Isolation from *Klebsiella* and Characterization of Two *rsc* Genes That Activate Colanic Acid Capsular Biosynthesis in *Escherichia coli*

By PHILIPPA ALLEN,^{1,2} C. A. HART² AND J. R. SAUNDERS^{1*}

Department of Microbiology¹ and Medical Microbiology², University of Liverpool, PO Box 147, Liverpool L69 3BX, UK

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Two genes, designated *rscA* (regulation of capsule synthesis) and *rscB*, that had been cloned from the chromosome of *Klebsiella aerogenes* (*K. pneumoniae*) capsular serotype K21 were capable of activating expression of colanic acid capsular polysaccharide in *Escherichia coli* K12. The *Klebsiella rscA* gene encoded a polypeptide of 23 kDa that was required for the induction of a mucoid phenotype at $\leq 30^\circ\text{C}$ but not at $\geq 37^\circ\text{C}$. The *Klebsiella rscB* locus encoded no apparent polypeptides and was not capable by itself of causing the overproduction of colanic acid. However, when present in the same cell with *rscA*, either *in cis* or *in trans*, *rscB* caused expression of mucoidy in *E. coli* at all growth temperatures. These findings are best explained if the *Klebsiella rscA* gene product acts as a positive regulator of colanic acid biosynthesis in *E. coli* and that activity of this protein is in turn subject to regulation by Lon protease. The *Klebsiella rscB* locus may exert its effect by preferentially binding a negative regulator of capsular biosynthesis, possibly Lon itself. DNA sequences homologous to the *Klebsiella* K21b *rscA* and *rscB* genes were found in the genomes of all other capsular serotypes of klebsiellae examined, including K2, K12, K36 and K43. However, there was no homology between such genes and the chromosome of *E. coli*. The ability of these *rsc* genes to induce a mucoid phenotype explains the apparent conjugative transfer from klebsiellae to *E. coli* of the ability to produce K21 or other *Klebsiella* capsular polysaccharides that are structurally and antigenically related to colanic acid.

INTRODUCTION

Klebsiellae are important nosocomial pathogens (Meers *et al.*, 1981) and can cause life-threatening bacteraemia in adults (de la Torre *et al.*, 1985) and in neonates (Morgan *et al.*, 1984). Characteristically *Klebsiella* species are surrounded by a hydrophilic polysaccharide capsule and at least 83 different capsular serotypes have been recognized (Nimmich, 1968). It has been reported that the ability to express a sero-specific capsular antigen can be transferred from some strains of *K. aerogenes* (*K. pneumoniae*) K21 to *Escherichia coli* K12 (Barr, 1981). Attempts in this laboratory to transfer the ability to elicit either a mucoid character or K21 antigen *per se* by conjugation to *E. coli* or other klebsiellae have proved unsuccessful. However, it has proved possible to transfer genes responsible for the expression of a mucoid phenotype by conjugation from a K36-producing strain of *K. aerogenes* to *E. coli* K12 (unpublished observations).

E. coli K12 is non-mucoid under normal laboratory conditions but some mutant strains are capable of synthesizing large amounts of the capsular polysaccharide colanic acid under certain conditions (Markovitz, 1977). Mutations at a number of loci, including the *lon* gene, result in copious synthesis of colanic acid and hence confer a mucoid phenotype. The *lon* gene of *E. coli* codes for a tetrameric protein with subunits of 94 kDa (Schoemaker & Markovitz, 1981). This protein exhibits multiple enzymic activities and probably acts as a repressor of the enzymes involved in colanic acid synthesis (Trisler & Gottesman, 1984).

Abbreviations: CIE, countercurrent immunoelectrophoresis; MMS, methyl methanesulphonate.

Colanic acid cross-reacts strongly with a number of *Klebsiella* antigens including the K21 polysaccharide (Henriksen, 1954). The *Klebsiella* K21 serotype does not form a homogeneous population but consists of strains producing one or other of two chemically distinct types of capsular polysaccharide which we have referred to as K21a for capsules with the structure reported by Choy & Dutton (1973) and a novel type referred to as K21b (unpublished observations). During an investigation of the genetic regulation of the production of K21b-type capsular polysaccharide we noted that some *Klebsiella* DNA fragments can activate colanic acid synthesis in *E. coli* K12. This paper describes the isolation and characterization of two *Klebsiella rcs* (regulation of capsule synthesis) genes capable of producing this response.

METHODS

Bacterial strains and plasmids. *Klebsiella aerogenes* strain U53/1 (redesignated 1L918) and an *E. coli* transconjugant, designated 1L920, were obtained from J. G. Barr, Victoria Hospital, Belfast, N. Ireland (Barr, 1981). Other *Klebsiella* strains were clinical isolates obtained from the Royal Liverpool Hospital. *E. coli* DH1 (*recA*, *hsd* r_k^- , m_k^+) (Hanahan, 1983) was used as the recipient in cloning experiments. *E. coli* C600 (*recA*⁺) (Appleyard, 1954) was used in methyl methanesulphonate (MMS) sensitivity experiments. Plasmids pLV59 (O'Connor & Humphreys, 1982), pBR322 (Bolivar *et al.*, 1977) and pLG339 (Stoker *et al.*, 1982) were used as cloning vectors.

Isolation of DNA. Chromosomal DNA was prepared according to the method of Marmur (1961). Plasmid DNA was isolated by the bulk preparation method of Humphreys *et al.* (1975).

Restriction enzyme digestion and ligation of DNA. Restriction enzymes (obtained from P and S Biochemicals, Liverpool, or from Boehringer) were used according to the supplier's recommendations. DNA fragments were typically ligated at a concentration of 100 $\mu\text{g ml}^{-1}$ at 16 °C overnight; 0.2 U of T4 DNA ligase was added for each μg of DNA.

Agarose gel electrophoresis. DNA samples were run on horizontal agarose slab gels containing ethidium bromide in Tris/borate buffer (89 mM-Tris, 89 mM-boric acid, 2 mM-EDTA). DNA samples were mixed with loading buffer [50 mM-EDTA, 4 M-urea, 8% (w/v) Ficoll, 0.1% (w/v) bromophenol blue] and gels were run at 100 V for several hours or 20 V overnight. DNA was visualized using a 302 nm transilluminator.

Transformation. Transformation of CaCl_2 -treated *E. coli* and *Klebsiella* with plasmid DNA was by the method of Saunders *et al.* (1984).

Hybridization of DNA. DNA from agarose gels was transferred to nitrocellulose filters according to the method of Southern (1975). Probe DNA (1 μg) was labelled with [α - ^{35}S]dATP (10 μCi , 370 kBq) by nick translation with DNA polymerase I. The filter-bound DNA was hybridized overnight at 65 °C with 2 $\times$ SSC (0.3 M-NaCl, 0.03 M-trisodium citrate), 0.3% (w/v) sodium dodecyl sulphate (SDS), 100 μg denatured calf thymus DNA and 1 μg denatured probe DNA. The filter was then washed at 65 °C in 2 $\times$ SSC containing 1% (w/v) SDS, dried and exposed to X-ray film overnight.

MMS sensitivity. Strains were grown in LB overnight and then appropriately diluted before spreading on LB agar plates and LB-MMS plates (containing 0.05%, v/v, MMS). Plates were incubated overnight and colony counts were subsequently performed.

Capsular serotyping. This was done by using counter-current immunoelectrophoresis (CIE) as described by Palfreyman (1978) and by immunofluorescence. Strains were also serotyped independently by the Coventry Public Health Laboratory using CIE and Quellung reactions as described by Casewell (1972).

Polysaccharide extraction. Exopolysaccharide was extracted from mucoid strains according to the method of Wilkinson *et al.* (1955). Bacteria were grown on a minimal medium consisting of 0.4 g NH_4Cl , 7.5 g Na_2HPO_4 , 3.0 g KH_2PO_4 , 0.5 g NaCl, 0.2 g $\text{Mg}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 10 g agar in 1 l distilled water. The cells were then harvested and resuspended in distilled water. The capsules were removed from the cells by boiling for 10 min and the suspension was centrifuged for 30 min at 20 000 g. The supernatant was retained and the capsular polysaccharide was precipitated with 2 vols acetone. The polysaccharide was resuspended to form a 0.5% (w/v) solution in acetate buffer [4% (w/v) sodium acetate, 2% (w/v) acetic acid] and was then deproteinized by shaking 100 ml volumes with 20 ml chloroform and 4 ml butanol. The polysaccharide was then reprecipitated with 2 vols acetone, dialysed extensively against tap water, precipitated and freeze dried. Purified polysaccharide (5 ml) was hydrolysed in 1 ml HCl (2 M) for 3 h at 100 °C, rotary evaporated and resuspended in 20 μl distilled water. The hydrolysates were applied to Whatman 3MM chromatography paper and run in butanol/acetic acid/water (4:1:5, by vol.) solvent. The papers were developed with alkaline silver nitrate to allow detection of the sugars.

Isolation and labelling of minicells. Minicells were isolated from *E. coli* DS410 and its plasmid-bearing derivatives essentially by the method of Halliwell & Sherratt (1976). Minicells were purified on sucrose gradients and

resuspended in minicell labelling medium containing 0.01% (w/v) methionine assay medium, 1% (w/v) glucose and M9 minimal salts solution. Minicells (2×10^8) were preincubated before the addition of [35 S]methionine (30 μ Ci, 1.1 MBq) and further incubated for 60 min. After labelling, the minicells were washed and resuspended in final sample buffer [10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, 3% (w/v) SDS, 0.0625 M-Tris, 0.01% (w/v) bromophenol blue pH 6.8]. The sample was then boiled and analysed by SDS-PAGE.

SDS-PAGE. SDS-PAGE of proteins was done on 10–17% (w/v) gradient vertical slab gels according to the method of Laemmli (1970). The gels were run typically with a current of 10 mA for 1 h and then at a constant current of 20 mA until the tracking dye reached the bottom of the gel. The gel was stained with Coomassie blue, dried down on to Whatman 3 MM paper and exposed to X-ray film.

DNA sequencing. Cloned DNA was cut in single or double digestions with a number of restriction enzymes and was then ligated to appropriately cleaved M13 mp8 or mp9 DNA (Messing & Vieira, 1982). Ligation mixtures were transfected into *CaCl*₂-treated *E. coli* JM103 cells. M13 recombinants were identified as white plaques on plates containing X-gal and IPTG. Preparation of single-stranded template and sequencing reactions have been described elsewhere (Walker *et al.*, 1986). Samples were boiled for 3 min before loading on polyacrylamide gels (6%, w/v). The gels were typically run at 30 mA until the tracking dye reached the bottom of the gel. The gel was fixed, washed and then dried at 80 °C for 60 min. The dehydrated gel was then exposed to X-ray film overnight. The entire sequence of the cloned DNA was determined by sequencing a series of overlapping M13 mp8 or mp9 subclones.

RESULTS

Cloning of Klebsiella DNA in pLV59

The plasmid pLV59 (O'Connor & Humphreys, 1982) is a derivative of pACYC184 that carries a chloramphenicol-resistance determinant and the *Eco*RI restriction/modification genes. The *Eco*RI methylase gene carried by this plasmid is temperature sensitive, being functional at 28 °C but not at 37 °C. Hence, the vector is lethal to its host at 37 °C and above. Cloning of a DNA fragment into the *Bgl*II site inactivates the *Eco*RI restriction gene and permits cells containing the hybrid plasmid to survive at 37 °C. Partial *Sau*3A digests of total *Klebsiella* K21b (1L918) DNA were cloned into the *Bgl*II site of pLV59 and the ligation mixture was used to transform *E. coli* DH1. Two recombinant clones were isolated that conferred a mucoid phenotype on *E. coli* grown at 37 °C. The clones possessed recombinant plasmids with DNA inserts of 14 kb (pLV210) and 11.5 kb (pLV211) respectively. Restriction analysis of plasmid DNA indicated that a 9 kb region of inserted DNA was shared by both pLV210 and pLV211. Subcloning of *Bgl*II/*Bam*HI fragments of pLV210 into pLV59 localized the *Klebsiella* genes responsible for the mucoid phenotype to a 2.7 kb segment. The recombinant plasmid carrying this fragment was designated pLV212. In addition, subcloning of partial *Sau*3A digests of pLV210 into pLV59 allowed the isolation of clones that possessed a mucoid phenotype when grown at ≤ 30 °C but not at 37 °C. These clones were found to contain identical species of plasmid DNA with an insert size of 1.2 kb. One of these clones was designated pLV213. *Sau*3A digests of pLV210 DNA were also cloned into pBR322, which is compatible with pLV59. Recombinants were transformed into cells containing pLV213 and selection was made for clones that expressed the mucoid phenotype at all growth temperatures. Plasmid DNA was extracted from mucoid strains and pBR322 recombinant plasmids were isolated. Restriction analysis of the plasmid DNA identified two types of pBR322 recombinant clones, containing inserts of 1.2 kb (pLV214) and 0.39 kb (pLV215). The insert DNA of pLV214 was identical to that of pLV213 and *E. coli* cells containing pLV214 possessed a mucoid phenotype when grown at ≤ 30 °C but not at 37 °C. However, the restriction map of the insert DNA of pLV215 differed from that of pLV213 and *E. coli* cells containing pLV215 alone did not exhibit the mucoid phenotype at any growth temperature. The restriction maps of these plasmids are shown in Fig. 1. Insert DNA from pLV213 was also cloned into the low-copy-number (6–8 copies per chromosome) plasmid vector pLG339. The resulting recombinants also expressed the mucoid phenotype when grown at ≤ 30 °C but not at 37 °C. pLV213 when transformed into *K. aerogenes* 1L919, a non-mucoid derivative of the K21b strain 1L918, failed to induce biosynthesis of colanic acid.

Fig. 1. Restriction maps of *Klebsiella* DNA capable of inducing a mucoid phenotype in *E. coli*. The boxed areas refer to the putative coding regions of *rcsA* (pLV213) and *rcsB* (pLV215) deduced from the primary DNA sequence. B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; P, *Pst*I.

Table 1. Capsular serotyping of clones using CIE, Quellung reaction and immunofluorescence

Strain	Reactions*		
	CIE	Quellung	Immunofluorescence
<i>K. aerogenes</i> 1L918	K21	K21	K21
<i>E. coli</i> DH1(pLV210)	NT	K21	K21
<i>E. coli</i> DH1(pLV211)	NT	K21	K21
<i>E. coli</i> DH1(pLV213)	NT	K21	K21
<i>E. coli</i> DH1(pLV59)	NT	NT	NT

NT, not typable.

* Results were identical when the tests were repeated on two separate occasions.

Serotyping of mucoid clones

Capsular serotyping indicated that the *E. coli* clones elicited K21-specific material by immunofluorescence but not by CIE (Table 1). In independent tests by the Coventry Public Health Laboratory the clones were shown to exhibit K21 serospecificity with Quellung reactions but not with CIE. These clones have also been tested with antisera raised against the *E. coli* M antigen and found to react both with *Klebsiella* K21 and *E. coli* M (colanic acid) antisera (F. Ørskov & I. Ørskov, personal communication).

Examination of capsular polysaccharide extracted from mucoid clones

Capsular polysaccharide was extracted from *K. aerogenes* 1L918 and mucoid *E. coli* clones to determine whether the recombinants were producing K21 antigen or colanic acid. Purified polysaccharide was hydrolysed and examined by paper chromatography. The capsular polysaccharide of the mucoid *E. coli* clones contained glucuronic acid, galactose, glucose and fucose in accordance with the reported structure for colanic acid (Garegg *et al.*, 1971) rather than K21b polysaccharide, which contains glucuronic acid, galactose and rhamnose (unpublished observations). In addition, the mucoid transconjugant reported by Barr (1981) to have received K21-specific genes appeared to produce colanic acid rather than K21b capsular polysaccharide.

MMS sensitivity of mucoid clones

lon mutants produce altered Lon protease with a decreased ability to carry out *in vitro* ATP-dependent proteolysis (Chung *et al.*, 1983). *lon* mutants also exhibit several other phenotypic characteristics, including sensitivity to DNA damage (Howard-Flanders *et al.*, 1964) and decreased lysogenization by phage λ (Walker *et al.*, 1973). To determine whether the *E. coli*

Fig. 2. Southern transfer hybridizations of DNA cleaved with *Bgl*II and *Bam*HI from *klebsiellae* and mucoid *E. coli* derivatives using pLV213 DNA as a probe. Tracks: (1) pLV213, (2) plasmid DNA extracted from *Klebsiella* K21b 1L918, (3) 1L918 chromosomal DNA, (4) total DNA from *Klebsiella* 1L919, (5) total DNA from *E. coli* transconjugant 1L920, (6) total DNA from *K. aerogenes* K36 1L884, (7) total DNA from *E. coli* DH1. In track 4, bands of homology additional to the 2.7 kb fragment are attributable to partial digestion of *K. aerogenes* 1L919 DNA.

clones carrying *Klebsiella* DNA were mucoid due to the generation of a *Lon*⁻ phenotype, the clones were tested for MMS sensitivity. *lon* mutants are unable to induce the SOS repair system and are thus sensitive to the SOS-inducing agent MMS. pLV213 and pLV212 were transformed into *E. coli* C600, which is *recA*⁺ and thus competent to repair DNA damage. Strains carrying either recombinant plasmid were MMS-resistant. Furthermore, carriage of these plasmids did not render the host cells liable to filamentation following UV-irradiation nor reduce plaquing efficiency of λ phage (data not shown). Thus neither of these cloned segments of the *K. aerogenes* chromosome renders *E. coli* *Lon*⁻.

Hybridization of cloned DNA

To determine the location of the cloned 1L918 genes, plasmid and chromosomal DNA preparations isolated from *K. aerogenes* 1L918 were probed with labelled insert DNA from pLV213. Chromosomal DNA preparations isolated from 1L919 (a spontaneous non-mucoid derivative of the K21b strain), the *K. aerogenes* K36 strain 1L884, the mucoid *E. coli* transconjugant (1L920) and *E. coli* K12 (DH1) were also included. The hybridization data shown in Fig. 2 demonstrate that the cloned mucoidy genes from 1L918 are located on a 2.7 kb *Bgl*II-*Bam*HI fragment of chromosomal DNA rather than on plasmid DNA. The results also indicate the presence of these genes in the non-mucoid *Klebsiella* derivative and in the K36 *Klebsiella* strain. However, the genes appeared absent in the *E. coli* transconjugant and in *E. coli* K12. DNA homologous to the cloned DNA from pLV213 was also present in *Klebsiella* serotypes K2, K12 and K43 (data not shown).

DNA sequencing of pLV213 and pLV215

The sequence of the 1.2 kb insert of pLV213 was determined by the dideoxy chain termination method using overlapping subfragments subcloned in M13 mp8 and mp9. The sequence of the cloned DNA in pLV213 is shown in Fig. 3. Only one open reading frame of 1165 nucleotides with recognizable transcription initiation and termination sites was evident. The sequence of the 0.39 kb insert of pLV215 was also determined and contained one possible short coding region apparently of 220 bp (Fig. 4).

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1      GATCAACCTGTACGCAACCGGCCAGCGCCACCACCATGCCAGCAAAAAATAACTTTTT
59    CATCATTATCCACACAAGCCGATAAAAAAGTATTGTGGCATTAAAGCGTTAAGCCTCCACCC
122   CCACCGCAAAGGGATAAGCGAGCAAGCCAGCAACCGCTAACGTGGGTTTCATTTGAAGTAAGG
185   AAATTCTGAAAGTAAAGAATACTGGGCGCGTAACCATAGCATCTATGGGCACTTTTTGTTTT
248   TAATTCGGTCACACTACCGGTTCTTGACTTTACTTTAAGAGTTTTCTGGCAATTATATGCA
311   TAGATGCGGAATAGTTTAAATGGAGCTAATGGGTTCTTTCTAAACCTACTATTATTATCGCCG
374   CAAGGACTGCTTCGCACAGCCAGTGCGAAGTGATATCGTTACGTGTTGATTGAGGATGGGTC
437   ATG TCA ACG ATG ATT ATG GAT TTG TGC AGC TAT ACC CGG TTG GGA TTG
      Met Ser Thr Met Ile Met Asp Leu Cys Ser Tyr Thr Arg Leu Gly Leu
485   ACG GGA TAT CTG ACC AGT CGG GGA ATT AAA AAA CAG GAA ATC GTT GAG
      Thr Gly Tyr Leu Thr Ser Arg Gly Ile Lys Lys Gln Glu Ile Val Glu
533   GTC AAC AGT GCT GCG GAT CTG CAG AAA CAC TGT ACG TCG TGT TGC CCG
      Val Asn Ser Ala Ala Asp Leu Gln Lys His Cys Thr Ser Cys Cys Pro
581   GCG GTG GTG TTT CTG AAT GAA GAC TGT TTC GTG CAT GAT GAT GAA AGT
      Ala Val Val Phe Leu Asn Glu Asp Cys Phe Val His Asp Asp Glu Ser
629   AAT GGC ATT ATT CGC CAG ATC ATT ACG CAA AAC CCG GCG ACG CTG TTT
      Asn Gly Ile Ile Arg Gln Ile Ile Thr Gln Asn Pro Ala Thr Leu Phe
677   GTT ATC TTT ATG TCG CTG GCG AAC ATC CAT TTT GAC CGC TAT TTG CGG
      Val Ile Phe Met Ser Leu Ala Asn Ile His Phe Asp Arg Tyr Leu Arg
725   GTA CGG AAG AAT CTG CTA ATC AGT TCA AAA TCG ATA ACC CCA AAA GAC
      Val Arg Lys Asn Leu Leu Ile Ser Ser Lys Ser Ile Thr Pro Lys Asp
773   CTT GAT GTT ATT CTG GTT AAT TAT CTT AAA TAC AAA AAC ACC AGT GTA
      Leu Asp Val Ile Leu Val Asn Tyr Leu Lys Tyr Lys Asn Thr Ser Val
821   GGC CAG TTA ACT TTA CCG ACA TTG TCA CTG AGT AAA ACA GAA TCA AAT
      Gly Gln Leu Thr Leu Pro Thr Leu Ser Leu Ser Lys Thr Glu Ser Asn
869   ATG CTG CAA ATG TGG ATG GCC GGG CAT GGT ACT TCG CAA ATC TCA ACG
      Met Leu Gln Met Trp Met Ala Gly His Gly Thr Ser Gln Ile Ser Thr
917   CAA ATG AAC ATC AAA GCG AAG ACG GTA TCG TCG CAT AAA GGC AAT ATT
      Gln Met Asn Ile Lys Ala Lys Thr Val Ser Ser His Lys Gly Asn Ile
965   AAA AAG AAA ATA CAA ACG CAT AAT AAG CAG GTG ATT TAT CAT ATC GTT
      Lys Lys Lys Ile Gln Thr His Asn Lys Gln Val Ile Tyr His Ile Val
1013  CGG CTG ACC GAA AAC ATC ACC TCC GGT ATT CAG GTA AAT ATG CGC TGA
      Arg Leu Thr Glu Asn Ile Thr Ser Gly Ile Gln Val Asn Met Arg
1061  AACAACTGGCGGCGCTCCCGCCAGTTCAGTTTTACGAGACCTCTTTTCCACGAAAATCCCA
1124  TCCGGGTGGCCTATTTTATTAAAAACAGATC

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Fig. 3. Nucleotide sequence of the region containing the *rcs* gene carried by pLV213 and the predicted amino acid sequence of the *rcsA* protein. The suggested Shine–Dalgarno sequence is boxed and sequences sharing homology with the *E. coli* consensus –10 promoter sequence are underlined. A classical *E. coli* –35 promoter region was not apparent.

Insertional inactivation of plasmid DNA

The cloned *rcs* gene represented by the 1165 bp open reading frame specified by pLV213 was inactivated by the *in vitro* insertion of *Cla*I fragments of λ DNA into the single *Cla*I site present within the putative coding region. The resulting ligation mixture was transformed into *E. coli* K12 and plasmid DNA was extracted from clones that possessed a non-mucoid phenotype at

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1          GATCGCAGGCGATCGCGCTATCGAATGAAACCGGTTT
38 GCGGCTAAGCCTTCTCACATCAGCCTTGCAGCCGATAAGTCATCAAGCCCAAC
93 ATTCTCAATATTTTTCGTCATGAATATCTTGTTCATGCAAATAATTGAGGATA
148 ATG TTT CAA CGC ATC GGA TTT CCC GGT GTA ACG AAT TTT CAA
    Met Phe Gln Arg Ile Gly Phe Pro Gly Val Thr Asn Phe Gln
190 GTG CTT CTT GCA TTA GCA AGT TTG ATC CCG ACT CCT GCG AGT
    Val Leu Leu Ala Leu Ala Ser Leu Ile Pro Thr Pro Ala Ser
232 CGG GAT TTT TTT CGC CTG CCG CTA ACG GTT GGC TTC ACT GGC
    Arg Asp Phe Phe Arg Leu Pro Leu Thr Val Gly Phe Thr Gly
274 GCT GAA ATC GTG AAT ATC GAG TTC GAA TGC CTC CGC CAG CTC
    Ala Glu Ile Val Asn Ile Glu Phe Glu Cys Leu Arg Gln Leu
316 ACG CCA GGT ATG TGA CCCGCGCCATCGATGCTGGCGCGCTGGCGCGCTG
    Thr Pro Gly Met
366 CGGGTGTCCAGCGTTCGATGGACTTCGCTTCGCTGATC

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Fig. 4. Nucleotide sequence of the region containing the *rcsB* locus carried by pLV215 and the predicted amino acid sequence of a possible *rcs* protein. A putative Shine-Dalgarno sequence and a possible — 10 promoter sequence are shown as referred to in Fig. 3.

<30 °C. Two of these insertionally inactivated clones, designated pLV216 and pLV217, were subsequently subjected to analysis in minicells.

Minicell analysis of plasmid DNA

Minicells were utilized to determine the nature of any proteins specified by the recombinant plasmids pLV213 and pLV215. These plasmids were transferred to the minicell-producing *E. coli* strain DS410 and plasmid-encoded protein synthesis was examined in purified minicells. Four proteins with apparent molecular masses of 17, 19, 23 and 40 kDa were shown to be expressed by pLV213 in addition to the polypeptides encoded by the vector. Examination of protein synthesis in derivatives in which the putative *rcs* gene had been inactivated by insertion of DNA fragments revealed the loss of the 23 kDa polypeptide species (Fig. 5). This protein has a molecular mass equivalent to a polypeptide translated from the predicted open reading frame of the sequenced *rcs* gene in pLV213. The remaining polypeptides specified by the insert probably represent read-through translation products from vector sequences. The cloned DNA in pLV215 could potentially specify a polypeptide of approximately 7000 Da. However, this recombinant plasmid did not appear to specify any proteins other than those attributable to the vector itself.

DISCUSSION

Cloning of total DNA from *K. aerogenes* K21b using the plasmid vector pLV59 has allowed the isolation of *E. coli* clones expressing a mucoid phenotype. The clones were of two types, able to express a mucoid phenotype at ≤ 30 °C or at all growth temperatures. We have found that such clones produce *E. coli* M antigen (colanic acid) rather than K21b polysaccharide. Additionally, a supposed *E. coli* transconjugant that had been mated with *K. aerogenes* K21 (Barr, 1981) was shown to be producing colanic acid rather than *Klebsiella* K21 polysaccharide. These findings indicate that the presence of certain heterologous DNA segments may induce the synthesis of the colanic acid polysaccharide in *E. coli* and explain the apparent *in vivo* transfer to *E. coli* of the ability to elicit K21 or structurally related capsular antigens. Mucoid *E. coli* clones bearing *Klebsiella* DNA sequences are not sensitive to the SOS-inducing agent MMS and thus do not appear to have a Lon⁻ phenotype. This would suggest that the genes we have cloned do not induce mucoidity because they specify a defective Lon analogue. However, the regulation of

Fig. 5. Autoradiogram of SDS-PAGE of proteins obtained from minicells carrying *rsc* genes. Minicells were isolated from *E. coli* DS410 carrying pLV59 (track 1), pLV213 (track 2), pLV216 (track 3) and pLV217 (track 4). The 'large' polypeptide band specified by pLV216 but not by pLV217 is probably a λ protein.

colanic acid synthesis in *E. coli* is complex and other regulatory genes, notably *capS* and *capT*, have been described (Markovitz, 1977). Mutations in these gene loci result in the production of large amounts of colanic acid in *E. coli* but do not confer the other phenotypes of *lon* mutants. More recently, Trisler & Gottesman (1984) isolated and characterized a number of *E. coli* genes necessary for the synthesis of colanic acid. Mud *cps::lac* fusions were used to identify three regulatory genes in the *E. coli* chromosome for synthesis of this polysaccharide (Gottesman *et al.*, 1985). These regulatory genes only effect over-production of colanic acid and do not show other *Lon*⁻ phenotypes. Two of these *E. coli* genes, designated *rscA* and *rscB*, act as positive regulators of colanic acid biosynthesis. The *lon* gene product is presumed to regulate capsule synthesis indirectly by regulating the availability of one of the positive regulators. In addition, the *E. coli rscA* gene when cloned on a multicopy plasmid induces mucoidy in *lon*⁺ cells (Gottesman *et al.*, 1985). The cloned fragment of the *Klebsiella* chromosome carried by pLV212 also produces this effect. This *Klebsiella* DNA was divisible into two regions, one specified in pLV213 and the other in pLV215. The *Klebsiella* DNA in pLV213 encodes a single polypeptide of 23 kDa, expression of which in *E. coli* results in mucoidy at temperatures of 30 °C or below. Disruption of the large open reading frame in pLV213 results in simultaneous loss of this protein and the ability to induce colanic acid biosynthesis. The production of this capsular polysaccharide seems to require the elevated synthesis of a number of different *E. coli* enzymes, including phosphomannose isomerase, UDP-glucose pyrophosphorylase and galactose epimerase (Trisler & Gottesman, 1984). Therefore it seems unlikely that the 23 kDa polypeptide possesses an enzymic activity that relieves a single deficiency in the biosynthesis of colanic acid. It seems more reasonable to suppose that this protein is the product of a *Klebsiella* gene, which we have called *rscA*, that is capable of regulating the colanic acid regulon. Capsular polysaccharide

synthesis results at all growth temperatures if the dosage of this *Klebsiella rcsA* is increased. This effect could be explained if the protein specified by the *rcsA* gene is actively competing with a repressor of colanic acid synthesis, such as Lon protease, and if this repressor were to be less readily available at lower growth temperatures. There is some evidence to support this notion as synthesis of colanic acid in *E. coli* is favoured at low growth temperatures (Markovitz, 1977) and *lon* strains show less *cps::lac* expression at 39 °C than at 30 °C (Gottesman *et al.*, 1985). However, the *rcsA* gene specified by pLV213 is present as only a single copy in pLV212 but this recombinant is able to induce the mucoid phenotype at all growth temperatures. A second locus that we have designated *rcsB* carried by the *Klebsiella* DNA in pLV212 and isolated in pLV215 is additionally required to give a mucoid phenotype in *E. coli* at 37 °C. The presence of pLV215 alone in *E. coli* does not result in mucoidy at any growth temperature. The cloned DNA in pLV215 does not appear to specify a protein. Moreover, it is possible that this DNA may preferentially bind and thus decrease the availability of a repressor such as Lon. Lon protein is known to exhibit DNA-binding activity (Zehnbauer *et al.*, 1981) although specific DNA-binding sequences have not been identified. Our results are perhaps best explained if the protein specified by the *Klebsiella rcsA* gene acts fortuitously or otherwise as a positive regulator of colanic acid synthesis and that activity of this regulatory polypeptide is subject in turn to regulation by Lon protease. If this hypothesis were correct then increasing the dosage of *lon* should prevent the activation of colanic acid synthesis by the cloned *Klebsiella* genes.

DNA sequences homologous to the *rcsA* and *rcsB* genes from *K. aerogenes* K21b were found to be present in all serotypes of klebsiellae so far tested, including those of serotypes K2, K12, K36 and K43. Furthermore, additional *rcs* genes that show no homology to *rcsA* and *rcsB* can be detected in cosmid gene libraries of the *K. aerogenes* genome (unpublished observations). Neither *rcsA* nor *rcsB* is capable of inducing colanic acid biosynthesis when returned to *K. aerogenes*. However, their possible role in the regulation of serospecific *Klebsiella* capsular polysaccharides is currently under investigation.

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