Polysialic acid export in *Escherichia coli* K1: the role of KpsT, the ATP-binding component of an ABC transporter, in chain translocation

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The polysialic acid (polySia) capsule of *Escherichia coli* K1 is a key virulence determinant of the organism, allowing it to evade host defenses. The proteins necessary for expression of the capsule are encoded by the 17 kb *kps* gene cluster. This cluster contains two genes, *kpsM* and *kpsT*, that are required for polySia transport across the cytoplasmic membrane. KpsM is a hydrophobic integral inner membrane protein, while KpsT is a peripheral inner membrane protein that binds ATP. They belong to the ATP-binding cassette (ABC) superfamily of transporters. To study the role of KpsT in polySia translocation, we used PCR mutagenesis to isolate dominant negative mutations of plasmid-encoded *kpsT*. All mutations mapped to the same glutamic acid residue at position 150, adjacent to Walker motif B of KpsT. Wild-type (*kps*) cells harboring one such allele, E150G, did not transport polySia to the cell surface but accumulated intracellular polysaccharide and produced small colonies containing cells that grew as long filaments. The E150G protein still bound ATP as shown by 8-azidoATP photolabeling assays. We combined the E150G allele with each of five mutations isolated previously in *kpsT*. Mutations that disrupt ATP-binding (*K44E*) or alter protein stability (*C163Y, H181Y*) did not suppress. These studies have allowed the development of a working model for the role of KpsT in polySia chain translocation.

**Key words:** ABC-transporter/dominant negative mutation/*Escherichia coli* K1/KpsT/polysialic acid

Introduction

Although commonly regarded as a harmless commensal, *Escherichia coli* is an important human pathogen (Sussman, 1985). In addition to a variety of diarrheal diseases, *E.coli* is associated with serious invasive infections including pyelonephritis, sepsis and neonatal meningitis (Levine, 1984). The polysaccharide capsule of this organism is an essential virulence determinant in extraintestinal disease, enabling the bacteria to evade nonspecific host defenses (Cross, 1990; Moxon and Kroll, 1990). The K1 capsule is associated with 80% of isolates from cases of *E.coli* neonatal meningitis (Robbins et al., 1974), and this disease continues to portend a poor prognosis in infected neonates (McCracken et al., 1974; Unhanand et al., 1993).

The K1 capsule is an α2,8 linked homopolymer of sialic acid. Polysialic acid (polySia), although rare in prokaryotes, is found in a diverse range of biological contexts including the sea urchin embryo, the vitelline envelopes of fish eggs, the α-subunit of the sodium channel in adult rat brains, the surfaces of vertebrate neurons, and the embryonic form of the neural cell adhesion molecule (N-CAM) (Troy, 1992, 1995; Roth et al., 1993). Polysialylated N-CAM is also aberrantly expressed on human neuroblastomas and Wilms tumor (Roth et al., 1987, 1988). The genes involved in expression of the polysialic acid capsule of *E.coli* K1 have been cloned and characterized (Vimr et al., 1989; Boulnois and Roberts, 1990; Silver, 1994). The 17 kilobase *kps* gene cluster is divided into three functional regions (Boulnois et al., 1987; Vimr et al., 1989). The central region 2 genes encode proteins responsible for the synthesis, activation, and polymerization of polySia, while the flanking regions 1 and 3 are essential for transport of the polysaccharide from its cytoplasmic site of synthesis to the cell surface (Boulnois and Jann, 1989; Vimr et al., 1989; Boulnois and Roberts, 1990).

Region 3 of the *kps* cluster contains two genes, *kpsM* and *kpsT* (Smith et al., 1990; Pavelka et al., 1991). KpsM is a hydrophobic integral inner membrane protein containing six transmembrane helices (Pigeon and Silver, 1994), while KpsT is a hydrophilic peripheral inner membrane protein that binds ATP (Pavelka et al., 1994). Together, these proteins comprise a member of the ATP-binding cassette (ABC) superfamily of transport proteins also referred to as traffic ATPases (Ames et al., 1992; Higgins, 1992). ABC transporters are characterized by their structural organization and by the presence of Walker motifs A and B in the primary structure of the ATP-binding component (Walker et al., 1982). They have been described in a wide range of both prokaryotic and eukaryotic organisms and are involved in transport of a wide variety of substrates in both directions across cell membranes (Ames et al., 1992; Higgins, 1992). Cells harboring mutations in either *kpsM* or *kpsT* accumulate polysaccharide in the cytoplasm (Kröncke et al., 1990; Pavelka et al., 1991; Wunder et al., 1994), and our group and others postulated that KpsM and KpsT form a capsule specific transporter required for export of the polysaccharide across the cytoplasmic membrane (Smith et al., 1990; Pavelka et al., 1991). KpsM and KpsT are conserved among *E.coli* synthesizing serologically distinct capsules (Silver, 1994), and homologous proteins are involved in the export of capsular polysaccharides in *Haemophilus influenzae*, *Neisseria meningitidis*, and *Salmonella typhi* (Kroll et al., 1990; Frosch et al., 1991; Hashimoto et al., 1993).

Previous results obtained from site-directed and saturation mutagenesis of the ATP-binding consensus sequence (Walker...
motif A) of KpsT are consistent with the view that ATP-binding, and presumably hydrolysis, are important to KpsT function and capsule transport (Pavelka et al., 1991, 1994). In addition, we obtained biochemical evidence that KpsT binds ATP using 8-azidoATP photolabeling assays and proposed a secondary and tertiary structure model of the KpsT protein based on a model developed for other ABC-transporter proteins (Pavelka et al., 1994). The results obtained from chemical mutagenesis of kpsT are consistent with the model and revealed domains important for the protein’s function distinct from those involved with ATP binding (Pavelka et al., 1994).

In the present study, we sought dominant negative mutations in kpsT in an effort to gain insight into the molecular mechanism of polysial chain translocation. Our current view of capsular expression predicts that the membrane bound components of the cluster form a hetero-oligomeric complex involved in both polysial synthesis and translocation, and dominant negative mutations are a valuable tool in the study of proteins that function as part of multicomponent systems. The concept is based on the premise that proteins possessing domains involved in interactions with different molecules may form defective immunoligomeric aggregates if one of their domains is deleted or altered (Herskowitz, 1987). We report here the isolation and characterization of a dominant negative mutation in kpsT that inhibits polysial translocation in wild-type cells. Cells containing the dominant negative allele grow as long filaments. We also describe additional mutations in kpsT that suppress the dominant negative phenotype intragenically. These studies provide insight into the sequence of events involving KpsT in polysial chain translocation across the cytoplasmic membrane.

Results

Isolation of a ‘dominant negative’ mutation of kpsT

kpsT was mutagenized by PCR under conditions that increase the frequency of replication errors as described in Materials and methods. Mutagenized PCR products were cloned into a pBluescript KS+ vector under control of the lac promoter and transformed into EV36, a K12/K1 hybrid strain which contains the entire kps gene cluster (Vimr et al., 1989). Transformants were plated on ampicillin containing media that had been seeded with the K1-specific bacteriophage E to select for acapsular cells. Nine phase-resistant clones were isolated. Plasmid DNA was extracted from each and retransformed into EV36 to confirm that the phage-resistant phenotype was associated with the plasmid. Cells harboring each plasmid lacked precipitin halos on antiserum agar and were resistant to lysis by K1-specific bacteriophage. In contrast, EV36 harboring pSR340, which contains wild-type kpsT cloned into Bluescript KS+, remained halo− on antiserum agar and phage sensitive. Further, pSR340 readily complemented RS2436, a derivative of EV36 with a chromosomal deletion in the kpsT gene (Pavelka et al., 1994), while none of the dominant negative clones were able to complement the mutation in RS2436. Western blot analysis, however, demonstrated that cells harboring each clone made KpsT protein equivalent in size and amount to cells carrying pSR340 (data not shown).

The kpsT gene of the nine plasmids giving a dominant negative phenotype was sequenced. Four of the nine contained a single mutation, and in each case the mutation resulted in alteration of the glutamic acid residue at amino acid 150 (GAA codon from nucleotides 1221–1223 in Pavelka et al., 1991). In three clones, the residue was changed to glycine (GAA to GGA) and in one to aspartic acid (GAA to GAT). The five other clones had mutations affecting more than one residue, but each one also had a mutation in E150. One of the dominant negative clones, pSR577, coding for a single amino acid change relative to wild-type, E150G, was chosen for further study.

Characterization of the E150G allele

We previously demonstrated that KpsT binds ATP, and described a mutation in Walker motif A of the protein (K44E) that disrupted ATP binding (Pavelka et al., 1991, 1994) (Figure 1, lanes b and g). The E150G mutation is adjacent to Walker motif B of KpsT, and we assessed the ability of the mutant protein to bind ATP by 8-azidoATP photolabeling assays (Figure 1). KpsT containing the E150G mutation was derivatized by 8-azidoATP equivalent to the wild-type, suggesting that the mutation does not interfere with ATP binding (Figure 1, lanes f and h).

In the process of isolating the dominant negative mutation, we observed that EV36 harboring the E150G bearing plasmid, pSR577, not only lacked precipitin halos on antiserum agar but also grew to a smaller sized colony compared to EV36 harboring the wild-type plasmid, pSR340, or a plasmid containing the K44E mutation, pSR346 (Figure 2). Several observations suggest that this phenotype required polysial synthesis. First, colonies grew to equivalent size when incubated at 23°C. PolySia is expressed only at physiological temperatures and is not synthesized below 30°C (Bortolussi et al., 1983; Ørskov et al., 1984). Second, the strain EV138 harboring pSR340, pSR346, or pSR577 grew to colonies of equivalent size at 37°C. EV138, a derivative of EV36, carries a mutation in neuB, the gene encoding NeuAc synthase and thus is unable to synthesize either sialic acid or polymer (Vimr, 1992).

We have described other nonfunctional mutations in kpsT (Pavelka et al., 1991, 1994). These include mutations in Walker motifs A and B, K44E and G155R respectively; a conserved histidine, H181Y; a cysteine residue conserved among capsule transporters, C163Y; the helical domain, G84D; and the linker peptide, S126F (Pavelka et al., 1994). We examined whether any of these mutations, each residing in pBluescript KS+ (Pavelka et al., 1994), exerted similar dominant negative effects as observed with pSR577. Each was transformed into EV36. Cells harboring the K44E, G155R, C163Y, G84D, and S126F alleles looked equivalent to EV36 cells carrying pSR340 (K44E, for example, is shown in Figure 2) and thus had no dominant negative effect. EV36 cells harboring the H181Y allele did show a loss of precipitin halo production, but lacked the characteristic growth inhibition observed with EV36 harboring pSR577. These findings indicate that E150G is, in some way, unique.

Fig. 1. 8-AzidoATP photolabeling of plasmid encoded KpsT. Western blot of KpsT expressed from plasmids pSR340 (wild-type, lanes a and f), pSR346 (K44E, lanes b and g), and pSR577 (E150G, lanes c and h) following UV irradiation in the presence (e–h) and absence (a–d) of 8-azidoATP. Binding of the ATP analogue is indicated by an upward shift in mobility of the derivatized protein. The pKsT vector control is shown in lanes d and e.
**Fig. 2.** Halo and growth phenotypes of EV36 harboring plasmid encoded kpsT alleles. Antiserum agar plate of EV36 harboring pSR340 (A), pSR346 (B), and pSR577 (C). Colonies containing encapsulated cells are surrounded by precipitin halos.

**Electron microscopy**

We compared the morphology of EV36 harboring pSR577 to cells carrying pSR340 or pSR346 by scanning electron microscopy (SEM) (Figure 3). Long filaments were formed during growth of EV36 harboring pSR577 at 37°C (Figure 3C). Comparable filaments were not observed with cells harboring pSR340 or pSR346 (Figure 3A,B). These observations suggest that expression of the dominant negative allele of kpsT leads not only to a decreased colony size on agar plates but also to filamentation of the cells. EV36 harboring pSR577 did not exhibit filamentation when cells were grown at 23°C, a temperature nonpermissive for capsule expression (Bortolussi et al., 1983; Ørskov et al., 1984), suggesting that polySia synthesis is necessary for the observed growth effects.

EV36 harboring pSR340, pSR346, or pSR577 were also analyzed by transmission electron microscopy (TEM) (Figure 4). Cells carrying pSR577 contained electron lucent zones within the cytoplasm (Figure 4A,G), indicative of intracellular polysaccharide (Bronner et al., 1993; Pelkonen, 1990). Polymer accumulated around the periphery of the cells at the cytoplasm—cytoplasmic membrane interface (Figure 4A). In cross-section, these cells generally gave a 'bullseye' appearance (Figure 4G). Surprisingly, although data presented above show that only pSR577 prevented polySia translocation to the cell surface, EV36 carrying pSR340 or pSR346 also contained zones characteristic of intracellular polysaccharide accumulation. The zones within EV36/pSR340 were similar to those observed with pSR577 (Figure 4B,C). Cells carrying pSR346, however, accumulated polymer at discrete sites along the cytoplasmic membrane (Figure 4D,E) as was observed in cells with the K44E allele in place of the wild-type chromosomal kpsT gene (Pavelka et al., 1994).

**Intragenic suppression of the dominant negative phenotype of E150G**

The E150G mutation was incorporated into plasmids encoding the K44E, G84D, S126F, C163Y, or H181Y mutant alleles. Each plasmid containing the combined mutations was transformed into EV36 and the resultant phenotype assessed (Figure 5). K44E, G84D, and S126F provided complete suppression of the dominant negative phenotype as evidenced by precipitin halos that were equivalent to EV36 containing wild-type kpsT in trans and by the lack of a detectable difference in colony size. C163Y and H181Y failed to provide suppression of the dominant negative phenotype. The kpsT gene of each plasmid was sequenced to confirm that the intended two mutations were the only mutations present, and Western blot analysis showed that RS2436 (ΔkpsT) harboring each plasmid expressed a pro-
tein of equivalent size and amount to the wild-type protein expressed from pSR340 (data not shown).

Discussion

In *E. coli* K1, polySia is synthesized in the cytoplasm and transported through two membranes by a complex and poorly understood process. We have isolated a single amino acid change in residue E150 of the ATP-binding component, KpsT, that interferes with this process despite the presence of all wild-type components. E150 is adjacent to Walker motif B (Walker et al., 1982) and likely to be part of the nucleotide binding fold, yet the E150G mutant binds ATP. Mutational analysis of the ATP-binding component of the histidine permease, HisP, revealed that a mutation of the analogous E179 residue to an aspartic acid yielded a protein incapable of histidine transport but bound ATP equivalent to wild-type HisP (Shyamala et al., 1991). In contrast, HisP with a mutation in the adjacent D178 residue which is also widely conserved among ABC transporters did not bind ATP (Shyamala et al., 1991). These findings led the authors to propose that E179 is necessary for ATP hydrolysis since it is part of the nucleotide binding fold, but the protein still binds ATP when this residue is altered. Our data are consistent with this view. Further, since combining the K44E mutation on the same gene suppresses the dominant negative phenotype of E150G, our data suggest that ATP binding is essential for the dominant negative phenotype, and that a lack of ATP hydrolysis may be at the root of the defect.

In addition to the K44E/E150G double mutant, the G84D and S126F mutations were also able to suppress the dominant negative phenotype when present in cis. In our structural model of KpsT (Figure 5), which is based on the computer assisted Mimura model of ATP-binding components of ABC-transporters (Mimura et al., 1991), these residues belong to a 'helical domain' that interrupts the nucleotide binding fold (Pavelka et al., 1994). This domain is variable among ATP-binding components and is postulated to be important for interaction with the membrane components in an individual system (Mimura et al., 1991). The S126 residue is part of a short, conserved 'linker peptide' within the helical domain that is thought to act in signal transmission from the ATP-binding domain to the helical domain (Ames et al., 1992). Thus, in this model ATP-binding is signaled to the helical domain via the linker peptide, which then affects the membrane component to allow transport to occur. The finding that nonfunctional mutations in the helical domain and linker peptide suppress the dominant negative phenotype implies that the phenotype will only be present when both the signaling pathway and interactions via the helical domain with KpsM are intact. Taken together, the suppression data lend support to a model in which KpsT binds ATP generating a translocation competent KpsMT complex. The dominant negative phenotype is likely the result.
of a defect in subsequent ATP hydrolysis necessary to release KpsT, such that the mutant KpsT freezes the transport apparatus midway through the cycle forming a translocation incompetent complex, and subsequent translocation is thereby abolished. Thus, mutations in KpsT that interfere with ATP binding (K44E), ATP induced signaling to the helical domain (S126F), or interaction with KpsM (G84D) suppress the dominant negative phenotype by preventing steps necessary to form the complex. The finding that mutations in H181 and C163 do not suppress the phenotype suggests that these residues are involved in transport steps independent of formation of the presumed translocation incompetent complex.

The H181Y mutation, like E150G, inhibits polySia transport in EV36, but the lack of a detectable growth defect implies that the dominant negative phenotype in these cells is through a mechanism distinct from that of E150 mutations. Interestingly, mutations in the respective histidine residues in the MalK (H192) and HisP (H211) proteins resulted in complete loss of transport activity, but with no apparent defects in ATP binding or hydrolysis (Shyamala et al., 1991; Walter et al., 1992). The authors suggested that this residue is critical for conformational changes following ATP binding (Shyamala et al., 1991; Walter et al., 1992). We envision that the H181Y mutant protein, present in excess, is able to displace wild-type KpsT but, unlike E150G, does not freeze the translocation apparatus.

The surprising finding by TEM that polySia accumulates in EV36 harboring not only the dominant negative allele of kpsT but also the wild-type and K44E alleles suggests that the formation of the translocation incompetent complex in our model is not solely responsible for polySia accumulation. This finding supports the possibility of an interaction between polySia and KpsT. The initiation codon of kpsT overlaps the termination codon of kpsM, suggesting that KpsM and KpsT are normally expressed in equimolar amounts (Pavelka et al., 1991). The overexpression of KpsT within a capsule producing cell may increase the cytoplasmic pool of KpsT/polySia complexes. Since transport is likely to occur only at specific membrane sites occupied by KpsM, these additional KpsT/polySia complexes may make association with KpsM a rate limiting step in the transport process. Overabundance of KpsT may therefore act as a 'tether', keeping polySia in a cytoplasmic location. Interestingly, previous biochemical studies defined a membrane protein of approximately 20 kDa attached to polysaccharide in K1 cells (Weisgerber and Troy, 1990). The authors suggested that this protein may be involved in translocation of polySia through the membrane (Weisgerber and Troy, 1990). We believe KpsT, a 25 kDa protein, to be the molecule that attaches to the polySia and transports it through the cytoplasmic membrane in association with KpsM.

Although accumulation of polySia is observed in cells expressing each allele of kpsT, the cells are still quite competent for transport in the absence of the dominant negative allele, as no alteration in capsular phenotype is observed. This observation supports the notion of a translocation incompetent complex formed with the E150G allele that is not formed by wild-type or the K44E allele. The E150G allele may initiate transport but is unable to complete the transport cycle due to a presumed inability to hydrolyze ATP. It likely exerts its dominant negative effect by preventing access of wild-type KpsT to transport sites. The K44E allele lacks a dominant negative effect in our model because its inability to bind ATP prevents its entry in the cycle. Further, the observation that overexpression of the K44E allele results in accumulation of polySia at distinct membrane sites while overexpression of the wild-type

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**Fig. 5.** Intragenic suppression of E150G. (A) Secondary structure model of KpsT showing the approximate locations of mutations tested for their ability to suppress E150G (adapted from Pavelka et al., 1994). (B) Mutations and the proposed defect associated with each mutation (Pavelka et al., 1994) are listed as is the ability of each to suppress the dominant negative effect of E150G when present in cis. + indicates EV36 harboring a plasmid encoding the double mutation forms large colonies that are halo on antiserum agar; - indicates the same phenotype as plasmid encoding E150G alone.

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<tr>
<th>Mutation</th>
<th>Proposed Defect</th>
<th>Suppression</th>
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<tr>
<td>K44E</td>
<td>ATP Binding</td>
<td>+</td>
</tr>
<tr>
<td>G84D</td>
<td>Interaction with KpsM</td>
<td>+</td>
</tr>
<tr>
<td>S126F</td>
<td>Intramolecular Signaling</td>
<td>+</td>
</tr>
<tr>
<td>C163Y</td>
<td>Unknown</td>
<td>-</td>
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<tr>
<td>H181Y</td>
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or E150G allele results in greater accumulation of polySiA that coalesces to completely surround the cytoplasm suggesting that ATP-binding to KpsT may be an important signal involved in coupling polySiA synthesis and transport.

Recent studies with the ATP-binding components of periplasmic permeases, MalK and HisP (Baichwal et al., 1993; Schneider et al., 1994, 1995) as well as the sec-dependent protein translocation system of E.coli (Schätz and Beckwith, 1990; Wickner et al., 1991; Economou and Wickner, 1994; Kim et al., 1994) have contributed significantly to our understanding of ATP driven transport. Our data in combination with these findings suggest that there may be aspects of ATP-dependent transport in common among different systems such as ATP-dependent association with membrane components, marked conformational changes, and insertion of the ATP-binding component, possibly linked to substrate, into or through the membrane at specific sites defined by membrane components. Further study in these systems will be necessary to define more specifically the nature of these interactions and the specific series of events in a transport cycle.

Materials and methods

Bacteria, plasmids, bacteriophage, and media

E.coli strains used in this work were EV36 (p923 rpsL9 [arg + rba kpsT]; Virm et al., 1989), EV138 (p1104 ansA25 zgi-791::Tnl0, Virm, 1992), and RS2436 (EV36 ΔkpsT; Pavelka et al., 1994). Plasmids pKsP and pSK+ are 2.95 kb Bluestrip cloning vectors (Ap+ Stratagene). Plasmid pSR340 containing wild-type kpsT and pSR346 containing the K44E allele of kpsT have been described previously (Pavelka et al., 1991). Plasmid pSR501 is a 2.0 kb DraI-ClaI fragment containing kpsM and kpsT cloned into pSK+. Plasmid pSR577 is a 722 bp EcoRI-BamHI PCR fragment containing kpsT with the E150G mutation cloned into pKS+, downstream of the lac promoter. Induction of competence and transformations were performed as described previously (Sambrook et al., 1989). Bacterial cultures were grown in L-broth or L-agar supplemented with appropriate antibiotics (Sambrook et al., 1989) at either 23°C or 37°C, restrictive and permissive temperatures for capsule expression, respectively. Horse 46 (H.46) antisera agar plates were used to detect capsule expression as described previously (Silver et al., 1984). Bacteriophage E is specific for encapsulated E.coli K1 (Gross et al., 1977). Positive selection for capsule-transformants was obtained by seeding L-agar plates with bacteriophage E at a multiplicity of infection of approximately 10

DNA manipulation and sequencing

DNA manipulations were done as previously described (Sambrook et al., 1989). Plasmid DNA isolation was by the rapid alkaline extraction procedure as described previously (Is-Horowitz and Burke, 1981). Double stranded DNA for sequence analysis was prepared by polyethylene glycol precipitation (Krahl et al., 1988) and sequenced using an Applied Biosystems 373A DNA sequencer. Oligonucleotides used as primers for sequencing and the polymerase chain reaction (PCR) were synthesized by an Applied Biosystems 380B DNA synthesizer.

SDS-PAGE

Discontinuous sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of proteins was done as previously described (Pavelka et al., 1991).

Western blotting

Immunoblotting was performed as described previously (Aussel et al., 1987). Proteins were transferred to nitrocellulose filters electrophoretically using a semi-dry blotting apparatus (PolyBlot, American Biencenec, Emeryville, CA) as recommended by the manufacturer. Polyclonal rabbit KpsT antiserum was used as the primary antibody at a dilution of 1:1000 (Pavelka et al., 1994). Primary antibody was detected by the secondary goat anti-rabbit IgG conjugate to horseradish peroxidase (Boehringer Mannheim) at a dilution of 1:5000 and visualized with the chromogenic substrate 3'3',5,5'-tetramethylbenzidine (Kirkegaard and Perry Laboratories, Gaithersberg, MD) as recommended by the manufacturer.

8-azidoATP photolabeling assays

Photolabeling of KpsT with 8-azidoATP was performed on whole cell lysates of RS2436 harboring plasmid encoded kpsT alleles as described (Pavelka et al., 1994) using nonradioactive 8-azido-ATP (Sigma). Derivatization of KpsT was detected by an altered mobility on SDS-PAGE and visualized by immunoblotting.

PCR mutagenesis

kpsT was mutagenized by PCR under conditions which reduce the fidelity of the Taq polymerase according to the method recommended by Cadwell and Joyce (1992). Template for the reaction was plasmid pSR501. Primers used for amplification flanked the kpsT coding region and introduced unique EcoRI and BamHI restriction endonuclease sites (Pavelka et al., 1991). PCR products were visualized under long-wave UV light on a 0.7% agarose gel, and a 722 base pair fragment was recovered by the Elu-Quick DNA purification kit (Schleicher & Schuell, Keene, NH) according to the manufacturer’s instructions. The fragment was cloned into pBluescript KS+ under the control of the lac promoter using the new restriction endonuclease sites.

Construction of kpsT double mutations

Five separate double mutations in kpsT were constructed. The kpsT coding sequence contains a unique AgeI restriction site at codon 83 (Pavelka et al., 1991). The 415 bp AgeI/BamHI fragment from pSR577 containing the base change leading to substitution of glycine at glutamate acid 150 (E150G) was gel purified and recovered using the Elu-Quick DNA purification kit (Schleicher & Schuell, Keene, NH) and ligated into the same sites of pSR346 which encodes the K44E mutation (Pavelka et al., 1991). The resulting plasmid contained both mutations as confirmed by restriction analysis; both create novel Hinfl sites in the coding sequence. The remaining four double mutations were constructed by overlap extension PCR (Ho et al., 1989) in which mutagenic primers were synthesized that encoded the E150G mutation. Templates used in the PCR reactions were plasmids each containing previously described mutations in kpsT (Pavelka et al., 1994), such that the PCR product would contain the original mutation in cis to the E150G mutation. The initial reactions to make overlapping partial products were using the 5′ EcoRI site containing flanking primer with mutagenesis primer 5′-CCACACGACGTGATCCATCAAAG-3′ or the 3′ BamHI site containing primer with mutagenesis primer 5′-CTTTAGTGGAATGCTGGT-3′. The resulting PCR products were gel purified and recovered using the Elu-Quick DNA purification kit (Schleicher & Schuell, Keene, NH) and subjected to a second PCR reaction using the flanking primers described above. Parameters for these reactions were as previously described (Pavelka et al., 1991). The 722 base pair fragment containing each double mutation was isolated and ligated into the unique BamHI and EcoRI sites of pBluescript KS+ as described above. Sequence analysis was performed on the entire kpsT coding sequence for each clone to confirm the presence of both mutations and to rule out any unintended mutations resulting from PCR error.

Preparation of bacteria for electron microscopy

For scanning electron microscopy, overnight cultures were pelleted by centrifugation and resuspended into Tyrode’s buffer containing a 1% final concentration of glutaraldehyde and incubated for 90 min at room temperature. Samples were then gently loaded onto a polylysine-coated, 3 μm pore size cyclopore membrane cell culture cup (Falcon Catalog #3096) and gently drawn through the filter by gentle vacuum. Filters were critical point dried in CO2 and sputter coated with platinum before examination in a Field Emission Scanning Electron Microscope (Hitachi S4500) at 5 kV accelerating voltage and photographed. For transmission electron microscopy, bacteria were grown to midlogarithmic phase and processed as previously described (Pavelka et al., 1994, Wunder et al., 1994). Samples were examined at 75 kV with a Hitachi 11-E-1 electron microscope.

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