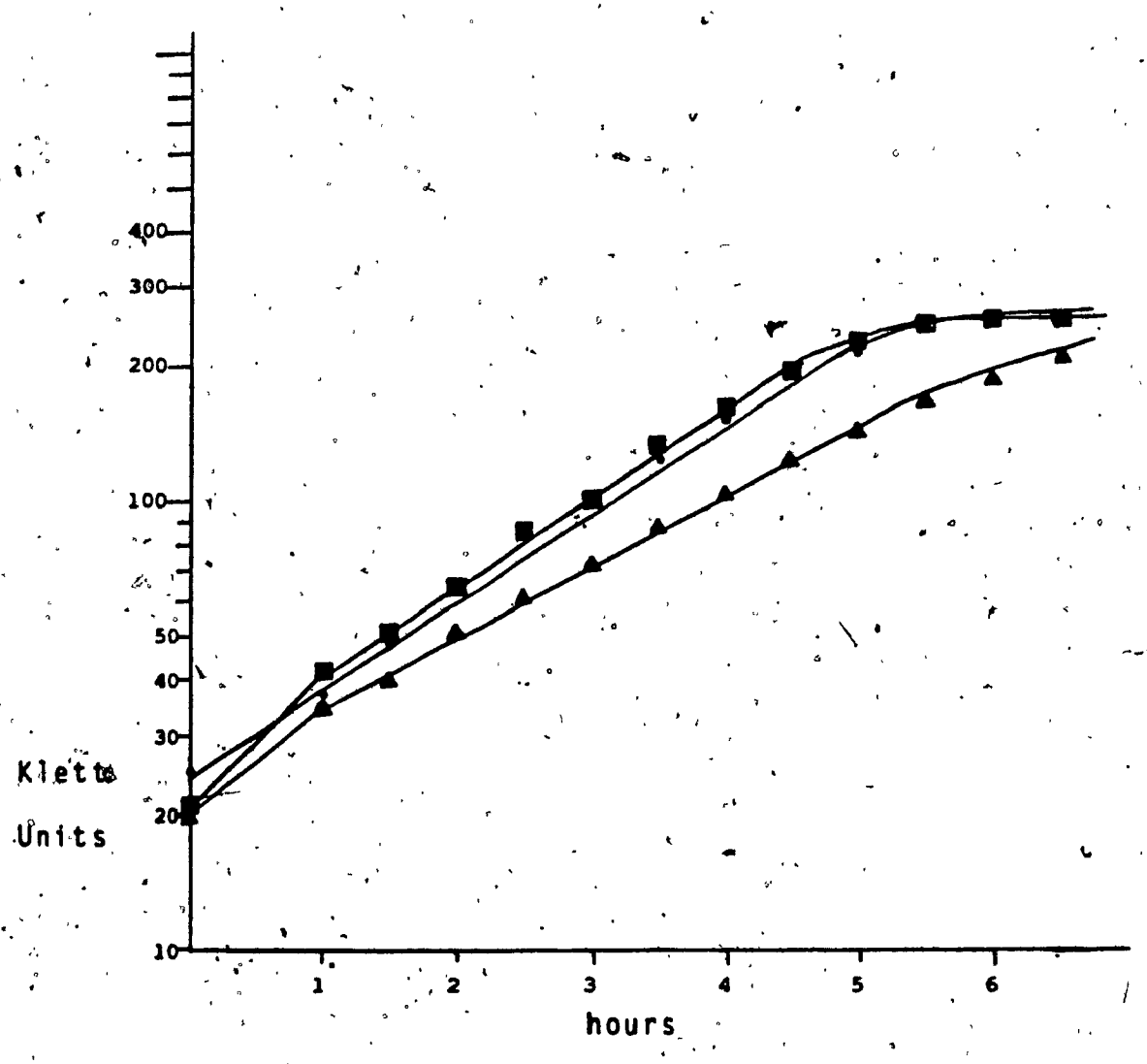
A. Growth curves

The mucoid phenotype of ZP10 varies with the growth medium. Growth curves were obtained to establish whether growth media had an effect on the doubling time of ZP10 with respect to the parent CU. As seen in Figure 4, CU and ZP10 had very similar growth curves with generation times of 1.6 hours in MMNG at 30° C, whereas M6 showed a slower growth rate, with a generation time of 2 hours. Figures 5 and 6 show growth curves for CU and ZP10 at 30°C in LB and in MMNG containing casamino acids. The Mu d(Ap lac) insertion does

Figure 4.

GROWTH CURVES

CU, M6 and ZP10 in MMNG at 30° C

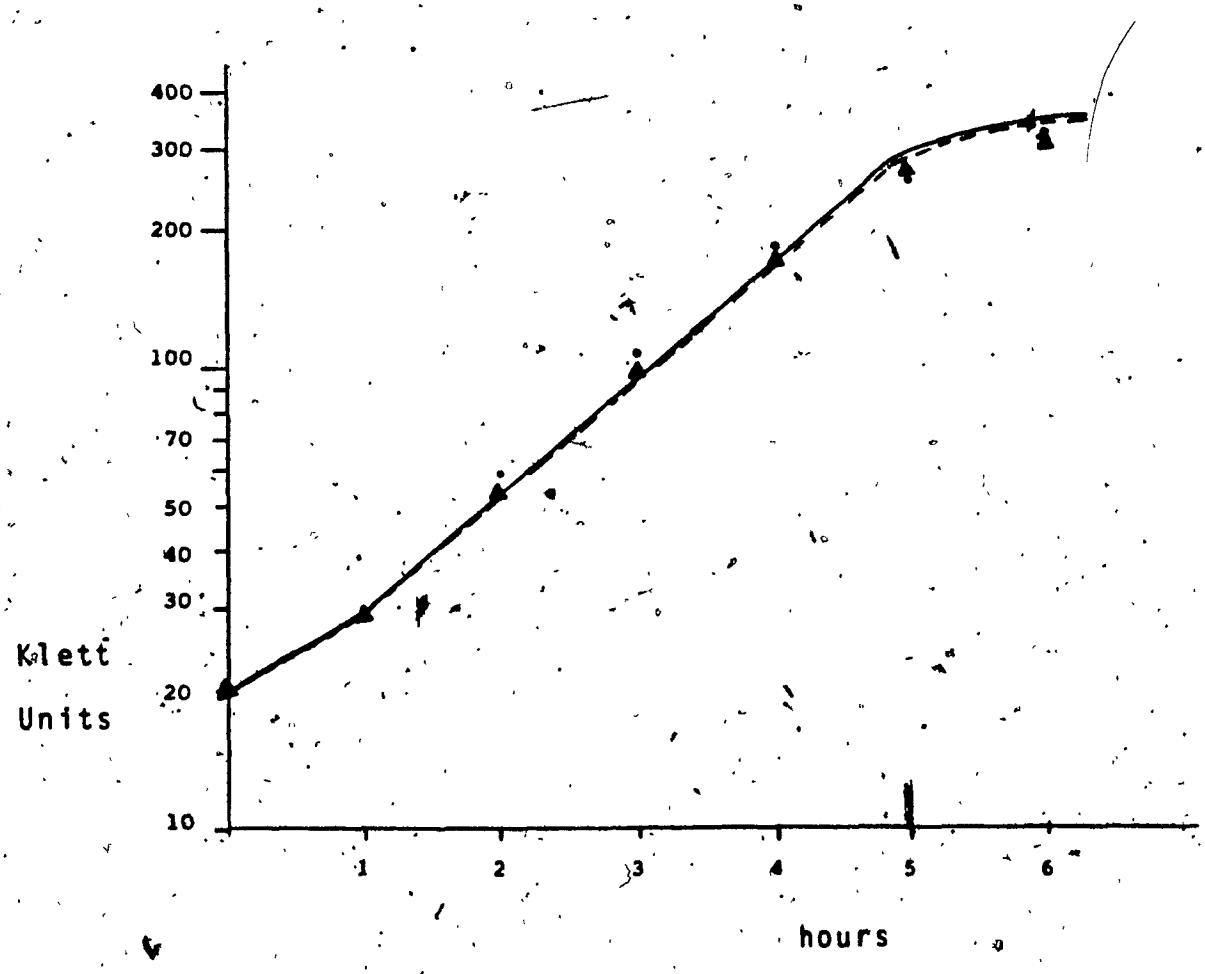


●CU. ▲M6. ■ZP10.

Figure 5.

GROWTH CURVES

CU and ZP10 in Luria Broth at 30° C.



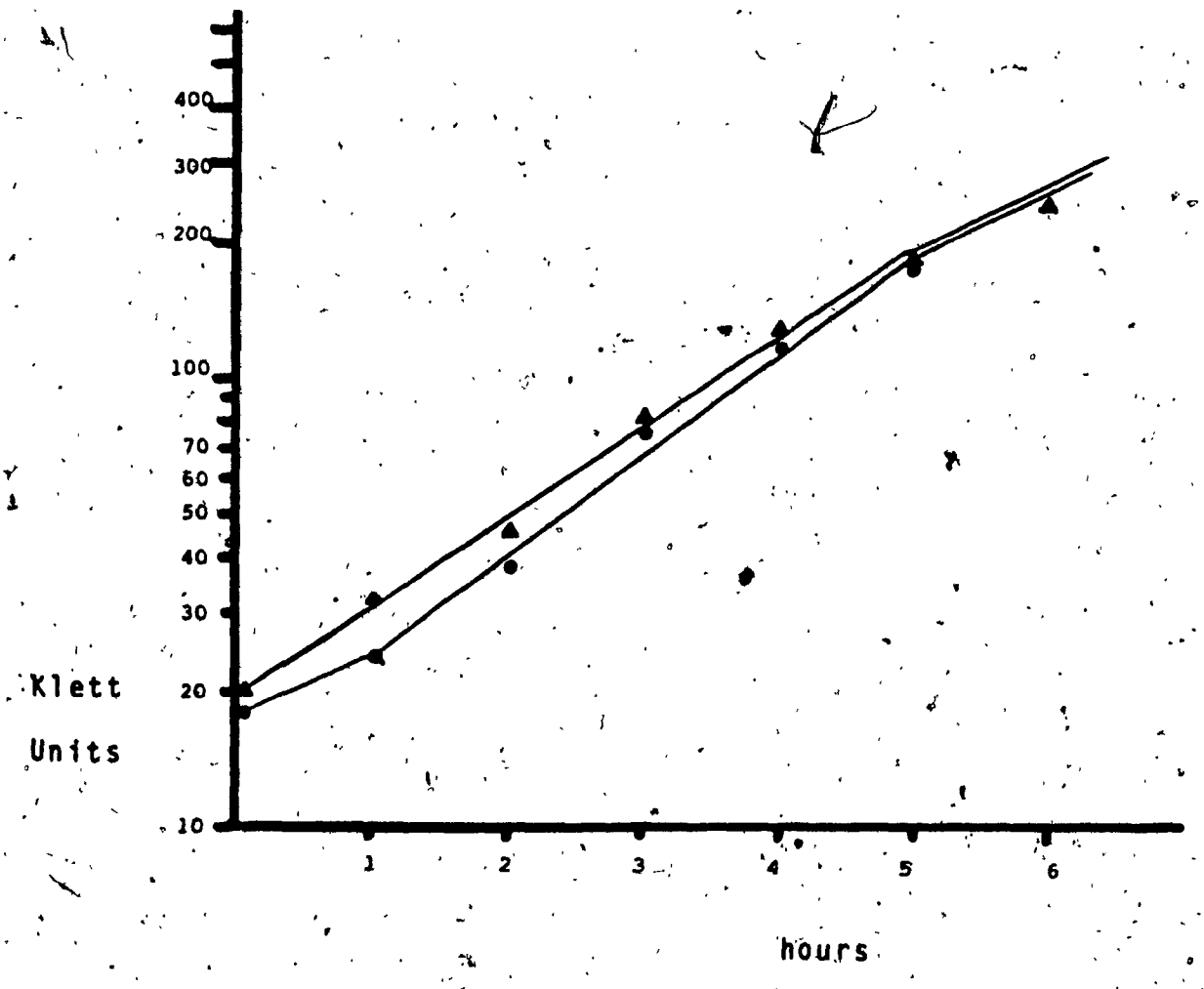
●CU. ▲ZP10.

Figure 6.

GROWTH CURVES

CU and ZP10 in MMNG + Casamino acids

30° C.



● CU. ▲ZP10.

not seem to have affected growth rates in the media used in these experiments.

B. β-Galactosidase activity

β-Galactosidase activity due to the Mu d(Ap lac) insertion, reflects the expression of the gene into which it has inserted. Unfortunately, in ZP10, the β-galactosidase values were very low, on the order of 4 units, and the value was not affected by the growth media (MMNG, LB, MMNG containing casamino acids). Strain ZP10 is mucoid on MMNG but not on LB and one might expect that this behaviour would be reflected in variations of the β-galactosidase gene expression; this, however, was not the case.

The activity of β-galactosidase remained unchanged in ZP10 when the organism was grown on different carbon sources: glucose, fructose, galactose, glycerol, glycerol and lactose, and mannose.

C. Growth media effects

Growth on plates containing various carbon sources yielded surprising results. As has been pointed out earlier M6, true to its lon genotype, is visibly mucoid on LB plates incubated at 30°C but not at 37° C. When M6 is grown on MMNG however, it is mucoid at both 30° C and 37°C. The carbon source, be it glucose, glycerol or pyruvate, has no influence on the mucoid appearance of M6.

CU remains non-mucoid when plated on the above media at

both 30° C and 37° C.

Mucoid production in ZP10 displays greater dependance on the composition of the growth medium. This mutant never appears mucoid on complex media. It is mucoid on MMNG irrespective of the temperature. The unusual behaviour of ZP10 becomes apparent when glucose-grown colonies are compared with glycerol-grown colonies. ZP10 is non-mucoid on MMN glycerol plates. It is very mucoid on MMN pyruvate plates. We are presently unable to account for ~~this~~ variation in mucoidy by ZP10.

D. UV sensitivity

In contrast to M6, CU and ZP10 survived a one minute exposure to UV radiation of 253.7 nm and were indistinguishable from unirradiated controls. Following exposure to the same dose of UV radiation, only a few single colonies appeared on the M6 plate.

E. Phosphomannose and phosphoglucose isomerase

Phosphomannose isomerase (PMI) and phosphoglucose isomerase (PGI) are two enzymes involved in colanic acid biosynthesis. In capR mutants such as M6, PGI is found at wild type levels, but PMI is derepressed and its activity is several times higher than that found in wild-type E. coli K-12 (36).

The activities of these enzymes were examined in CU,

M6 and ZP10, and the results are shown in Table 3. All three strains showed similar PGI values. ZP10 and its parent had identical PMI activities, whereas M6 clearly showed increased levels of enzyme activity. For comparison Table 3 also shows results obtained by Markovitz (36), who assayed these enzymes in different mucoid mutants: capR6, capR9 and capS.

III Isolation of ZP10 variants exhibiting high β-galactosidase activity

Strain ZP10 grows very slowly on lactose due to low levels of β-galactosidase, an enzyme required for growth on lactose as sole carbon source. To select for mutants with higher levels of β-galactosidase, ZP10 was grown overnight in a tube of LB and 0.1 mL of culture was plated on a MMN 0.2% lactose iv amp plate and incubated at 37°C for a few days. The first colonies to appear were picked and subcultured a number of times on lactose plates. The β-galactosidase activities of these mutants ranged from 430 to 1000 units.

These mutants were cultivated on a variety of media including MMN + glycerol and MMN + pyruvate, and the β-galactosidase activity under the different conditions was either within the 20% range of error of the assay, inconsistent or demonstrated no recognizable pattern. Furthermore their mucoid phenotype was not always expressed. They are of interest, however, in the discussion of the

Table 3. Phosphomannose isomerase and phosphoglucose isomerase activities in wild-type strains and in mucoid mutants

	1.			2.			
	CU	ZP10	M6	wild type	capR9	capR6	capS
phospho-mannose isomerase	0.39	0.34	1.8	1	5.0	4.7	1
phospho-glucose isomerase	1.1	1.1	1.2	1	1	1	-

1. CU, ZP10, M6 in units per mg of protein. 1 unit = umoles NADH/hour.

2. Wild type, capR6, capR9, capS : wild type enzyme activities arbitrarily set at 1 (after Markovitz (36)).

reasons for the low expression of the lacZ gene in the original ZP10.

IV Complex media factors

Growth on rich media has been reported to decrease polysaccharide output in a number of different organisms. The composition of LB and Casamino acids was examined with the hope of eventually determining whith component(s) inhibited mucoid production in ZP10 . Since M6 is non-mucoid on LB at 37°C it was of interest to know if the same components are responsible for inhibiting mucoid production in both strains.

Table 4 lists the ingredients in LB and in Casamino acid media. The amino acids present in these media were randomly divided into two groups. MMNG plates were prepared containing one or the other group of amino acids. Amino acid concentrations approximated those at which they are found in LB. By process of elimination, it was determined that the co-presence of arginine, glycine, leucine, isoleucine and valine, was sufficient to produce a non-mucoid phenotype in ZP10. The phenotypes of CU and M6 with respect to mucoidy, were not affected by these amino acids. At the time of this work, distinction between mucoid and non-mucoid phenotypes was done qualitatively as there was not a suitable means to quantify the polysaccharide being produced.

Whether the isoleucine and valine were required only as

Table 4.

List of some of the ingredients in Luria broth and in minimal medium containing nitrogen, glucose, and casamino acids (mg/100 mL).

	LB	MMNG Casamino acids
Arginine	36.9	38.0
Aspartic acid	94.5	4.9
Glutamic acid	222.0	51.0
Glycine	36.0	11.0
Histidine	24.7	23.0
Isoleucine	62.5	46.0
Leucine	53.0	99.0
Lysine	88.0	67.0
Methionine	28.0	22.0
Phenylalanine	52.0	40.0
Threonine	48.0	39.0
Tryptophan	18.9	8.0
Tyrosine	74.0	19.0
Valine	80.0	72.0
Ash	123.3	36.4
Total Nitrogen	177.3	153.9
Phosphorus	8.95	377.3
Potassium	3.21	618.1
Sodium	225.2	7.7
Magnesium	0.6	4.2
Calcium	1.16	0.39

Table 4 continued...

	LB	Casamino acids
Chloride	200.6	112.6
Pyridoxine	5.2	0.07
Biotin	1.06	0.10
Thiamine	1.93	0.12
Nicotinic Acid	150.5	2.7
Riboflavin	9.68	0.03

Calculated from the Difco Manual (9).

growth factors and to prevent growth inhibition by leucine, or whether they were actually instrumental in repressing the mucoidy was not determined.

V Polysaccharide Quantification

Colanic acid synthesized by overproducing mutants of E. coli is usually quantified in terms of the amount of non-dialyzable methylpentose present in the supernate of centrifuged cell cultures. While this method proved adequate for mutant M6, no methylpentose could be detected in the supernates of exopolysaccharide-producing ZP10. To eliminate the possibility that the polysaccharide produced by ZP10 did not contain methylpentose, total hexose in dialyzed supernates was assayed by the phenol-H_2SO_4 procedure (13). Strain M6 showed large quantities of methylpentose and hexose in its dialyzed supernate whereas ZP10 and CU did not (Table 5).

Following centrifugation, a mucoid-producing strain of E. coli appears quite different from a non-mucoid strain. When the supernatant is removed, one can see, and actually decant, a loose slime layer. The compact cell pellet which remains behind closely resembles the cell pellet of non-mucoid strains. Liquid cultures of M6 and ZP10 had the characteristic appearance of mucoid strains when cultivated under appropriate conditions. It was therefore unlikely, that ZP10 was mucoid on solid media but non-mucoid in liquid culture.

Table 5.

Methylpentose and hexose analyses of supernates.

µg/ml	CU	M6	ZP10
methylpentose trial 1.	4.6	161	8.2
trial 2.	1.3	151	4.0
hexose	20.8	372	18.5

20 mL cultures grown until a density of 160 Klett units were used.

The possibility that the polysaccharide produced by ZP10 was cell-bound rather than exported into the liquid medium was considered. Whole cell methylpentose determinations were attempted using the colorimetric procedure of Dische and Shettles (10), but this proved inadequate as even the non-mucoid parent, CU, gave a positive reaction. This was due to the first step in the assay which consisted of boiling the sample with sulfuric acid. A number of sugars including those of the cell wall form a brown color upon boiling with this reagent. The methylpentose assay was not specific enough to quantitatively distinguish the mucoid strains from the non-mucoid strains.

Attempts were made to separate any polysaccharide that might be adherent to the cell surface of ZP10. Resuspended cell pellets were boiled, vigorously vortexed, blended and sonified. However, none of these procedures resulted in the presence of a large amount of carbohydrate or methylpentose in the resuspension fluid.

If the exopolysaccharide could not be removed from the cell surface, a different way of quantifying exopolysaccharide, compatible with whole cells was necessary. Such a method would have to have a very low reactivity towards the cell wall sugars. Fucose is contained only in the exopolysaccharide of E. coli K-12; it is not present elsewhere in the cell. L-fucose dehydrogenase, which catalyzes the oxidation of L-fucose [equation (1)], was

considered a good candidate for our needs and a micro assay had recently been published.

$$\text{L-fucose} + \text{NAD}^+ \longrightarrow \text{L-fucono-1,5-lactone} + \text{NADH} \qquad \text{eq.(1)}$$

The assay is linear from 0 - 40 nmoles of L-fucose. L-fucose dehydrogenase also oxidises D-arabinose, D-xylose, L-xylose and L-galactose but much more slowly than L-fucose.

When the supernates of ZP10 liquid cultures were assayed with L-fucose dehydrogenase, no L-fucose could be detected.

A crude polysaccharide extract was made from the bacterial strains, as described in the methods. It was evident (Table 6) that in order to quantify the exopolysaccharide associated with ZP10, it would be necessary to isolate it.

It would appear that strain M6 excretes much more exopolysaccharide into the culture broth than does strain ZP10, though the amounts of material associated with the pellet and slime layer are similar for both strains. The non-mucoid parent strain CU1008 Δ lac, did not produce any detectable exopolysaccharide.

VI Exopolysaccharide Purification

Extraction of exopolysaccharide from the cell pellet and slime layer associated with ZP10 and M6 was performed using a method generally successful for removing capsular

Table 6.

Exopolysaccharide quantitation using L-fucose dehydrogenase

CU	M6	ZP10
0.0	264.0	246.0

nmol of L-fucose/20 mL of culture (all growth 1.85 Klett units).

polysaccharides from Gram-negative bacteria. Non-mucoid strain CU was used as a control. The exopolysaccharide contained in the supernatant of strain M6 was not extracted, as only exopolysaccharide associated with the cell was of interest. A flow chart of the purification is given in Figure 7.

The aqueous layer obtained following extraction with 45% phenol contained predominantly polysaccharide, lipopolysaccharide and nucleic acids and appeared to be devoid of protein (Appendix I). Lipopolysaccharide was removed by ultracentrifugation and quantified in terms of 2-keto-3-deoxyoctanoic acid (KDO). (Table 7). Nucleic acids were removed by digestion with RNase and DNAse and subsequent dialysis; the remainder were precipitated as Cetavlon salts.

Cetavlon forms complexes with polyanionic species (44) and these complexes have different solubilities in aqueous solutions of NaCl. Cetavlon-nucleic acid complexes are insoluble in $<$ 0.3 M NaCl; Cetavlon-acidic polysaccharide complexes are soluble at 0.25 M NaCl, and only precipitate in much more dilute solutions. Therefore, to remove any remaining nucleic acids, the extraction solution was made 0.25 M with respect to NaCl, and 4% Cetavlon added. No absorbance at 260 nm could be measured following removal of the precipitated nucleic acid-cationic detergent complex by centrifugation.

The supernate thus obtained was slowly diluted with

Figure 7. Polysaccharide extraction

	CU	M6	ZP10
I. g of acetone dried bacteria :	8.6	8.3	10.0
II. mg of lyophilized material following phenol extraction and dialysis:	750	780	690
III. mg/mL nucleic acid content in 1% solution of lyophilized Fraction (II).	2.8	2.0	2.0
IV. mg/mL nucleic acid content following nuclease digestion and dialysis.	0.79	0.62	0.62
V. mL of polysaccharide solution prior to Cetavlon addition.	111	97	114

Figure 7 continued...

VI.
Following Cetavlon treatment, OD 260 of solution was @ 0. (i.e. no nucleic acid present).

VII.
mL of water added to cause precipitation of the Cetavlon-polysaccharide complex:

CU	M6	ZP10
54	50	52

VIII.
addition of water to the supernatant of VII caused no further precipitation.

IX.
mg hexose recovered; as % of original lyophilisate.

	CU	M6	ZP10
mg	2.63	42.4	48.8
%	0.34	6.14	6.23

Table 7.

Polysaccharide purification

μmoles of KDO present in the pellet of phenol-extracted, ultracentrifuged aqueous phase.

	Total KDO recovered in μmoles
Cu	19.4
16	18.3
ZP10	17.3

water. According to Orskov and Orskov (40), dilution of a 0.25 M NaCl solution of Cetavlon-polysaccharide with 0.5-1 volume of water precipitates the M antigen, whereas 1.5-2 volumes of water are required to precipitate the K-antigen. Upon addition of 0.5 volumes of water, extraction solutions of M6 and ZP10 showed a heavy flocculence; at this same dilution, only a very slight cloudiness was observed in the extraction solution of CU. The mixtures were centrifuged; more water was added to the supernates but no further precipitation was observed.

The pellets were dissolved in 1 M NaCl and small aliquots removed and set aside and reprecipitated with ethanol. Polysaccharide in extracts from M6 and ZP10 could easily be spooled onto a stirring rod. The small aliquots were separately precipitated and used to quantify the recovery of exopolysaccharide. The amount of polysaccharide obtained using this procedure was 2.63, 42.4 and 48.8 mgs for CU, M6 and ZP10 respectively.

VII Polysaccharide analysis

A. IR spectra

IR spectra were obtained for the polysaccharides purified from M6 and ZP10 (Figures 8 and 9). The IR spectra are virtually identical and compare quite favorably with those of colanic acid published by Sapelli and Goebel (43) (Figure 10). However, the IR spectra do not permit any definitive structural determinations. For example, the D-

Figure 8. IR spectrum of the polysaccharide from M6

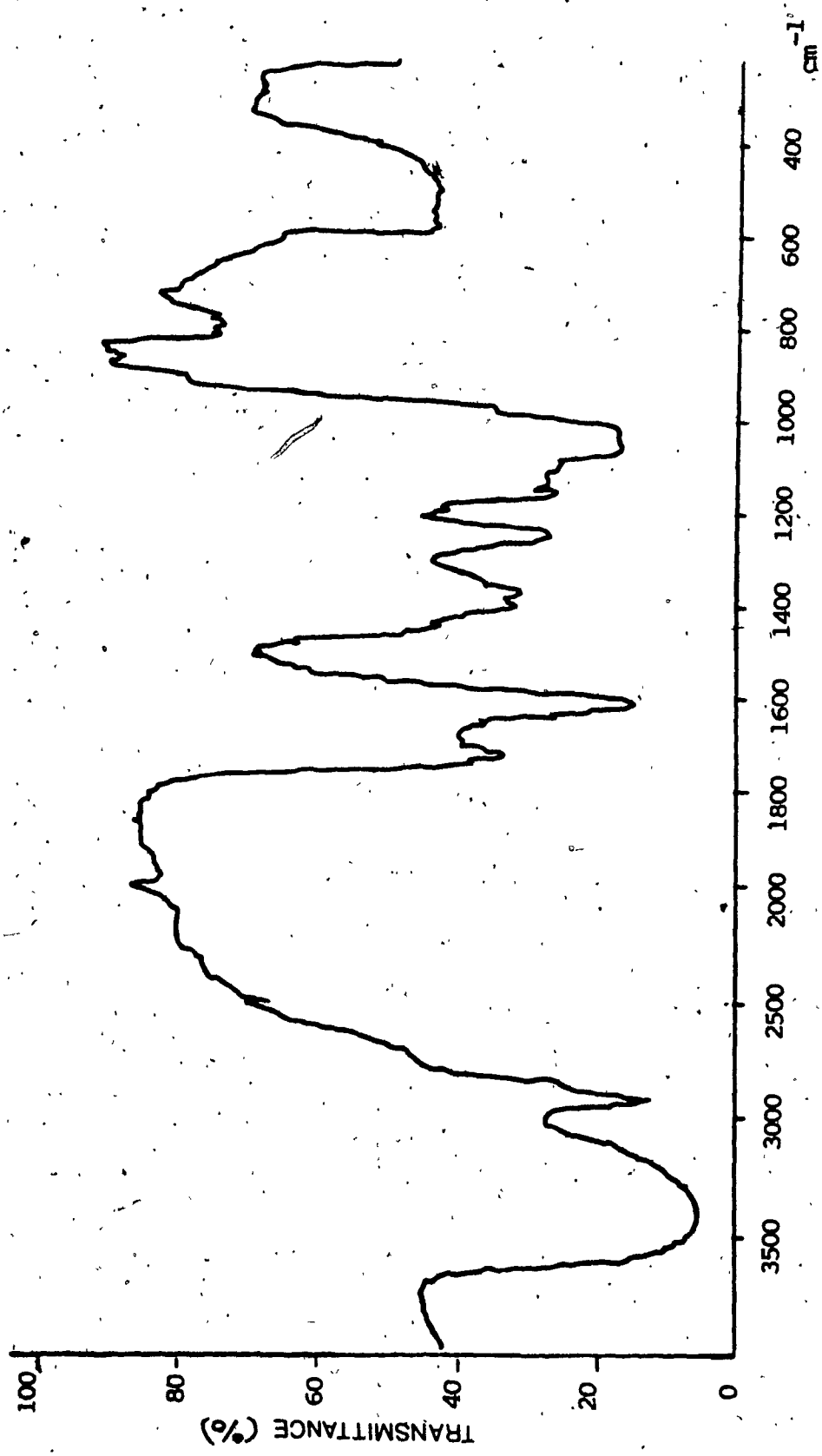
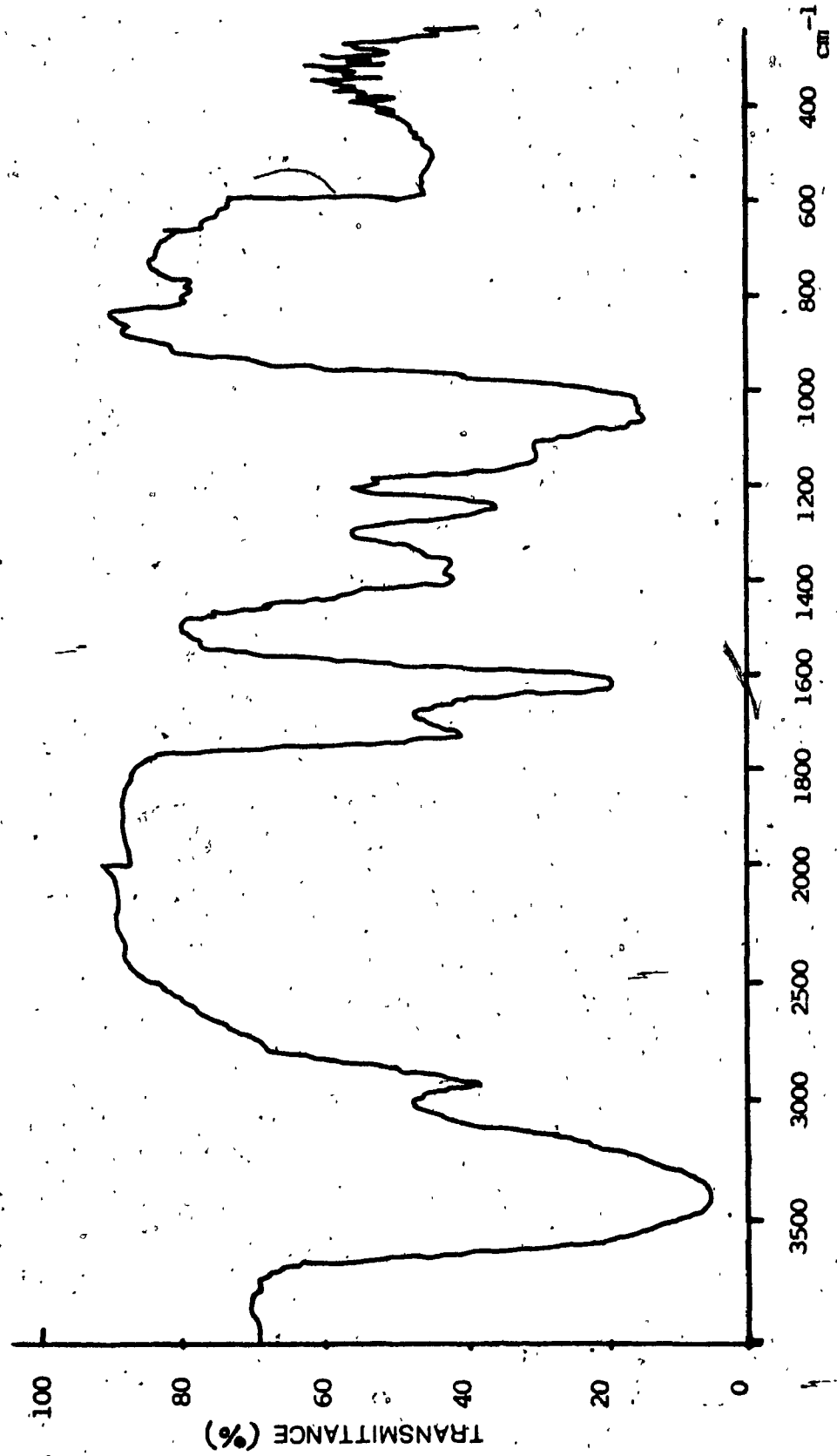


Figure 9. IR spectrum of the polysaccharide from ZP10



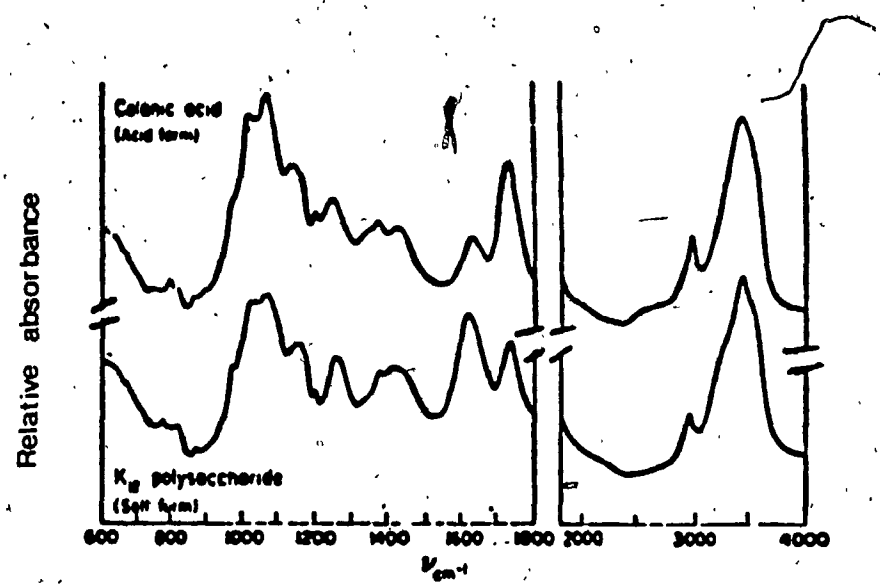


Figure 10. Published IR spectra of colanic acid. Sapelli and Goebel (43).

and L- forms of monosaccharides cannot usually be distinguished by IR (2). The IR spectra of the exopolysaccharides obtained from M6 and ZP10 resemble those obtained from polysaccharides containing uronic acid (2). The carbohydrate shows strong antisymmetric and symmetric stretching bands at 1370 and 1640 cm^{-1}. The band at 1720 cm^{-1} is diagnostic for the $\nu_{C=O}$ stretch of -COOH.

B. PMR

Numerous studies have been published on proton and carbon nmr analyses of complex carbohydrates (8). Pmr spectra of the purified polysaccharides of ZP10 and M6 as well as of the supernatant polysaccharide of M6, were taken for comparative purposes. Longer collection times under different conditions might have provided further structural detail. Solutions of these polysaccharides were extremely viscous and it was very difficult to prepare samples of appropriate concentrations for pmr analysis. All three exopolysaccharide samples showed very similar spectra in the δ2 - δ3.6 region, but differences became apparent in the δ4 - δ5 region. The resolution in this area was extremely poor so one can only say that on the basis of these pmr spectra, the exopolysaccharides from M6 and ZP10 though similar, are probably not identical.

VIII Genetic mapping

While many of the earlier results indicated that the

ZP10 mutation was not a lon mutation and that it was probably different from the other known mutations giving rise to antigen overproduction, it was necessary to confirm this by mapping the site of the Mu d(Ap lac) insertion. Conjugations were done to determine the general area of the insertion, and transductions were performed to more precisely locate the overproduction locus.

A. Conjugations

Two mutants of ZP10 were selected for conjugations. The first strain was nalidixic acid resistant (nalR); the nalR phenotype maps at either 48 (nalA) or 57 (nalB) minutes on the E. coli K-12 chromosome (1). The second strain was streptomycin resistant (strR) and a thymidine auxotroph (thy). The genes for strR and thy- phenotypes map at 76 and and 60 minutes respectively. ZP10 and its mutant derivatives are also auxotrophic for isoleucine and valine (ilv). The single mutation for this phenotype maps at 84 minutes.

The Hfrs used for the conjugations and their points of origin are listed in Table 8 . The results of the conjugations are shown in Table 9 and illustrated in Figure 11.

The first set of conjugations was done using Hfr 4294 and ZP10 nalR, with mating times of 0, 50 and 90 minutes. For mating times of 50 and 90 minutes the areas from the point of origin to 18 and 58 minutes respectively

Table 8.

List of Hfrs used to map the Mu d(Ap lac) insertion in *E. coli* K-12 strain ZP10.

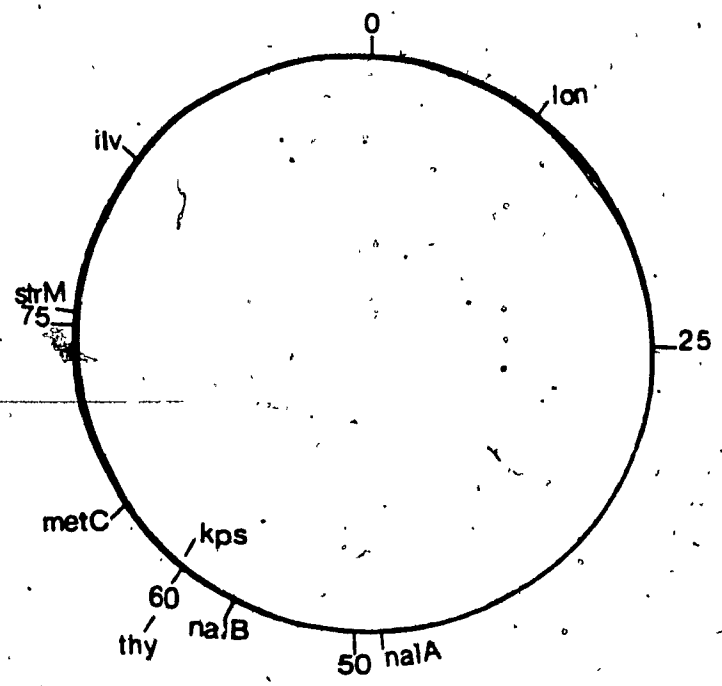
	Chromosomal markers	Point of origin	Map position
4242	thi-1	P042	pyrD pyrC 21 23
4294	thi-1	P068	argG tolC 68 66
4312	metB1	P03	argF lac 6 8
4316	thi-1		his cheC 44 41

Table 9.

Conjugation results

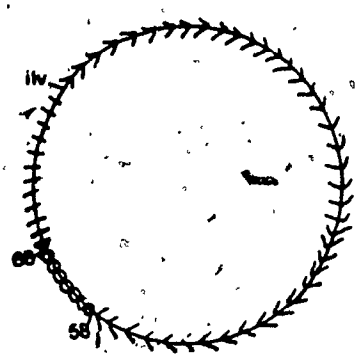
Mating	mating time (minutes) / # of conjugants examined			phenotypes
Hfr 4294/ ZP10 nalR	0	50	90	all ampR, nalR, mucoid
	0	85	130	ilv+
Hfr 4312/ ZP10 nalR	0	25	50	all ampR, nalR, mucoid
	0	66	0	ilv+, met+
Hfr 4242/ ZP10 strR thy-	0	25	50	19/23 ampR, mucoid
	23	26	150	17/26 ampR, mucoid
				53/150 ampR
				46/53 mucoid.
Hfr 4316/ ZP10 strR thy-	0	20	50	28/37 ampR, mucoid
	37	150	0	115/150 ampR, mucoid

Figure 11. Diagram of mapping by conjugations



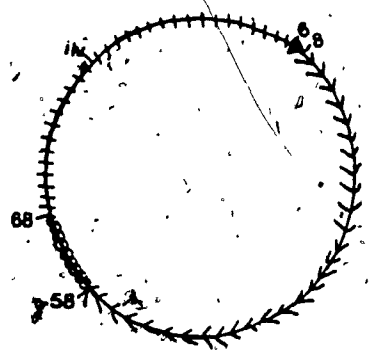
a. Relevant sites for conjugations.

Figure 11 continued...



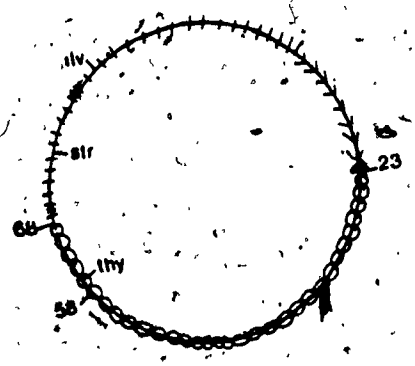
b. 4294/

ZP10 nalR



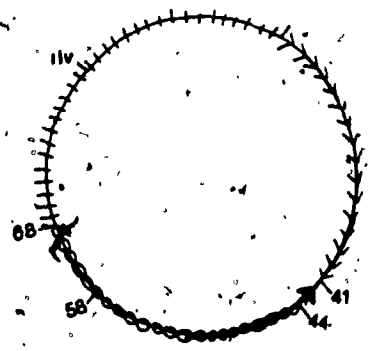
c. 4312/

ZP10 nalR



d. 4242/

ZP10 thy strR



e. 4316/

ZP10thy strR

does not contain the insertion

probably does not contain the insertion

contains the insertion

have been transferred. The colonies obtained from these conjugations were all ilv+ , nalR and ampR as well as mucoid. Thus, the area between Hfr 4294 point of origin at 67 minutes and ilv at 84 minutes definitely does not contain the insertion. If the conjugation bridges did not break during mating and the results of the 50 and 90 minutes are truly indicative of the portion of chromosome which had been donated, then the insert is between nalB at 57 minutes and the Hfr origin at 67 minutes. This conjugation was repeated and the results lead to identical conclusions. (The requirement for thiamine was not tested since traces of thiamine in the Bacto-agar would permit thiamine auxotrophs to grow).

The second set of conjugations was done using Hfr 4312, with a point of origin between 6 and 8 minutes, and ZP10 nalR. These conjugations were in the opposite direction to the first set. Sixty-six colonies were obtained when the mating time was 25 minutes and all retained the insertion, confirming the area between 7 and 84 minutes is free of the insertion. Mating times of 50 minutes gave no colonies probably due to the conjugation going beyond the nal gene.

The third set of conjugations mated Hfr 4242 and ZP10 thy- strR. While nalidixic acid plates inhibit further mating between these bacteria, some mating can occur on streptomycin plates. (One usually obtains more background colonies with str than with nal). The 0-time plate for the 4242 / ZP10 matings had 23 colonies; 19 of these retained

the insertion, while 4 did not. The same kind of pattern occurred at 25 minutes. Had further matings not taken place on the plates, no colonies would be expected at 25 minutes, since ZP10 would still be auxotrophic for thymine. Fifty minute matings produced 150 colonies, about a third of which retained the insertion. Since resistance to str was required for growth on these selective plates, the conjugation could not have gone further than 76 minutes (strR) but would have gone at least as far as thymine at 60 minutes. These results confirmed the insertion as being between 23 and 68 minutes.

The final set of conjugations between Hfr 4316 and ZP10 strR thy-, gave conjugates after mating times of 20 minutes but yielded no conjugates after mating times of 50 minutes. Again, colonies would have had to retain their resistance to str and no longer require thymine in order to grow on the selective medium. Taken together, the results of the conjugation studies indicated that the Mu d(Ap lac) insertion was certainly between [illegible] and 68 minutes and probably between 58 and 68 minutes. [illegible] ten minute region was further examined by transduction analysis.

B. Transductions

The curing of amino acid auxotrophy is a frequently-utilised procedure for selecting transductants. Genes lysA and serA are both in the region of insertion suggested by the conjugations. Phage were grown on ZP10 and used to

transduce E. coli K-12 strain MH420, a lys mutant and strain 6350, a serA mutant. Transductants were selected for lys+ or ser+ phenotypes by plating on MMNG. (Colonies which appeared after two days, were transferred to LB, purified, and tested on MMNG and MMNG amp).

There was no detectable linkage of Mu d(Ap lac) to lysA; however, serA, ampicillin resistance and the mucoid phenotype, are co-transduced 62 % of the time (Table 10). These results indicate that the Mu d(Ap lac) insertion is near serA. This region has not previously been reported as containing a locus causing overproduction of exopolysaccharide by E. coli K-12.

Table 10.

TRANSDUCTIONS

Phage grown on ZP10 used to transduce strains MH420 and 6350

	# Colonies*	# Amp R	# Mucoid	% Cotrans-duction
MH420				
Trial 1	155	-	-	-
Trial 2	145	-	-	-
6350				
Trial 1	111	39	39	35
Trial 2	157	121	121	77
Trial 3	162	122	122	75
Average				62

* Colonies were obtained during the initial selection procedure and are lys+ in the case of MH420 and ser+ for 6350.

DISCUSSION

A chromosomal mutation mapping near serA was generated by Mu d(Ap lac) insertion. This is the first time that such a locus has been implicated in overproduction of exopolysaccharide in E.coli K-12. In this discussion, four aspects of my research will be considered: a) the site of the insertion b) speculations on the nature of the actual mutation c) the nature of the polysaccharide d) the physical aspects of the insertion ie. orientation etc. While this project did not explore the possible roles of exopolysaccharides, it is appropriate to briefly address the question here and give some indication of why the study of antigens is important. Finally, I will suggest some further work which could be carried out on mutant ZP10.

a) The insertion site

Polysaccharide biosynthesis is a highly complex process involving a large number of enzymic reactions. From the evidence published to date, the structural genes for the enzymes involved in colanic acid (51) or K-antigen (46) production, occur in several distinct clusters and at least some of these genes are co-regulated. It was pointed out earlier that there is some overlap in the enzymes and monosaccharides involved in colanic acid and lipopolysaccharide synthesis; depending on their precise composition, a similar overlap may also apply to K-antigens. It is not unreasonable to suppose, then, that although

E. coli K-12 has not thus far been shown to produce K- or O-antigens, it may well have the structural genes required for their synthesis. In fact, a number of rfb genes, responsible for O-antigen synthesis in other E. coli strains, are found in E. coli K-12 even though no O-antigen is produced by this strain. Therefore, kps genes, involved in K-antigen synthesis, could be present but not normally expressed.

The genetic mapping results indicate that the Mu d(Ap lac) insertion is very close to serA. Transduction with the lysA- strain MH420 was done to determine if the insert was located between serA and lysA. The lack of cotransduction with lysA indicates that the insertion is closer to serA than to lysA. Due to the parameters of the transduction, these results do not localize the insertion on one side or the other of the serine gene. Phage P1 can package a maximum length of DNA equivalent to 2 minutes on the E. coli chromosome, whereas the Mu d(Ap lac) insertion is about 0.8 minutes long. This leaves a length of approximately 1.2 minutes for bacterial DNA. Inasmuch as lysA and serA are about 1.5 minutes apart (1), linkage to serA but not to lysA is possible, even if the insertion maps between the two loci.

None of the previous genetical work done on colanic acid biosynthesis has implicated linkage of an overproduction locus to serA. As reviewed in the introduction, mutation at a number of loci can lead to

overproduction of colanic acid. Since E. coli K-12 has been found to produce only this exopolysaccharide, it is possible that we have mapped yet another locus for colanic acid. However, linkage of kps genes to serA has been demonstrated in a number of K-antigen producing strains of E. coli, including K1 (46), K10 (41) and K54 (39). Until the actual structure of the ZP10 exopolysaccharide has been elucidated (see below), the possibility of K-antigen production by E. coli strain ZP10 cannot be dismissed.

b) Speculations on the nature of the actual mutation

A single mutation caused by the insertion of Mu d(Ap lac) rendered the non-mucoid parent, CU, mucoid. Wild-type strains of E. coli K-12 produce excess colanic acid when growing slowly at low temperatures (35) and therefore must have the structural genes required for colanic acid synthesis. Strain CU is mucoid when cultured on limiting nitrogen. Under normal growth conditions, we were able to isolate some exopolysaccharide from CU, indicating that colanic acid is normally produced in small quantities by this strain of E. coli K-12. If insertion mutant ZP10 makes excess colanic acid under circumstances where the parental strain does not, , what might be the cause of this?

Insertion mutagenesis usually causes substantial damage to the gene(s) affected. The resulting phenotypes are more likely due to a failure to produce a viable gene

product, than to a change in function of a gene product. A point mutation causing a mucoid phenotype, might be due to modification of an allosteric site on a (rate-limiting) enzyme such that the enzyme is no longer susceptible to feedback inhibition; such a mechanism for mucoidy is highly unlikely, however, in the case of insertion mutagenesis.

The insertion did not cause any recognizable pleiotropic effects in ZP10, suggesting that the mutation is specific to polysaccharide production. There are at least three possible models to account for overproduction of exopolysaccharide in ZP10:

i) insertion may have occurred in a gene coding for a repressor of colanic acid synthesis. A functional repressor protein can no longer be made, resulting in excessive colanic acid biosynthesis.

ii) the insertion may have damaged the promoter of a repressor protein such that the repressor gene can no longer be transcribed, again resulting in overproduction of colanic acid.

iii) a positive regulatory protein is made in larger quantities than normal due to the disruption of its regulation. Mutation at an <u>rcsA</u> like gene causing overproduction of the gene product illustrates this model. For reasons explained above, it is unlikely that insertion mutagenesis in the gene coding for a positive factor would cause it to be "super" functional.

The quantity of exopolysaccharide produced by ZP10 is

far less than that synthesized by M6, though the amount bound to the cells is similar for both strains. It has been postulated (49) that there are a limited number of attachment sites available for exopolysaccharide on the outer membrane; when these are filled, any further polysaccharide produced, will be excreted into the media. It is not known whether new exopolysaccharide replaces the old, the latter being dispersed into the media, or if the old remains attached and the new is excreted. Since ZP10 shows negligible quantities of polysaccharide in its supernate, it may not have saturated its attachment sites. This hypothesis is supported by data showing that ZP10 appears more mucoid when grown on pyruvate as a carbon source than on glucose and that this increase in mucoidy is accompanied by an increase in detectable methylpentose in the supernate (see Appendix II).

Strains M6 and ZP10 both make excess polysaccharide, but in very different amounts. Perhaps some feedback mechanism in ZP10 limits polysaccharide production to the maximum amount that can be accommodated by the attachment sites on the cell surface, while M6 lacks this feedback mechanism. This would imply that there is hierarchial control of exopolysaccharide synthesis.

Another explanation for the absence of exopolysaccharide in the supernate of mutant ZP10 is that the polysaccharide remains very tightly bound to the outer membrane. One might expect, then, that ZP10 would have

more cell-bound exopolysaccharide than M6. However, similar amounts of cell-bound polysaccharide were extracted from strains ZP10 and M6.

The question arises: if ZP10 and M6 both exhibit excessive production of exopolysaccharide, why does ZP10 make less exopolysaccharide than M6?

As discussed earlier, the pattern of derepression of colanic acid biosynthetic enzymes differ from one mucoid mutation to another. The mutation in strain ZP10 might derepress one or more of these enzymes, leading to a modest increase in colanic acid output, in comparison to the lon mutant. The system might be such that some or even only one of the key enzymes remain at wild-type levels, slowing down biosynthesis. As genes for K-antigen biosynthesis are known to be linked to serA, our localization of the Mu d(Ap lac) insertion in ZP10 raises the possibility that enzymes common to K-antigen and colanic acid biosynthetic routes are derepressed in this mutant due to insertion at a K-antigen control site. These enzymes, which in the past might have been used for both K-antigen and colanic acid production, are now being used exclusively for colanic acid synthesis in ZP10. According to this scenario, E. coli K-12 once elaborated K-antigens, but lost some of the genes while retaining a control site; in the absence of some elements necessary for K-antigen synthesis, these enzymes previously shared between the K-antigen and colanic acid biosynthetic pathways would now be utilized exclusively for

the synthesis of colanic acid.

If ZP10 is not making colanic acid, it might be producing a K-antigen. This would require a model where the structural genes for K antigen production are present in E. coli K-12. A previous mutation in a gene coding for a repressor would have caused permanent repression of the system. The Mu d(Ap lac) insertion might have damaged this gene causing derepression of the genes coding for the K-antigen enzymes. The insertion would reverse the original defect that had occurred in the wild type.

c) The nature of the polysaccharide

The IR spectra of the purified polysacchardie from E. coli K-12 strains M6 and ZP10 and the results of our chemical analysis are consistent with the exopolymer being a L-fucose containing polysaccharide. The IR spectra also indicated the presence of some uronic acids. The pmr spectra of M6 and ZP10 do show slight discrepancies and due to lack of resolution in the area where these occur, it has been difficult to evaluate their significance.

The cell-bound exopolysaccharide produced by M6 and ZP10 behaved identically during the purification. Both precipitated at NaCl concentrations in the region where colanic acid is known to precipitate as a Cetavlon complex (40). The critical salt concentration at which these complexes precipitate, is a function of the anionic groups in the polysaccharide and of the molecular weight of the

polymer. For a given anionic polysaccharide there is a linear relationship between the molecular weight of the species and the salt concentration at which it precipitates. (31). That complete precipitation of the polysaccharide-Cetavlon complex was obtained at the same salt concentrations for the M6 and ZP10 slime layers suggests that the two probably have similar molecular weights.

Immunological studies which are now being carried out for our laboratory, should determine whether the polysaccharide produced by ZP10 has the same serological characteristics as colanic acid.

Some K-antigens are composed of the same monosaccharides as are found in colanic acid. The existence of such overlap demands caution in interpreting the physical data. Cetavlon precipitation as an analytical tool has not been tested on all K-antigens and it is likely that this technique only distinguishes between the M antigen and some of the approximately 100 known K-antigens.

If the ZP10 exopolysaccharide is indeed colanic acid, then there is a locus close to serA which is instrumental in controlling its biosynthesis. If the polysaccharide is a K antigen, then these results indicate that linkage to serA is not exclusive to the low molecular weight antigens as has been proposed by Jann and Jann (24), because the Cetavlon precipitation indicates that the ZP10 polysaccharide is of a molecular weight comparable to that of colanic acid.

d) Physical aspects of the insertion.

Mutant ZP10 has very low β-galactosidase activity. There are a number of possibilities to explain this. If the orientation of the Mu d(Ap lac) DNA in ZP10 is reversed relative to that of the promoter of the gene into which it is inserted, then the lacZ gene would only be transcribed at very low levels. In that case, whatever transcription does occur would probably result from read through of the transcript from an adjacent gene.

Another reason for the very low β-galactosidase levels in ZP10 could be very inefficient transcription caused by destruction of the promoter by the insertion. This would also apply if the insertion occurred in a gene necessary for its own expression (51).

Low β-galactosidase levels would also be expected if the insertion site is in a regulatory gene. Regulatory gene products are usually only made in small amounts; that the β-galactosidase levels are very similar in ZP10 grown on glycerol (non-mucoid phenotype) and on pyruvate (highly mucoid phenotype) supports a regulatory gene site, provided the insertion is in the correct orientation.

While ZP10 expressed low levels of β-galactosidase, it was possible to obtain mutants with high enzyme activity. This only suggests that in order to obtain such mutants, a promoter has been affected but does not indicate whether it is the promoter of the exopolysaccharide related gene or an adjacent promoter.

e) The role of exopolysaccharides

Exopolysaccharides play a determining role in the virulence of micro-organisms. Exopolysaccharides, especially in capsular form can provide the bacterial cell with protection. The capsule of Streptococcus pneumoniae protect the organism against phagocytosis. Exopolysaccharides can hide other antigenic determinants of micro-organisms enabling the cells to circumvent a host's immune system. This phenomenon is known as "masking" (52). Exopolysaccharides can also enhance a pathogen's virulence by enabling it to tenaciously adhere to the host.

Cell-bound exopolysaccharides confer a negative charge on the cell surface, which can influence the uptake of ionized molecules. The entry of anionic compounds can be slowed while that of cationic species accelerated. Given the acidic character of many of the β-lactam antibiotics, for example, a negatively-charged capsule may render the bacterium resistant to these drugs (7).

The polysaccharides are highly hydrated and so may help keep the bacterium moist under adverse conditions. Many organisms produce these substances under nutrient limiting conditions.

f) Future work

Control of the biosynthesis of exopolysaccharides warrants further study because of the insights we can gain about the regulation of complex systems. The control,

assembly and export of polysaccharides, might be an important model for the production through cloning techniques, of complex products involving a number of genes.

It will be necessary to carry out a more rigorous analysis of the chemical structure of the ZP10 exopolysaccharide. If it is unequivocally proven to be colanic acid, the level of derepression of the biosynthetic enzymes should be determined. The pattern of derepression could then be compared with those of the known mutations.

Cloning and deletion techniques could be used to pinpoint the actual site near serA which causes the mucoid phenotype.

It has been proposed, that at least two loci are required for the expression of K-antigens (39). Complementation studies with strains which elaborate K-antigens could be used to see if this mutation is of any significance to their production.

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Appendix I

Polysaccharide purification: spectrophotometric analysis of protein content

Aqueous dilutions (1:80) of the ultracentrifugation supernate following digestion of nucleic acids and dialysis were assayed for protein content by the procedure of Kalckar (13).

$$\text{mg protein/mL} = (1.45\ OD\ 280) - (0.74\ OD\ 260)$$

	CU	M6	ZP10
OD 260	0.427	0.338	0.337
OD 280	0.200	0.162	0.159
mg/mL protein	0	7.3×10^{-4}	0

Appendix II

Methylpentose assay performed on supernates of pyruvate-grown cells of *E. coli* K-12

	CU	ZP10	M6
ug/mL methylpentose	1.7	21.6	160

Performed on 20 mL cultures grown in 0.4% pyruvate, to a density of 180-190 Klett units.