A New Regulatory Locus of the Maltose Regulon in *Klebsiella pneumoniae* Strain K21 Identified by the Study of Pullulanase Secretion Mutants

By MICHAEL G. KORNACKER,^{1,2*}† ALAN BOYD,¹‡
ANTHONY P. PUGSLEY² AND GRAHAM S. PLASTOW^{1,3}

¹The Leicester Biocentre, University of Leicester, University Road, Leicester LE1 7RH, UK

²Unité de Génétique Moléculaire, Institut Pasteur, 25 rue du Dr Roux,
75724 Paris Cedex 15, France

³Dalgety PLC, Group Research Laboratory, Station Road, Cambridge CB1 2JN, UK

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This study has shown that *Klebsiella pneumoniae* strain K21 differs from the previously characterized and closely related *K. pneumoniae* strain PAP996 in that expression of the pullulanase gene (*pulA*) and other genes of the maltose regulon is partially independent of exogenous inducer (maltose/maltotriose). Mutants of strain K21 which are defective in pullulanase synthesis and/or secretion were isolated following Tn10 mutagenesis. Three phenotypic classes of mutants were identified. Class I mutants were defective in the surface localization and secretion of pullulanase. Class II mutants did not secrete detectable levels of pullulanase but were able to export pullulanase to the cell surface. Class II mutants also expressed pullulanase and other maltose-regulated genes at markedly lower levels than those found in the parent strain under non-inducing conditions. The single class III mutant was intermediate between K21 and class I mutants; most of the cell-associated pullulanase was localized at the cell surface whilst a significant amount was secreted into the medium. Mapping indicated that all but three of the Tn10 insertions were adjacent to, and at either side of, *pulA*. One class II mutant carried a Tn10 insertion in or close to *malT* whereas in the remaining class II mutants the insertions were located at least 4 kb upstream of *pulA* in a region which may define a new regulatory locus of the maltose operon.

INTRODUCTION

Pullulanase (EC 3.2.1.41) hydrolyses (1→6) α -linkages in (1→4) α -, (1→6) α -linked glucans and is synthesized and secreted by strains of the Gram-negative bacterium *Klebsiella pneumoniae* but not by *Escherichia coli* (Konishi *et al.*, 1979). Pullulanase synthesis is maltose-inducible (Bender & Wallenfels, 1966; Michaelis *et al.*, 1985; Chapon & Raibaud, 1985; Raibaud & Richet, 1987) and the cognate gene, *pulA*, is thus a component of the maltose regulon whose genes are required for the uptake and metabolism of maltose and maltodextrins. In *E. coli*, and at least in part also in *K. pneumoniae* (Woehner & Woerber, 1978*b*; Bloch & Raibaud, 1986*a*), the maltose regulon includes the linked *malT* and *malPQ* genes, the linked *malk*–*lamB*–*malM* and *malEFG* operons (Schwartz, 1987) as well as the *malS* gene (Freundlieb & Boos, 1986). Expression of all genes is positively regulated by the product of the *malT* gene, which is interchangeable between *E. coli* and *K. pneumoniae* (Michaelis *et al.*, 1985; Bloch & Raibaud, 1986*a*). Expression of at least some genes of the maltose regulon is enhanced by another positive regulator, the complex of the *crp*

† Present address: Unité de Génétique Moléculaire, Institut Pasteur, 25 rue du Dr Roux, 75724 Paris Cedex 15, France.

‡ Present address: Department of Biochemistry, University of Edinburgh Medical School, Hugh Robson Building, George Square, Edinburgh EH8 9XD, UK.

gene product with cAMP (Chapon, 1982), rendering the regulon subject to catabolite repression by glucose (Guidi-Rontani & Gicquel-Sanzey, 1981). In addition to the classical component genes of the maltose regulon, *K. pneumoniae* also encodes the *malT*-inducible *malX* operon, which includes some pullulanase secretion genes (d'Enfert *et al.*, 1987b) and an additional maltose-inducible but *malT*-independent maltose uptake system (Schwartz, 1987; Bloch & Raibaud, 1986b).

Pullulanase has a predicted molecular mass of approximately 117 kDa (Katsuragi *et al.*, 1987). In most strains, pullulanase is first exported to the cell surface where it accumulates until it is slowly released into the medium (Woehner & Woebner, 1978a; d'Enfert *et al.*, 1987b). Functions required to export pullulanase to the cell surface and to release it into the medium have recently been shown to be encoded by genes on either side of *pulA* on the chromosome of *K. pneumoniae* strain UNF5023 (d'Enfert *et al.*, 1987b).

In this report we describe the isolation and characterization of three classes of Tn10 insertion mutants of *K. pneumoniae* strain K21 defective in the synthesis and/or secretion of pullulanase. This strain is unusual in that a large proportion of the pullulanase it produces is rapidly released into the medium and does not appear to pass through a membrane-anchored transition state (Kornacker *et al.*, 1988). Our results essentially confirm but also complement the findings of d'Enfert *et al.* (1987b). In addition, we show that strain K21, in contrast to all other previously characterized strains of *K. pneumoniae*, is partially constitutive for the expression of genes of the maltose regulon. We also present evidence for the existence of a new regulatory locus of the maltose regulon. This locus is located upstream of *pulA* and its inactivation results in the same phenotype as that resulting from inactivation of the *malT* locus.

METHODS

Media and culture conditions. Bacterial strains are listed in Table 1. Bacterial cultures were grown in Luria broth (Miller, 1972) or M63 medium (Miller, 1972) containing Casamino acids (0.4%) and glycerol (0.4% or 0.8% depending on the presence or absence of additional sugars, respectively) and supplemented with maltose or glucose (both 0.4%) as indicated. *E. coli* and *K. pneumoniae* strains were grown at 37 °C and 28 °C, respectively. Antibiotic selection was as appropriate. The amounts used per ml were: chloramphenicol (Cm), 300 µg; ampicillin (Ap), 100 µg; tetracycline (Tc), 10 µg; kanamycin (Km), 25 µg.

Southern analysis. Chromosomal DNA from mutants was isolated as described by Plastow *et al.* (1986) and digested to completion using appropriate restriction enzymes. DNA was then separated on a 0.8% agarose gel.

Table 1. *Bacterial strains, bacteriophages and plasmids*

Strain/phage/ plasmid	Characteristics	Source/reference
<i>E. coli</i>		
5K	F ⁻ <i>thi thr leuB6 lacY tonA21 supE44 λ⁻ r_K m_K</i>	Laboratory stock
MC4100	F ⁻ <i>araD139 Δ(argF-lac)U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR</i>	Laboratory stock
<i>K. pneumoniae</i>		
K21	λ ^r Ap ^r <i>pulA</i> ⁺ prototrophic	Laboratory stock
PAP996	A non-mucoid derivative of ATCC15050	Pugsley <i>et al.</i> (1986)
UNF5023	<i>his</i>	d'Enfert <i>et al.</i> (1987b)
Phage		
λ ₈₄₀	C ₁₈₅₇ , P _{am} ; carries a derivative of Tn10	Way <i>et al.</i> (1984)
Plasmids		
pACYC184	Cloning vector	Chang & Cohen (1978)
pKGW	Positive selection vector	Kuhn <i>et al.</i> (1986)
pUN121	Positive selection vector	Nilsson <i>et al.</i> (1983)
pMG1	Derivative of plasmid pHCP2 (Clement <i>et al.</i> , 1982) which carries the Km ^r marker of pUC-4K in its <i>Pst</i> I site	This study
pGSP1	Derivative of pACYC184 carrying <i>pulA</i> on the cloned chromosomal E ₁ -E ₃ fragment of strain K21 (Fig. 3)	This study
p9MT	Derivative of pBR322 carrying the Km ^r marker of pUC-4K in the <i>Pst</i> I site and the <i>malT</i> gene of pOM25 (Cole & Raibaud, 1986) inserted between the <i>Eco</i> RI and <i>Sal</i> I site	This study

Southern blot procedures were essentially as described by Maniatis *et al.* (1982), using the probes indicated in the text. Probes were radiolabelled by the method of Feinberg & Vogelstein (1983). Hybridizations were carried out overnight at 55 °C (using the *malT* probe) or 65 °C (using the *lamB* probe and those used for mapping Tn10 insertions) and were washed with 6 × SSC or 4 × SSC, respectively (1 × SSC was 0.15 M-NaCl, 0.015 M-sodium citrate).

Construction of a λ -sensitive (λ^s) derivative of strain K21. A λ^s derivative of strain K21 was constructed as described for *Erwinia* spp. by Salmond *et al.* (1986), except that plasmid pMG1 (Table 1) was used instead of plasmid pHCP2 (Clement *et al.*, 1982), since plasmid pHCP2 only specifies resistance to Ap, to which strain K21 is resistant.

Conjugal plasmid transfer into strain K21. Plasmids pMG1 and p9MT were transferred into strain K21 essentially as described by Salmond *et al.* (1986), except that K21-transconjugants were isolated by selecting for Km-resistant prototrophs on M9-glucose plates (Miller, 1972).

Cloning of chromosomal DNA fragments from strain K21. Chromosomal DNA from strain K21 was isolated, digested to completion with *Eco*RI, *Hind*III or *Bgl*II and ligated to pACYC184, pUN121 or pKGW (each digested with the corresponding enzyme), respectively. For the ligation with pUN121, chromosomal DNA of 6 kb and greater was isolated on a 20–40% (w/v) sucrose gradient (Maniatis *et al.*, 1982). All ligation mixtures were transformed into *E. coli* 5K. A clone (carrying pGSP1) able to ferment pullulan was identified by its purple colour when transformants from the appropriate ligation were screened on MacConkey plates containing pullulan as the sole carbon source. Recombinant clones obtained from the other ligations were screened by colony hybridization (Maniatis *et al.*, 1982), using the 6 kb *E*₁–*E*₂ fragment (see Fig. 3) as a probe.

Enzyme assays. Pullulanase was assayed essentially as described by Hope & Dean (1974) except that incubations were at 37 °C in sodium phosphate buffer (0.1 M, pH 7.0). Total cellular activity was assayed using sonicated and lysed cells (using 1% octyl polyoxyethylene), which give identical results (data not shown). Activity is defined as nmol maltotriose liberated min⁻¹ (mg protein)⁻¹. Maltose uptake by maltose permease was measured by following the incorporation of [U-¹⁴C]maltose (185 mCi mmol⁻¹, 6.8 GBq mmol⁻¹; Amersham) into cultures grown to OD₆₀₀ = 0.5 in M63 minimal medium supplemented with Casamino acids and glycerol with or without maltose (all at 0.4%). A 2 ml sample of each culture was washed and resuspended in 0.9 ml M63 medium containing Casamino acids (0.4%). After incubation for 3 min at 37 °C, 0.1 ml unlabelled maltose (10⁻³ M, 10⁻⁴ M and 10⁻⁵ M) containing 10⁵–10⁶ c.p.m. of [U-¹⁴C]maltose was added. Samples (0.1 ml) were taken at intervals and the incorporation of [U-¹⁴C]maltose was determined by scintillation counting. One unit of activity is defined as the uptake of 10³ c.p.m. min⁻¹ (mg protein)⁻¹. Amylomaltase was assayed essentially as described by Raibaud *et al.* (1985) except that GOD-PAP reagent (Boehringer, grade II) contained 0.5 mM 4-aminophenazone, 0.6 mM chlorophenol, 12 units glucose oxidase ml⁻¹ and 6 units peroxidase ml⁻¹ in 0.2 M-Tris, 0.2 M-sodium phosphate, pH 8.0. Activity is defined as nmol glucose liberated min⁻¹ (mg protein)⁻¹. The halo assay was as described by Morgan *et al.* (1979). The red pullulan test was as in d'Enfert *et al.* (1988). Pullulan-reactive red was prepared as in Rinderknecht *et al.* (1967). α -Amylase activity in *K. pneumoniae* was detected by streaking bacterial strains on LB plates (Miller, 1972) containing 0.4% amylose. These plates were incubated until confluent growth occurred and then stained with iodine vapour. Staining and the absence of staining were interpreted to be due to the inability or ability, respectively, of bacteria to degrade amylose. Plates streaked with *E. coli* (strain MC4100), which synthesizes a periplasmic α -amylase (Freundlieb & Boos, 1986), gave a negative result. The areas directly under the colonies of *K. pneumoniae* did not stain. This positive result together with the absence of haloes surrounding those colonies was therefore assumed to be due to α -amylase that was located on the cell surface but was not secreted.

Other methods. Immunofluorescence and immunoprecipitation experiments were as described by d'Enfert *et al.* (1987a). Cell envelopes were isolated as described by Churchward & Holland (1976) or Pugsley & Oudega (1987). Envelope fractionation was carried out with Sarkosyl according to Filip *et al.* (1973). SDS-PAGE analysis was as described by Laemmli (1970), using 0.8% *N,N'*-methylene bisacrylamide. Proteins were stained with Coomassie Brilliant Blue. Protein was determined by the Lowry technique. Transposon mutagenesis was as described by Way *et al.* (1984) except that the multiplicity of infection was 10²–10³ in order to obtain sufficient Tc-resistant colonies to allow large-scale screening for pullulanase export mutants.

RESULTS

Pullulanase production by K. pneumoniae strain K21

Several lines of evidence suggest that strain K21 is closely related to *K. pneumoniae* strain PAP996, a derivative (Pugsley *et al.*, 1986) of the previously studied strain ATCC 15050 (Michaelis *et al.*, 1985; Chapon & Raibaud, 1985; d'Enfert *et al.*, 1987a). Firstly, total cell protein extracts of strain K21 gave SDS-PAGE profiles which were almost identical to those obtained with strain PAP996 but differed markedly from those obtained with strain UNF5023

Fig. 1. Comparison of protein profiles of strains of *K. pneumoniae* (lane 1, strain K21; lane 2, strain PAP996; lane 3, strain UNF5023). Strains were grown in M63 medium containing glycerol and maltose. Protein in cell lysates was separated by SDS-PAGE (10%, w/v, acrylamide). Pullulanase (Pull.) is indicated by arrows. Pullulanase of strain UNF5023 has a slightly lower apparent molecular mass than pullulanase of strains K21 and PAP996.

(d'Enfert *et al.*, 1987*b*) (Fig. 1) and other klebsiellae (not shown). Secondly, we have cloned a 6 kb *Eco*RI fragment encoding the *pulA* gene of strain K21 and found that its restriction endonuclease map is almost identical to that of the corresponding region of the ATCC 15050 chromosome (Michaelis *et al.*, 1985). Furthermore, the DNA sequence of the 5' end of the K21 *pulA* gene, its promoter region and the 5' end of the divergently transcribed *malX* gene (Chapon & Raibaud, 1985; G. S. Plastow, unpublished results), are almost identical to those of strain ATCC 15050 and differ markedly from those of strains UNF5023 (d'Enfert *et al.*, 1987*b*) and W70 (Katsuragi *et al.*, 1987). However, strain K21 differs from all previously characterized strains of *K. pneumoniae* (d'Enfert *et al.*, 1987*a*; Takizawa & Murooka, 1985) in that high levels of pullulanase are produced in the absence of maltose.

To study this property in more detail, we examined total cell protein extracts of strains K21 and PAP996 grown in the presence of maltose or glucose. These tests showed that glucose repressed the synthesis of pullulanase and at least two other proteins (molecular masses 48 kDa

Fig. 2. Comparison by SDS-PAGE analysis (10%, w/v, acrylamide) of (a) synthesis of maltodextrin phosphorylase (MalP), maltose porin (LamB) and maltose-binding protein (MalE), and (b) secretion of pullulanase in strains K21, PAP996 and linked (TK1) and unlinked (TK23) class II mutants of strain K21. Strains carrying additional plasmid-borne copies of *malT* are indicated by (+). Bacteria were grown in Luria broth containing glucose (G) or maltose (M) as indicated. The amounts of cells and culture supernatants loaded were equivalent to 0.1 ml and 5 ml of the original culture volume, respectively. The position of the *malP* gene product has been deduced by comparison with *E. coli* (Bloch & Raibaud, 1986a). Maltose-binding protein is largely obscured by other protein. Pullulanase is not visible in whole-cell lysates.

and 40 kDa) to a far greater extent in strain PAP996 than in strain K21 (Fig. 2). The latter two proteins correspond to the equivalents in *K. pneumoniae* of the maltose-inducible *E. coli lamB* and *malE* gene products, respectively, since they could be specifically immunoprecipitated using polyclonal antibodies against the respective *E. coli* proteins, and they were found by cell fractionation to be localized in the outer membrane and non-envelope fractions, respectively (not shown). The low degree of repression exerted by glucose in strain K21 suggests that maltose-independent production of pullulanase by strain K21 resulted from constitutive expression of the entire maltose regulon. This was confirmed by comparing the inducibility of the *malQ* gene product, amylomaltase, in strains K21 and PAP996; induction in strain K21 was only fourfold when grown in medium containing maltose (Table 2), whereas induction was at least 50-fold for

Table 2. *Determination of pullulanase (pulA) and amylomaltase (malQ) activities in strains K21 and PAP996*

Strains were grown to OD₆₀₀ = 3 in M63 medium containing glycerol and supplemented with sugars as appropriate. Activities were determined using sonicated whole cultures.

Strain	Carbon source	Units of activity*	
		Pullulanase	Amylomaltase
K21	Glycerol	68.9	86.2
	Glucose	66.3	90.3
	Maltose	108.0	345.0
PAP996	Glycerol	<1.3	<5.0
	Glucose	<1.3	<5.0
	Maltose	63.7	232.5

* Units of activity are defined in Methods.

strain PAP996. Similar results were obtained when pullulanase was assayed (Table 2). Furthermore, although stationary-phase cultures of strains K21 and ATCC 15050 contain similar amounts of pullulanase under inducing conditions, in the absence of inducer pullulanase synthesis can only be detected for K21 (not shown). Since the *pulA*, *malQ*, *lamB* and *malE* genes are in different operons of the maltose regulon (Schwartz, 1987), these results suggest that expression of the entire maltose regulon of strains K21 is partially constitutive.

Isolation and characterization of mutants of strain K21 that are defective in pullulanase synthesis and secretion

As part of our study of pullulanase synthesis and secretion by strain K21, mutants defective in secretion were isolated by λ ::Tn 10 mutagenesis. A λ derivative of strain K21 was constructed as described in Methods, and was subjected to transposon mutagenesis using λ_{840} (Way *et al.*, 1984). Approximately 5000 colonies were screened for defects in pullulanase secretion and/or synthesis using a plate haloassay (Morgan *et al.*, 1979). A total of 22 mutants able to synthesize pullulanase but defective in its secretion were isolated. They represent at least 18 different Tn10 insertions as judged by Southern blot analysis (see below).

All of the Tn10 insertion mutants that were isolated were defective in the secretion of pullulanase as determined by pullulanase assays and by the pullulanase-reactive-red plate test (d'Enfert *et al.*, 1988) (Table 3). However, they fell into three classes with respect to the level of synthesis of pullulanase and other components of the maltose regulon, growth on maltose, immunofluorescence and immunoprecipitation experiments and the proportion of pullulanase found on the cell surface (Table 3).

Although class I mutants synthesized almost wild-type levels of pullulanase, most of the activity was 'cryptic' (i.e. not detectable with intact cells), with very little activity present on the cell surface. These mutants are therefore defective in cell-surface localization of pullulanase. In addition, there was no detectable secretion of pullulanase into the culture medium.

Class II mutants exhibited levels of pullulanase that were less than 5% of those seen in the parent strain K21. Pullulanase assays suggested that class II mutants were capable of exporting most of the pullulanase made to the cell surface. Pullulanase was not detectable in the culture medium as judged by pullulanase assays (Table 3) and SDS-PAGE analysis of concentrated culture media (not shown), even though amounts lower than 5% of the amount secreted by wild-type K21 would have been detectable. The low amounts of pullulanase synthesized presumably prevented its detection on the cell surface by immunofluorescence (Table 3). Class II mutants were further distinguished by their slow growth on maltose as the sole carbon source, by the absence of an apparently maltose-inducible α -amylase activity on the cell surface and by an altered profile of total cell protein as determined by SDS-PAGE analysis (Fig. 2). The reduced production of maltose porin and maltose-binding protein (see above, Fig. 2) was particularly noticeable. Class II mutants were also strongly impaired in maltose uptake (Table 3),

Table 3. Comparison of properties of strain K21 and the three classes of Tn10 insertion mutants

For details see text. All assays were repeated at least once.

	K21	Class I	Class II	Class III
Units* of pullulanase activity	65	44.2–59.9	< 3	50.8
Percentage of pullulanase				
In culture medium	60	0	0	40
On cell surface	40	10–20	60–100	36
Cryptic†	0	80–90	0–40	24
Immunofluorescence with anti-PulA	+++	—	—	+++
Units* of maltose uptake				
With 10 ⁻⁵ M-maltose	28.3	ND	1.2	ND
With 10 ⁻⁶ M-maltose	98.7	ND	1.2	ND
Units* of amylomaltase (<i>malQ</i>) activity	317.5	210–257.5	< 5	231.0
Cell surface α -amylase activity	+++	+++	—	+++
Growth on maltose	+++	+++	+	+++
Immunoprecipitation				
LamB protein	+++	+++	—	+++
MalE protein	+++	+++	—	+++
Plate tests				
Halo assay	+++	—	—	++
Pullulan red	+++	—	—	+

ND, Not determined.

* Units of activity and uptake are defined in Methods.

† Cryptic activity is defined as pullulanase activity that is not accessible by assaying whole cells resuspended in fresh medium.

presumably due to low or absent expression of maltose permease genes. The pleiotropic nature of the class II mutants suggested a defect in the induction of all maltose-inducible proteins.

Only one class III mutant was isolated. In the halo assay this mutant produces a smaller halo than the parent strain. The class III mutant synthesized near-normal levels of pullulanase, and was shown to contain 'cryptic' activity. However, in contrast to the class I mutants, the class III mutant localized most of the cell-associated pullulanase to the cell surface. In addition, a significant amount of pullulanase (up to 40% of total activity in some experiments, see Table 3) was found in the culture medium, indicating that secretion in this mutant is impaired but not completely abolished.

Mapping of the sites of Tn10 insertion in mutant strains

To determine if any of the genes identified by the Tn10 insertions were linked to the *pulA* gene, the 6 kb E₁–E₂ fragment (Fig. 3) of pGSP1 carrying *pulA* (see Methods) was used as a probe in Southern blot analysis of genomic DNA isolated from Tn10 insertion mutants. This allowed the Tn10 insertions to be mapped with respect to known restriction sites inside and outside this fragment. Of the 22 mutants isolated, 12 class I mutants and the single class III mutant carried insertions within the *Eco*RI fragment. There were five and four class I insertions, respectively, at each side of the unique *Bam*HI site (B₂) (Fig. 3), with the class III mutant mapping in the E₁–B₂ fragment. The remaining three insertions (class I mutants TK13, TL14 and TK61) have not been mapped with respect to this *Bam*HI site. In each case the size of the novel *Eco*RI fragments generated by insertion of the transposon suggests two possible insertion sites: one outside *pulA* and one within it. Since all of the mutants synthesized pullulanase and since Tn5 insertions (Michaelis *et al.*, 1985; G. S. Plastow, unpublished results) and deletions (Takizawa & Murooka, 1985) throughout the entire length of *pulA* prevent synthesis of an active pullulanase, we conclude that the Tn10 insertions must be located outside *pulA*. At least one class I mutant, TK66 (see below), carries an insertion within the 6 kb *Eco*RI fragment but clearly is not in the E₁–G₂ restriction fragment that includes *pulA* (Fig. 3). Furthermore, Southern blot analysis (data not shown) shows that strain K21 carries only one copy of the *pulA* gene; the

Fig. 3. Positions of *Tn10* insertions in the *pulA* region of strain K21. The vertical broken lines indicate chromosomal regions to which mutants have been mapped. Mutants are referred to by their class and are listed in parentheses underneath. Mutants TK13, TK14, TK61 and unmapped mutants (TK23, TK42, TK48) are not shown. The positions of *pulA* and *malX* are indicated by the solid bars (the size of *malX* is not known). Their directions of transcription are indicated by the arrows. The positions of restriction sites outside the 6 kb *EcoRI*₁–*EcoRI*₂ fragment were deduced by Southern blot analysis and by restriction analysis of the chromosomal DNA fragments of strain K21 that were isolated in this study (see text). Restriction sites are: B, *Bam*HI; G, *Bgl*II; E, *Eco*RI; H, *Hind*III.

continuing ability of the *Tn10* insertion mutants to synthesize pullulanase cannot therefore be due to the presence of other *pulA* genes.

To see whether the remaining *Tn10* insertions were adjacent to the 6 kb *Eco*RI fragment, DNA flanking *pulA* was cloned into pKGW as two *Bgl*II fragments of 6.5 kb (*G*₁–*G*₂) and 10 kb (*G*₂–*G*₃). In addition, a 22.5 kb *Hind*III fragment (*H*₁–*H*₂) was cloned into pUN121 (Fig. 3) (see Methods). These fragments, or subclones thereof, were used as probes in further mapping experiments. Seven additional *Tn10* insertions were mapped (Fig. 3): (a) using *Bgl*II-digested chromosomal DNA of mutant TK66 (class I) and the *G*₂–*E*₂ fragment as a probe, the insertion in this mutant was assigned to within the *G*₂–*E*₂ fragment; (b) the insertion in mutant TK60 (class I) was assigned to within the *E*₂–*B*₃ fragment following hybridization of the *B*₂–*E*₂ and *E*₁–*E*₂ probes to *Bam*HI- or *Eco*RI-digested chromosomal DNA, respectively; and (c) the insertions within one class I mutant (TK45) and four class II mutants (TK1, TK3, TK4, TK18) were assigned to within the *B*₃–*G*₃ fragment following hybridization of the *B*₂–*E*₂ and *G*₂–*G*₃ probes to *Bam*HI- and *Bgl*II-digested DNA, respectively. The four class II mutants are probably identical since they were isolated from the same mutagenesis experiment and could not be distinguished by Southern blot analysis. Two class I mutants (TK42 and TK48) and another class II mutant (TK23) did not map within the *B*₁–*H*₂ fragment (Fig. 3).

The mapping data for the class I mutants indicate that genes required for secretion of pullulanase are located on either side of *pulA*. However, unlike strain UNF5023 (d'Enfert *et al.*, 1987b) at least one of these genes (see mutants TK42/48) is not adjacent to *pulA*.

The class III mutant may carry an insertion in a locus downstream of *pulA* that is distinct from the locus inactivated in the class I mutant carrying *Tn10* insertions in the same region. Alternatively, its phenotype may result from partial inactivation of a locus that is completely inactivated in the adjacent class I mutants.

The mapped class II mutations are not in malT, cya, crp or maltose transport genes

In *E. coli*, inactivation of *malT*, the maltose transport genes, *cya* and *crp* are known to affect expression of all components of the maltose regulon in the way seen for our *K. pneumoniae* class II mutants (Schwartz, 1987). Using the *malT* gene of *E. coli* as a probe in Southern blot analysis, it was found that genomic DNA of the unmapped class II mutant TK23 gave rise to a pattern of hybridizing fragments that was consistent with a *Tn10* insertion in or close to *malT* (Fig. 4). As expected, hybridization was not detectable in digests of the *H*₁–*H*₂ fragment.

Using *lamB* of *E. coli* as a probe, it was found that the gene for the maltose porin of *K. pneumoniae* does not map within the *H*₁–*H*₂ fragment. This almost certainly rules out the possibility that the mapped class II *Tn10* insertions are located in any of the maltose transport genes. This was confirmed by the finding that synthesis of pullulanase, maltose porin and

Fig. 4. Southern blot demonstrating *Tn10* insertion in or close to *malT* of class II mutant TK23. Chromosomal DNA from strain K21 or class I mutant TK42 served to identify the wild-type hybridization pattern. Chromosomal DNA was digested with *EcoRI* or *BamHI*, respectively. λ_{840} carries a derivative of *Tn10* which has a single *EcoRI* site and no *BamHI* site. Insertion in an *EcoRI* or *BamHI* fragment carrying *malT* will therefore result in the generation of two hybridizing bands or one hybridizing band whose increase in size relative to the wild-type corresponds to the size of the *Tn10* derivative, respectively. The amounts of DNA loaded were approximately 1–2 μ g of chromosomal DNA, 200 ng of plasmid pHi (not shown) or 3 ng of probe (the 3.5 kb *EcoRI*–*SalI* fragment of pOM25; Cole & Raibaud, 1986) (not shown). No hybridization was detectable in digests of plasmid pHi (not shown). Hybridization to the probe gave a strong signal (not shown). E, *EcoRI*; B, *BamHI*.

maltose-binding protein (and the secretion of pullulanase) could all be restored in the mapped class II mutants by introducing a multiple-copy-number plasmid carrying *malT* (see Table 1) which cannot substitute for structural components of the maltose regulon (Fig. 2) (see Discussion). Class II mutants exhibited normal expression of the *lac* operon (not shown). Since expression of the *lac* operon is dependent on *cya* and *crp*, this rules out these genes as sites for the *Tn10* insertions in the linked class II mutants. It follows that the *pulA*-linked class II mutants carry *Tn10* insertions in a new regulatory element of the maltose regulon.

DISCUSSION

Our analysis of pullulanase secretion mutants obtained by *Tn10* mutagenesis of *K. pneumoniae* strain K21 essentially agrees with that of d'Enfert *et al.* (1987b), who studied pullulanase secretion by a different approach, i.e. the mutagenesis of a cloned fragment in *E. coli*. We also find that genes for export of pullulanase to the cell surface and for its secretion are genetically linked to *pulA* in the chromosome of strain K21, as is the case for *K. pneumoniae* strain UNF5023 (d'Enfert *et al.*, 1987b). In fact, class I *Tn10* insertions of strain K21 are phenotypically similar or identical to transposon insertions in regions B and C (located downstream and upstream of *pulA*, respectively) in the region of the UNF5023 chromosome studied by d'Enfert *et al.* (1987b). However, strain K21 differs from strain UNF5023 in that at least one class I pullulanase secretion gene does not map close to *pulA*. This may explain why *E. coli* carrying the chromosomal H_1 – H_2 fragment of strain K21 does not secrete pullulanase although export to the cell surface does occur (Kornacker *et al.*, 1988). Nevertheless, it remains to be established why

the *E. coli* strain is able to expose pullulanase on the cell surface whereas mutants TK42 and TK48 are not. Interestingly, we were unable to isolate mutants of strain K21 with Tn10 insertions leading to a *pulA*⁻ phenotype even though a large number of Tn10 insertions were clustered around *pulA*.

Class II mutants are defective in the expression of the maltose regulon. Mutants with such a phenotype were not isolated by d'Enfert *et al.* (1987*b*). We do not know whether the Tn10 insertions in the class II mutants that map adjacent to *pulA* are in a gene or allele that is unique to strain K21 or to *K. pneumoniae* because mutants with transposon insertions in the same locus would not have been isolated in the study by d'Enfert *et al.* (1987*b*) if the corresponding gene was also present in *E. coli*. The phenotypes of all class II mutants are identical whether they carry Tn10 insertions in *malT* or in the locus that is located upstream of *pulA*. As expected, a wild-type phenotype can be restored in the *malT*::Tn10 mutant by (over)production of MalT from a multicopy plasmid. However, this is also the case in the *pulA*-linked class II mutants. With respect to the latter, this may be equivalent to the suppression by overproduction of MalT of the effects on maltose regulon expression that are caused by catabolite repression (Chapon, 1982) or the fact that maltose-regulon expression is maltose-independent in *E. coli* strains carrying multiple copies of *malT*. This suggests that the locus affected in the *pulA*-linked class II mutants encodes a positive regulator of the maltose regulon analogous to that encoded by the *crp* gene. Indeed, if the Tn10 insertions in the linked class II mutants affected expression of a negative regulator, an increase in expression of the maltose regulon would be predicted and this is clearly not the case. The ability of *malT* to substitute for this regulator probably rules out that the latter is involved in the expression of the alternative *malT*-independent maltose-uptake system that has recently been identified in *K. pneumoniae* (Bloch & Raibaud, 1986*b*). The restoration by *malT* of pullulanase secretion in all class II mutants demonstrates that expression of pullulanase-secretion genes is not subject to polar effects by the Tn10 insertions in these mutants. It is not known why class II mutants fail to secrete the residual levels of pullulanase from the cell surface.

The locus affected in the *pulA*-linked class II mutants could theoretically correspond to *malM*, a maltose-inducible gene of unknown function that has been identified in *E. coli* (Gilson *et al.*, 1986). However, this is unlikely since, at least in *E. coli*, *malM* is linked to *lamB*. We also consider it unlikely that the Tn10 insertions are in genes such as *malI* that are required for the synthesis of endogenous inducers because we do not observe induction of the maltose regulon by maltose as is the case for *malI*::Tn10 strains of *E. coli* (Ehrmann & Boos, 1987). In addition, we were unable to demonstrate by Southern blot analysis any hybridization of the H₁-H₂ fragment with the chromosomal fragment of *E. coli* that carries *malI* (unpublished data). We therefore suggest that the *pulA*-linked class II Tn10 insertions are in a previously unknown regulatory locus of the maltose regulon. The reason for the partial constitutivity of strain K21 remains to be established, but may be related to the fact that strain K21 is an industrial strain that has been selected for efficient production of pullulanase.

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