Journal of Industrial Microbiology (1995) 15, 352–360
© 1995 Society for Industrial Microbiology 0169-4146/95/$12.00

Biosynthesis of the polysialic acid capsule in *Escherichia coli* K1

E Vimr[1,2], S Steenbergen[1] and M Cieslewicz[1]

[1]*Department of Veterinary Pathobiology;* [2]*Department of Microbiology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA*

The extracellular polysaccharides elaborated by most or all bacterial species function in cell-to-cell and cell-substratum adhesion, cell signaling, and avoidance or inhibition of noxious agents in animal hosts or free-living environments. Recent advances in our understanding of exopolysaccharide synthesis have been facilitated by comparative approaches in both plant and animal pathogens, as well as in microorganisms of industrial importance. One of the best understood of these systems is the *kps* locus for polysialic acid synthesis in *Escherichia coli* K1. The genes for sialic acid synthesis, activation, polymerization and translocation have been identified and assigned at least tentative functions in the synthetic and export pathways. Initial studies of *kps* thermoregulation suggest that genetic control mechanisms will be involved which are distinct from those already described for several other exopolysaccharides. Information about the common as well as unique features of polysialic acid biosynthesis will increase our knowledge of microbial cell surfaces which in turn may suggest novel targets for therapeutic or industrial interventions.

Keywords: capsule biosynthesis; virulence factors; polysialic acid; sialic acid; genetic organization

Introduction

The intensively investigated species *Escherichia coli* and *Salmonella enterica* [34] are not usually studied by researchers as other than planktonic microorganisms. However, from an ecological perspective, these microorganisms may lead Jekyll-and-Hyde existences by alternating between life in the wild (outside animal hosts) and life in the gastrointestinal tracts of warm- or cold-blooded animals. Our working hypothesis is that these two environments are, if not fundamentally distinct, then at least different enough from each other to have required the evolution of separate strategies ensuring bacterial growth in either environment. The animal niche has probably not changed substantially since at least the middle Jurassic some 200 million years ago [26]. This broad yet relatively constant environment may have resulted in evolution of gastrointestinal microorganisms such as *E. coli* that are exquisitely adapted to environmental duality [26], quite unlike the poor doctor of Rober Louis Stevenson's novel who could only survive by choosing to live in one environment. It is also known that *S. enterica* and *E. coli* form biofilms on solid medium after hyperflagellation and altered lipopolysaccharide synthesis induced in response to environmental cues (Jean Petter, USDA ARS, Athens, GA, personal communication; [21a]), and that the resulting swarming behavior may be a virulence factor to assist spread of microorganisms in their animal hosts [2]. Therefore, biofilm formation may be a more common and useful phenotype in either environment than was previously recognized [66], consistent with the advantages offered by a sessile community over the vicissitudes of a planktonic existence [5,13].

Traditional views of microbial biofilms imply that extracellular polysaccharides provide relatively nonspecific glues which cement the community while simultaneously functioning to repel noxious intruders [21]. While this may be true of many polysaccharides, there is no ready explanation for the diversity of exopolysaccharides (more than 75 different structures) produced by different serotypes of *E. coli* [24]. Similar carbohydrate diversity in mammals is now known to function as part of the molecular 'zip codes' directing cell migration. These cellular post offices operate via recognition of carbohydrate ligands on one cell by lectins (carbohydrate-binding proteins) on the apposing cell [46]. Since recent results indicate that bacterial lectins may not be primarily responsible for adhesion to animal cell surfaces [42], we speculate that one of the functions of some microbial lectin/polysaccharide pairs may be the formation of microbial consortia in which carbohydrate diversity provides specificity and thus opportunity for different serotypes or species to interact through homo- and hetero-contacts (Figure 1). Protein-carbohydrate (lectin-like) interaction of the types diagrammed in Figure 1 has been observed and is designated coaggregation or coadherence in oral streptococci [25]. In contrast, some bacterial polysaccharides clearly function as anti-recognition molecules that inhibit or repulse the many elaborate host disease-fighting mechanisms encountered during some infections [43]. The K1 antigen or polysialic acid capsule virulence factor of certain invasive *E. coli* strains is a homopolymer of α2,8-linked sialic acid residues which both mimics host antigens, thus contributing to immune tolerance, and inhibits complement-fixation and phagocytosis [43]. The high density of negative cell-surface charge undoubtedly contributes to avoidance mechanisms conferred by poly-

Correspondence: E Vimr, 2522 VMBSB, 2001 South Lincoln Ave, Urbana, IL 61801, USA
Received 28 November 1994; accepted 20 April 1995

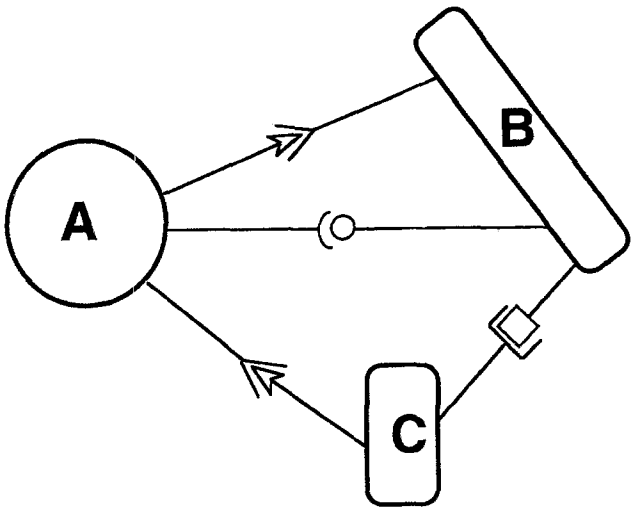

Figure 1 Carbohydrate diversity in biofilms. Cells A, B and C may be one, two or three species. Lectins (female bits) recognize specific carbohydrate epitopes (male bits) on the complementary cell surface

sialic acid and a few other negatively charged capsular polysaccharides [6]. The increasing incidence of antibiotic resistance and the lack of truly effective vaccines for many diseases warrants the identification of virulence factors and the steps involved in biosynthesis of these factors which may offer new targets for therapeutic interventions. We suggest that the synthesis, export and genetic regulation of capsular polysaccharides may be such targets. Better understanding of the basic mechanisms of polysaccharide biosynthesis may also be useful in some industrial applications [30].

K1 and other acidic polysaccharides of *E. coli* are thermoregulated cell structures that are maximally expressed at host body temperatures but not at environmental temperatures below about 25° C [36]. Capsules, as highly hydrated structures, could help bacteria resist desiccation in the wild, consistent with the observed direction of capsule thermoregulation. Since most capsular polysaccharides of *E. coli* are not associated with virulence [24], we assume that the evolution of capsule diversity has been driven by the rigors of life outside animal hosts. Furthermore, whatever the genetic mechanisms of capsule diversification, occasionally polysaccharides are produced which increase survival and thus invasiveness in extraintestinal host compartments. Thus, with the caveat that not all polysaccharides may directly participate in biofilm formation, this review focuses on the biosynthesis and expression of the K1 antigen in *E. coli* as a model of bacterial capsular polysaccharide synthesis in general. The ease of genetic manipulation with *E. coli* and the wealth of physiological information indicate that the choice of this species for investigating basic aspects of polysaccharide synthesis, modification, export, and regulation is valid.

Genetic organization of capsule loci

Taking advantage of the capacity of cosmid vectors for long inserts, Silver and colleagues were the first to clone the *kps* (*k*apsel *p*olysaccharide *s*ynthesis) genes necessary for K1 antigen synthesis [44]. Since then, a variety of capsule gene clusters from both Gram-positive and Gram-negative animal and plant pathogens have been cloned and investigated to varying degrees. These studies clearly support capsule gene clustering as a common theme in polysaccharide biosynthetic systems. Recognizing that the functional analysis of the *kps* cluster would be facilitated by the availability of simple genetic techniques, we constructed *E. coli* K-12/K1 hybrids that stably express the polysialic acid capsule [58–60]. This system led to the initial isolation and characterization of most of the *kps* genes. A central cassette (region 2) of *neu* (*N*-acetyl*neu*raminic acid) genes (Figure 2) encodes enzymes for sialic acid synthesis (*neuB* and *C*), activation (*neuA*), polymerization (*neuS*), and less well-defined functions for initiation or translocation of polysialic acid (*neuD* and *E*). Flanking this region on one side is region 3 [37,38,41,45], which codes for an ATP-binding cassette-type (ABC-type) transporter [3], and region 1 genes with poorly defined functions located to the other side of region 2 (*kpsFEDUCS*) [61]. In contrast to the capsule loci in other species (Figure 2), regions 2 and 3 of *kps* are transcribed in the opposite direction from region 1, suggesting that at least two distinct systems may be required to regulate *kps* expression. Transcription of regions 1 and 2 converges at an intergenic sequence located between *neuS* and *kpsS* [57]. This sequence probably represents a recombinant junction, because different region 2 cassettes share essentially common region 1 genes [9], all of which map at 64 minutes near *serA* [8,54]. Mechanisms to account for such diversity at a single locus are not understood, since *kps* clusters have never been detected on plasmids or phages which could account for the range of different regions 2. A separate origin of K1 *kps* region 2 from region 1 is, however, perhaps further indicated by the up to 80% A + T content of region 2 DNA compared to the more *E. coli*-like 50% content of region 1 [48].

Although limited progress has been made in the interesting area of capsule gene cluster diversity, we have detected nonfunctional *kps* DNA in *E. coli* strain B by Southern hybridization with K1 *kps*-specific probes [57]. This strain has no detectable sialic acid or polysialyltransferase activity, suggesting that although region 2 DNA is probably present, point mutations or small deletions may have accumulated which functionally silenced the region. That up to fifteen genes may be required for capsule expression (Figure 2), and the increased A + T content of region 2, are consistent with the relatively high spontaneous mutation rate (10^{-4} to 10^{-5} per bacterium per generation) measured for K1 *E. coli* [58]. Single mutations almost anywhere in the 17-kbp *kps* locus would frequently result in an acapsular phenotype, and if there was no subsequent selection against these mutants, further changes would continue to accumulate at the same spontaneous mutation rate. We have designated this situation the derelict hypothesis and have suggested how, together with the opportunity for intra- and interspecies recombination, this hypothesis could explain the evolution of *E. coli* K92, which synthesizes a rare polysialic acid composed of alternating α2,8-2,9 linkages [16,48,57]. Similar arguments have been advanced for the *rfb* locus for O-antigen biosynthesis [32 and therein].

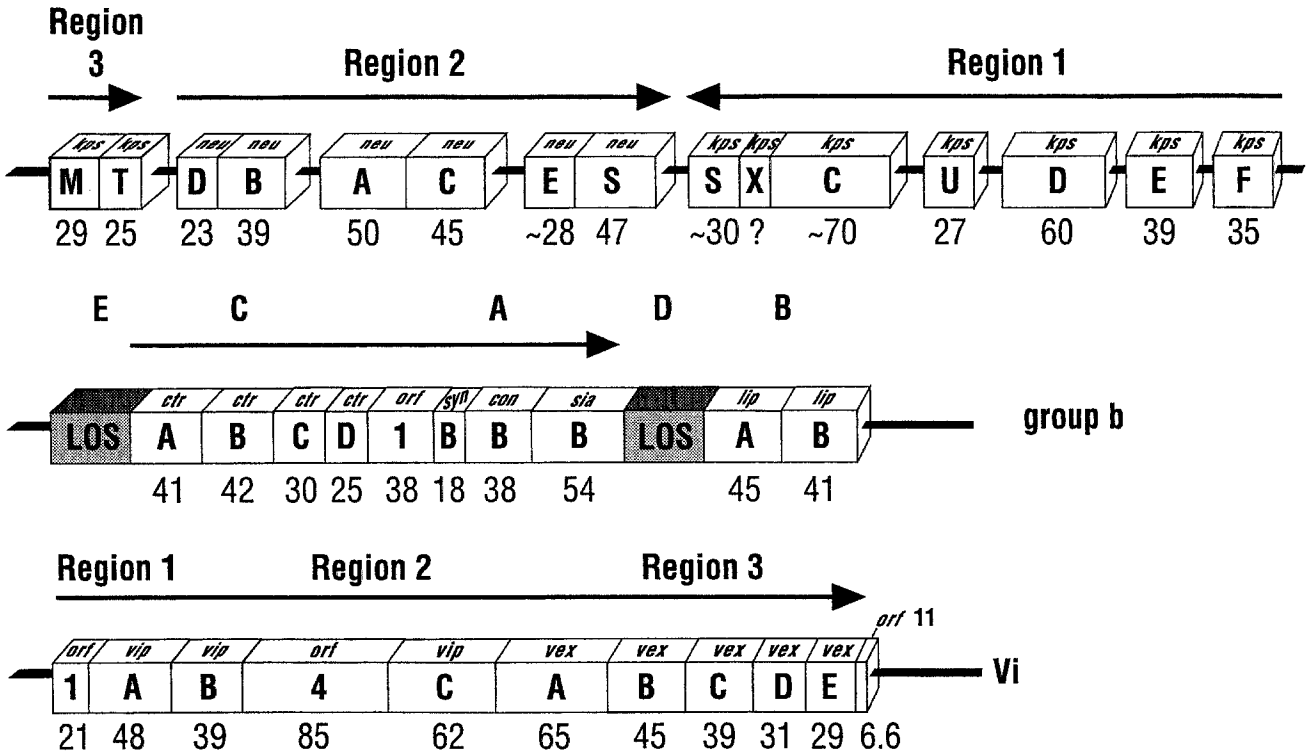

Figure 2 Genetic organization of *kps* and comparison to *ctr* and *viaB* capsule loci. The top line (open boxes) shows the known or suspected *kps* genes required for polysialic acid expression in *Escherichia coli* K1 (see Table 1 for references). Shown below *kps* are the *Nesseria meningitidis ctr* [18] and *Salmonella typhi viaB* [22] loci required for expression of the group B and Vi antigens, respectively. Numbers below each gene give the sizes of kilodaltons of the encoded polypeptides. Note that the size (18 kDa) of the *synB* gene product is likely to underestimate the true mass of the meningococcal CMP-sialic acid synthetase [19]. Arrows indicate transcription direction of the various regions, which are indicated by the numbers or letters above each locus. LOS refers to the group B meningococcal DNA involved in lipooligosaccharide synthesis, which interrupts *ctr* from *lipA,B* [18]

Capsule synthesis genes

Spontaneous or transposon-induced K1 capsule-negative mutants were selected as survivors after infection with the lytic K1 capsule-specific phage K1F [40,61a]. Simple assays for sialic acid activation or polymerization, and *in vivo* complementation of capsule synthesis by exogenously added sialic acid were used to identify the biosynthetic region 2 genes *neuA, B, C,* and *S* (Table 1). Homologs of these genes were subsequently identified in group B meningococci (Table 1) and renamed *siaA, B, C,* and *D* [15]. Since group B meningococcal biosynthetic homologs were previously designated *neu* [19,49], following the precedent in the literature [60], the proposal of *sia* would seem to be without merit and should be discouraged in future publications to facilitate comparisons between similar loci. What appears clear from the studies summarized in Table 1 is that *neuB* codes for the enzyme that condenses *N*-acetyl-

Table 1 K1 *kps* genes and proteins: their functions and homologs in other systems

Gene	Function	Homolog(s)[a]	Refs
neuA	CMP-sialic acid synthetase	SynB	[17,60]
neuB	sialic acid synthase	ConB	[4,17,60]
neuC	ManNAc-6-phosphate phosphatase	Orf1	[18,49]
neuD	transacetylase (?)	unknown	[4,60]
neuE	PSA translocase coupler	unknown	[48,57]
neuS	polysialyltransferase	SiaB	[6,18,47,48]
kpsM	traffic ATPase	CtrC, VexB	[17,22,37]
kpsT	traffic ATPase	CtrD, VexC	[17,22,38]
kpsF	regulatory (?)	GutQ	[12,57]
kpsE	translocation	CtrB, VexD	[12,17,22]
kpsD	translocation	unknown	[64]
kpsC	translocation	LipA, LpsZ	[6,18,41,60]
kpsU	CMP-KDO synthetase	KdsB	[10]
orfX	regulatory (?)	unknown	[57]
kpsS (?)	translocation	LipB	[18,48,60]
omp (?)	translocation (?)	CtrA, VexA	[18,22,57]

[a]Homologs refer to gene products encoded by the capsule loci for group B *Neisseria meningitidis* and *Salmonella typhi* Vi diagrammed in Figure 2

mannosamine (ManNAc) and phosphoenolpyruvate to yield sialic acid and inorganic phosphate, and that *neuA* activates sialic acid by the addition of CMP from CTP to form the nucleotide sugar CMP-sialic acid, which is then polymerized by the polysialyltransferase encoded by *neuS*. These steps are shown diagrammatically in Figure 3. Indirect results suggest *neuC* codes for ManNAc-6-phosphate phosphatase [49] or, possibly, *N*-acetylglucosamine → ManNAc epimerase [15]. The biosynthesis of ManNAc is not understood in *E. coli* and is an area that should be investigated, since it could offer a new target for therapeutic intervention. Homologs of *neuD* and *E* have not been detected in the meningococcal system. The biochemical functions of *neuD* and *E* gene products is thus of great interest because their presence in K1 *kps* suggests that polysialic acid synthesis in group B meningococci could involve a diffferent mechanism despite the occurrence of several functionally and structurally homologous gene products [15].

A potential applied aspect of this work is the overproduction of CMP-sialic acid in mutants with defects in *neuS* [47]. Since *E. coli* appears to lack CMP-sialic acid hydrolase [59], the only metabolic fate of the nucleotide sugar is polymerization by NeuS and recycling of CMP. In the absence of NeuS, bacteria accumulate 0.02–0.05 M concentrations of CMP-sialic acid, which could be used for the industrial scale production of this important sialoconjugate precurser by simple fermentation techniques.

Acapsular K1 mutants with defects in *neuD* do not synthesize polysialic acid [4,45], suggesting some as yet undefined role in polymer initiation or elongation *in vivo*. In contrast, *neuE* mutants synthesize polysialic acid but do not export it to the outer membrane [57]. This indicates that polysialic acid synthesized in the absence of *neuE*$^+$ does not productively engage the polysaccharide transport apparatus, suggesting NeuE may couple capsule synthesis to translocation. The presence of a potential polyprenyl binding site [1] in the predicted membrane anchor of NeuE

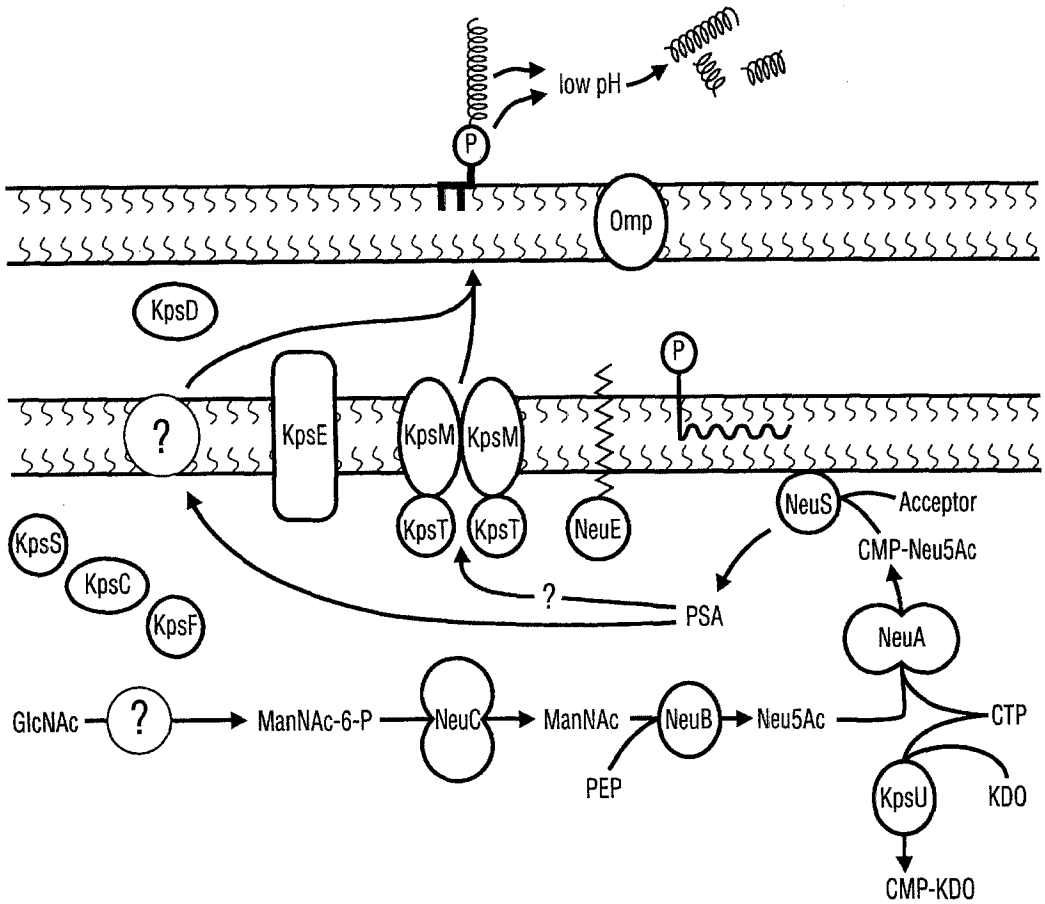

Figure 3 Working model of polysialic acid biosynthesis in *Escherichia coli* K1. Cytoplasmic *N*-acetylglucosamine (GlcNAc) is converted by an unknown enzyme or enzymes to *N*-acetylmannosamine-6-phosphate (ManNAc-6-P), which is then dephosphorylated by NeuC. ManNAc plus phosphoenolpyruvate (PEP) is condensed to yield *N*-acetylneuraminic (sialic) acid (Neu5Ac) by sialic acid synthase (NeuB). Sialic acid is activated to CMP-Neu5Ac by CMP-sialic acid synthetase (NeuA) and then polymerized by NeuS to yield poly-α2,8-linked sialic acid (PSA). CMP-KDO (keto-deoxy octonate) is synthesized by KpsU, although this step may not be important for PSA synthesis or export. The possible functions of NeuE, KpsC, D, E, and F are described in the text and Table 1. The Neu E membrane anchor is indicated by the zig zag. Undecaprenyl phosphate is indicated by the bold, wavy line shown embedded in the cytoplasmic membrane (narrow, wavy lines). The orientation of the phosphate head-group of undecaprenal shown in the periplasm is purely hypothetical. KpsM and T are shown as heterodimers interacting in 1 : 1 stoichiometry with the inner membrane. Curved arrows indicate the translocation of PSA across the inner membrane and periplasm to an extracellular location, where capsular polymers (helices) may be anchored via esterification to phosphatidic acid (bold lines) in the external leaflet of the outer membrane. Capsule sloughing [50] is shown by hydrolysis of PSA at low pH

further suggests that undecaprenyl phosphate may be involved in polymer translocation or the assembly of a functional polymerization-translocation apparatus [48,50–53,57]. Although there is no direct evidence that this motif binds undecaprenyl phosphate, every protein known or suspected of interacting with polyisoprenoids contains the motif consensus sequence [63]. We have recently succeeded in overproducing NeuE as a fusion to maltose binding protein and shown that the putative polyprenyl binding motif functions, as expected, as a membrane anchor (Steenbergen and Vimr, unpublished results). It will now be possible to determine the role of NeuE in polymer export.

Capsule modification and export

During or shortly after polymerization, capsular polysaccharides are exported to the external surface of the cell. In Gram-negative bacteria, polysaccharides must traverse two membranes and the periplasmic space to reach their external destination, indicating that components of the export apparatus may be needed in all three extracytoplasmic compartments. Since a range of structurally distinct *E. coli* capsule loci, defined by different regions 2, share region 1 and 3 genes [9], at least a subset of the common genes is likely to function in capsule export. Other functions could include post-polymerization modifications, such as acetylation or lipidation, and genetic regulation of region 1 or 3 expression [43,60]. Comparison of K1 regions 1 and 3 to other capsule loci (Figure 2) reveal three or four shared components (Table 1). By analogy to ABC-type transporters, the finding that different capsule loci include this cassette, plus one additional inner and one outer membrane polypeptide, has led to the hypothesis shown in Figure 4. Hydrolysis of ATP by KpsT may drive polysialic acid export through an inner membrane pore formed by dimers of KpsM [37]. Translocation to the outer membrane would then be coupled in the periplasm via KpsE to an outer membrane porin (Omp)-type protein, such as CtrA or VexA in meningococcal and *S. typhi*, respectively (Table 1). However, unlike the *ctr* or *vex* systems (Figure 2), *kps* does not code for any *bona fide* outer membrane component. Omp's are β sheet-rich proteins that facilitate solute diffusion by forming pores with relatively small atomic radii [33]. It is thus hard to envision how the known *E. coli* Omp's could function in the export of highly hydrated, negatively charged polysaccharide chains through size-selective pores (Figure 4). The possibility that protein K, an Omp associated with capsulated *E. coli*, functions as the specific outer membrane component for polysialic acid export is unlikely because *E. coli* K-12-K1 hybrids do not express this porin, yet clearly produce an effulgent capsule [60]. Although the model shown in Figure 4 is pleasing in its essential mimicry of a range of other export systems [28], and because diverse capsule systems share homologs of KpsM, T, and E, there is a tendency to assume that the ABC system must function as the inner membrane transporter. However, in the absence of an *in vitro* system which reconstitutes polysialic acid export, or genetic complementation of region 3 with an unrelated ABC system, only correlative evidence supports this model. Although *E coli* K-12/K1 inverted vesicles prepared from a mutant defective in CMP-sialic acid synthetase (*neuA*) were capable of *de novo* polysialic acid synthesis upon addition of the nucleotide sugar precursor, there was little apparent translocation of the polymer [57]. Even if an *in vitro* system can be developed, the results must be shown to be relevant to the *in vivo* process. Toward this goal, we have developed an *in vivo* system that permits studies of polysialic acid synthesis and transport by adding exogenous sialic acid to double mutants (*neuB nanA*) defective in sialic acid synthesis and degradation [55].

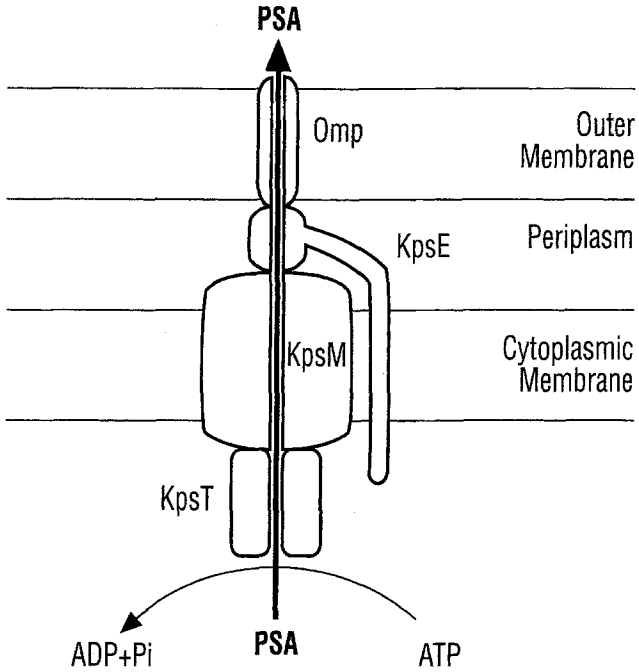

Figure 4 Hypothetical model of the polysialic acid translocase complex. KpsM and T define an ATP-binding cassette-type transporter in the inner membrane. Polysialic acid transport is coupled to hydrolysis of ATP and extrusion of the polymer to the surface through a transmembrane pore formed by KpsM, E and an undefined outer membrane porin (Omp). The diagram was adapted from the models and ideas about traffic ATPases given in Ref 28

Assuming that KpsM and T are the transporter, Frosch and Müller [17] suggested that the common signal recognized by the pore, which in *E. coli* would have to transport the many structurally distinct polymers synthesized by any given serotype, is phosphatidic acid esterified to the reducing end of polysaccharides [20]. Others have speculated that keto-deoxy octonate (KDO), phospholipid, or both KDO and phospholipid provides the common export signal [9,10,39]. Indirect evidence for the signaling role of lipidation was cited as altered electrophoretic or chromatographic mobility of polysialic acid isolated from *lipA* or *lipB* group B meningococcal mutants [17], As shown in Table 1, we have detected primary structure similarity between LipA and KpsC, and LipB and KpsS. In unpublished experiments directly assessing lipidation in *kpsT,S,C,E,* and *F* mutants, we found that each strain synthesized a pool of phospholipase-sensitive, low electrophoretic mobility polysialic acids, strongly suggesting that none of the mutations confers an absolute defect on lipidation of polysialic acid. We suggest that the previous negative results [17] do not provide adequate evidence to exclude

this particular modification, especially in mutants that may synthesize less than wild type levels of polysialic acid.

If KpsS and C are not directly involved with lipidation, what might be their function(s) in polysialic acid export? By analogy with *kpsD* mutants that accumulate periplasmic polysialic acid [64], it is possible that KpsS and C bind polysaccharide in the cytoplasm as a way of 'chaperoning' chains to the translocation apparatus. These polypeptides would presumably recognize some common secondary structural feature of acidic polysaccharides. Alternatively, the similarity of KpsC to LpsZ (Table 1) would be consistent with a possible polysaccharide-modification function [39]. LpsZ has been shown to modify *Rhizobium meliloti* LPS, a phenotype that corrects the nitrogen-fixing-negative (Fix^-) phenotype in alfalfa nodules induced by *exoA* mutants, which have defects in acidic exopolysaccharide production [62]. LpsZ is thought to modify LPS by adding additional sugar residues so that the altered molecule functions as an inducer of the Fix^+ phenotype normally signaled by the acidic exopolysaccharide. Structural similarity of KpsS to LipB (Table 1) suggests that these proteins perform similar roles in K1 *E. coli* and group B meningococci, although as noted above, we do not think this role is lipidation. In this case, it may be significant that no KpsC (LipA) or KpsS (LipB) homologs have been detected in the *viaB* locus (Figure 1). Therefore, either genes mapping outside of *viaB* perform the same function as *kpsC* and *S*, or the Vi antigen does not require the products of these genes for its export or assembly.

Polysialic acid in *E. coli* K1 strains is known to go through form variation in which carbon-9 is acetylated on a variable number of sialic acid residues after polysialic acid has been synthesized [23,35]. This modification affects immunogenicity and glycosidase (neuraminidase) sensitivity, both of which could be important depending on whether the bacteria are in the intestinal tract or the bloodstream of an infected host [35]. The enzyme(s) required for acetylation have not been identified, but since unacetylated chains are exported, this modification presumably plays no role in K1 synthesis or translocation. Jann and Jann and their colleagues suggested that KDO addition is important for export of the K5 capsule [9,39], but this modification has not been reported in polysialic acid. In addition to phosphatidic acid, it has been suggested that oligosialic acid chains may be linked to undecaprenyl phosphate [29,50–53], which could play a role in synthesis or export of polysialic acid. Unfortunately, direct evidence that any of these modifications have an effect on polysialic acid capsule expression is lacking, nor have any K1 *kps* or other gene products been unambiguously linked with catalyzing a particular modification. In contrast, CMP-KDO synthetase is clearly encoded by *kpsU* (Table 1) but it may be a non-essential region 2-like gene 'hitchhiking' in region 3, since KpsU is apparently not required for K1 expression nor have we been able to detect its synthesis in maxicell experiments (Steenbergen and Vimr, unpublished results). The role of polysaccharide modification in capsule expression and function clearly requires more detailed analysis.

An alternative explanation for the function of some region 1 and 3 gene products is that they play a role in assembling a functional polymerization apparatus. Our original observation that defects in region 1 or 3 genes exert epistatic effects on polymerization [48,59,60] has been confirmed in the K5 *E. coli* system [39]. Direct feed-back inhibition of unexported polysialic acid on the expression of *neuS* is unlikely because the exogenous activity of the polymerase with colominic acid acceptors remains intact [60], showing that *neuS* is not down-regulated. Competitive inhibition is also unlikely because endogenous polysialic acid synthesis is unaffected by the presence of exogenous acceptors [27]. Furthermore, unlike the membrane-associated polymerases for cellulose [11] or hyaluronic acid [14] biosynthesis, which efficiently synthesize polysaccharides *in vitro*, polysialic acid synthesis decays rapidly with time and displays nonlinear kinetics [27]. It is therefore possible that region 1 and 3 gene products provide the molecular context for coupling polysialic acid synthesis to export, and that this coupling is partially disrupted by cell fractionation. The observation that azido-ATP inhibits polysialic acid synthesis *in vitro* is very interesting [51], since one or more *kps* gene products might energize polymerization instead of translocation of polysialic acid. Since polymerization *per se* should not require energy beyond that provided by the activated sugar nucleotide precursors, an energy requirement for polymerization could imply a coupling between synthesis and export. That NeuS appears to be a processive sialyltransferase is consistent with this notion [47,60], and with the idea that energy might be required for extrusion of the polysaccharide [51]. The isolation of mutants with conditional defects in translocation would seem to offer an experimental system for investigating the dynamics of polysialic acid synthesis and export. The extracytoplasmic synthesis of teichoic acid may be an important variation in some polysaccharide biosynthetic systems which would obviate the requirement for a separate translocation system. In this case the polymerase might shuttle between the cytoplasm, where it picks up sugar nucleotide precursors, and the external site of polymerization [7]. Although a sugar nucleotide transporter has never been identified in bacteria, there remains the formal possibility that at least some polysaccharides may be assembled extracytoplasmically from transported nucleotide precursors. It is also possible that some polymerases span the membrane, in which case polymerization and export could occur within the same protein channel. There is no evidence, however, that NeuS is a membrane-spanning enyme.

Regulation of capsule expression

The only environmental condition known to influence K1 capsule expression dramatically is temperature. Using *E. coli* K-12-K1 hybrid strains, Merker and Troy [31] showed that sialic acid synthase was probably synthesized at low temperature, but that the enzyme was catalytically inactive and could be reactivated by shifting cells to the capsule-permissive temperature of 37° C. Both sialic acid and the synthase activity were detected when cells were shifted to the permissive temperature in the presence of chloramphenicol or actinomycin, suggesting that neither transcription nor translation was required for reactivation. These results indicate that region 2 is transcribed at low temperature but that the supply of precursor sialic acid is regulated by a

post translational mechanism that probably involves the synthase. Troy and his colleagues [50] had previously shown that NeuS was also present in cells grown at low temperature and that the absence of endogenous polymerase activity was caused by the failure to assemble or synthesize other components of a sialyltransferase complex required for *de novo* polysialic acid synthesis. Since mutants with defects in some region 1 or 3 genes synthesize less polysaccharide than wild type and have decreased polymerase activity [60], it is reasonable to conclude that these regions are thermoregulated.

To determine the mechanism of region 1 thermoregulation, we isolated a chromosomal *lacZ* operon fusion to *kpsE* [12]. β-Galactosidase activity in cells grown at 19° C was 10–20 times less than cells grown at 37° C, showing that *kpsE* and, perhaps, all of region 1 expression was thermoregulated. Sequence analysis and primer extension studies have so far failed to identify a *kpsE* promoter within 100 base pairs of the predicted translational start site [12, and unpublished results]. However, these studies led to the identification of a new open reading frame, designated *kpsF*, that was shown to code for a 35-kilodalton polypeptide with homology to GutQ [12]. GutQ is a putative ATP-binding protein of unknown function encoded by *gutQ* of the glucitol utilization operon in *E. coli* K-12 [65]. The homology (49% primary structure identity) between KpsF and GutQ implies these proteins play similar, perhaps regulatory roles in controlling the expression of their respective operons. An *aphT* cassette coding for kanamycin resistance was cloned into *kpsF* and recombined by allelic exchange into the chromosomal *kps* locus [12]. The resulting acapsular *kpsF::aphT* mutants accumulated polysialic acid in the cytoplasm, indicating that *kpsF* might be the first gene of the region 1 operon and that the cassette exerts polarity on downstream region 1 expression. Alternatively, KpsF may be required to activate other *kps* genes. Experiments are in progress to determine whether the *kpsF* promoter is thermoregulated. Unlike the regulation of *viaB* [22] or *alg* [30], for example, which involves regulatory loci mapping outside of the respective capsule gene clusters, no loci unlinked to *kps* have yet been unambiguously shown to affect polysialic acid synthesis or export, or *kps* gene expression, although such loci are likely to exist. Isolation and characterization of reporter fusions in region 1, such as *kpsE::lacZ*, should allow us to determine the mechanism of capsule thermoregulation.

Conclusions

Microorganisms have gone to extraordinary lengths in expressing sialoglycoconjugates at their cell surfaces, where these macromolecules function in avoidance or subversion of host immune mechanisms [56]. In addition to *E. coli* K1 and K92, several serotypes of *N. meningitidis* have evolved or acquired by horizontal genetic exchange an equally elaborate biosynthetic pathway for the synthesis, activation, polymerization, modification, translocation and regulation of sialic and polysialic acid. Significant progress has been made in identification of the genes and enzymes for precursor synthesis and polymerization of sialic acid, suggesting that specific inhibitors might be useful for prophylaxis as well as for treatment of acute illness caused by these invasive pathogens. By focusing on the unique biochemistry involved in the assembly of specific virulence factors, it may be possible to avoid the inherent problems of standard antibiotic drug therapy and the ineffectiveness or potentially dangerous side effects of many vaccines. Another potential therapeutic target is the shared export mechanism used by different microorganisms for the translocation of capsular polysaccharides. Although the translocators in Gram-negative bacteria appear to include an inner membrane ABC-type system, it is unclear whether polysaccharides enter the transporter during or after polymerization. We also have little idea how polymerizaton is initiated and terminated or whether the biosynthetic enzymes interact physically with other components of the system. A final area that is being investigated is the thermoregulation of capsule expression. Unlike other capsule systems, *kps* does not appear to be regulated by any of the global control systems that have been commonly investigated. The physiological explanation for this apparent difference can only be obtained by determining the actual mechanisms of K-antigen thermoregulation.

Acknowledgements

Work from the authors' laboratory was supported by a research grant (AI23039) from the National Institutes of Health. M Cieslewicz was supported in part by an Arnold O Beckman award from the University of Illinois Research Board.

References

1 Albright CF, P Orlean and PW Robbins. 1989. A 13-amino acid peptide in three yeast glycosyltransferases may be involved in dolichol recognition. Proc Natl Acad Sci USA 86: 7366–7369.
2 Allison CL, L Emody, N Coleman and C Hughes. 1994. The role of swarm cell differentiation and multicellular migration in the uropathogenicity of *Proteus mirabilis*. J Infect Dis 169: 1155–1158.
3 Ames GF-L, CS Mimura, SR Holbrook and V Shyamala. 1992. Traffic ATPases: a superfamily of transport proteins operating from *Escherichia coli* to humans. Adv Enzymol 65: 1–47.
4 Annunziato PW, LF Wright, WF Vann and RP Silver. 1995. Nucleotide sequence and genetic analysis of the *neuD* and *neuB* genes in region 2 of the polysialic acid gene cluster of *Escherichia coli* K1. J Bacteriol 177: 312–319.
5 Anwar H and JW Costerton. 1992. Effective use of antibiotics in the treatment of biofilm-associated infections. ASM News 58: 665–672.
6 Bayer ME and MH Bayer. 1994. Biophysical and structural aspects of the bacterial capsule. ASM News 60: 192–198.
7 Bertram KC, IC Hancock and J Baddiley. 1981. Synthesis of teichoic acid by *Bacillus subtilis* protoplasts. J Bacteriol 148: 406–412.
8 Boulnois G, R Drake, R Pearce and I Roberts. 1992. Genome diversity at the *serA*-linked capsule locus in *Escherichia coli*. FEMS Microbiol Lett 100: 121–124.
9 Boulnois GJ and K Jann. 1989. Bacterial polysaccharide capsule synthesis, export and evolution of structural diversity. Mol Microbiol 3: 1819–1823.
10 Bronner D, V Sieberth, C Pazzani, IS Roberts, GJ Boulnois, B Jann and K Jann. 1993. Expression of the capsular K5 polysaccharide of *Escherichia coli*: biochemical and electron microscopic analyses of mutants with defects in region 1 of the K5 gene cluster. J Bacteriol 175: 5984–5992.
11 Cannon RE and SM Anderson. 1991. Biogenesis of bacterial cellulose. CRC Crit Rev Microbiol 17: 435–447.
12 Cieslewicz MJ, SM Steenbergen and ER Vimr. 1993. Cloning, sequencing, expression, and complementation analysis of the *Escherichia*

coli K1 *kps* region 1 gene, *kpsE*, and identification of an upstream open reading frame encoding a protein with homology to GutQ. J Bacteriol 175: 8018–8023.
13 Costerton JW and HM Lappin-Scott. 1989. Behavior of bacteria in biofilms. ASM News 55: 650–654.
14 DeAngelis PL and PH Weigel. 1994. Immunochemical confirmation of the primary structure of streptococcal hyaluronan synthase and synthesis of high molecular weight product by the recombinant enzyme. Biochemistry 33: 9033–9039.
15 Edwards U, A Müller, S Hammerschmidt, R Gerardy-Schahn and M Frosch. 1994. Molecular analysis of the biosynthesis pathway of the α-2,8 polysialic acid capsule by *Neisseria meningitidis* serogroup B. Mol Microbiol 14: 141–149.
16 Egan W, T-Y Liu, D Dorow, JS Cohen, JD Robbins, EC Gotschlich and JB Robbins. 1977. Structural studies on the sialic acid polysaccharide antigen of *Escherichia coli* strain Bos-12. Biochemistry 16: 3687–3692.
17 Frosch M and A Müller. 1993. Phospholipid substitution of capsular polysaccharides and mechanism of capsule formation in *Neisseria meningitidis*. Mol Microbiol 8: 483–493.
18 Frosch M and U Edwards. 1993. Molecular mechanism of capsule expression in *Neisseria meningitidis* serogroup B. In: Polysialic Acid, from Microbes to Man (Roth J, U Rutishauser and FA Troy II, eds), pp 49–57, Birkhaüser, Basel.
19 Ganguli S, G Zapata, T Wallis, C Reid, G Boulnois, WF Vann and IS Roberts. 1994. Molecular cloning and analysis of genes for sialic acid synthesis in *Neisseria meningitidis* group B and purification of the meningococcal CMP-NeuNAc synthetase enzyme. J Bacteriol 176: 4583–4589.
20 Gotschlich EC, BA Fraser, O Nishimura, JB Robbins and TY Liu. 1981. Lipid on capsular polysaccharides of Gram-negative bacteria. J Biol Chem 256: 8915–8921.
21 Gristina AG, M Oga, LX Webb and CD Hobgood. 1985. Adherent bacterial colonization in the pathogenesis of osteomyelitis. Science 228: 990–993.
21a Harshey RM and T Matsuyama. 1994. Dimorphic transition in *Escherichia coli* and *Salmonella typhimurium*: surface-induced differentiation into hyperflaggelate swarmer cells. Proc Natl Acad Sci USA 91: 8631–8635.
22 Hashimoto Y, N Li, H Yokoyama and T Ezaki. 1993. Complete nucleotide sequencing and molecular characterization of ViaB region encoding Vi antigen in *Salmonella typhi*. J Bacteriol 175: 4456–4465.
23 Higa HH and A Varki. 1988. Acetyl-coenzyme A: polysialic acid *O*-acetyltransferase from K1-positive *Escherichia coli*. J Biol Chem 263: 8872–8878.
24 Jann K and B Jann. 1987. Polysaccharide antigens of *Escherichia coli*. Rev Infect Dis 9: S517–S526.
25 Jenkinson FF. 1994. Adherence and accumulation of oral streptococci. Trends Microbiol 2: 209–212.
26 Koch AL. 1976. How bacteria face depression, recession, and derepression. Perspect Biol Med 20: 44–63.
27 Kundig FD, D Aminoff and S Roseman. 1971. The sialic acids. XII. Synthesis of colominic acid by a sialyltransferase from *Escherichia coli* K-235. J Biol Chem 246: 2543–2550.
28 Lewis K. 1994. Multidrug resistance pumps in bacteria: variations on a theme. Trends Biochem Sci 19: 119–123.
29 Masson L and BE Holbein. 1985. Role of lipid intermediate(s) in the synthesis of serogroup B *Neisseria meningitidis* capsular polysaccharide. J Bacteriol 161: 861–867.
30 May TB and AM Chakrabarty. 1994. *Pseudomonas aeruginosa*: genes and enzymes of alginate synthesis. Trends Microbiol 2: 151–157.
31 Merker RI and FA Troy. 1990. Biosynthesis of the polysialic acid capsule in *Escherichia coli* K1, cold inactivation of sialic acid synthase regulates capsule expression below 20° C. Glycobiology 1: 93–100.
32 Nelson K and RK Selander. 1994. Intergeneric transfer and recombination of the 6-phosphogluconate dehydrogenase gene (*gnd*) in enteric bacteria. Proc Natl Acad Sci USA 91: 10227–10231.
33 Nikaido H and M Vaara. 1987. Outer membrane. In: *Escherichia coli* and *Salmonella typhimurium*, Cellular and Molecular Biology (Neidhardt FC, JL Ingraham, B Magasanik, KB Low, M Schaechter and HE Umbarger, eds), pp 7–22, American Society for Microbiology, Washington, DC.
34 Old DC. 1992. Nomenclature of *Salmonella*. J Med Microbiol 37: 361–363.
35 Ørskov F, I Ørskov, A Sutton, R Schneerson, W Lin, W Egan, GE Hoff and JB Robbins. 1979. Form variation in *Escherichia coli* K1 determined by *O*-acetylation of the capsular polysaccharide. J Exp Med 149: 669–685.
36 Ørskov F, V Sharma and I Ørskov. 1984. Influence of growth temperature on the development of *Escherichia coli* polysaccharide K antigens. J Gen Microbiol 130: 2681–2684.
37 Pavelka MS Jr, LF Wright and RP Silver. 1991. Identification of two genes, *kpsM* and *kpsT*, in region 3 of the polysialic acid gene cluster of *Escherichia coli* K1. J Bacteriol 173: 4603–4610.
38 Pavelka MS Jr, SF Hayes and RP Silver. 1994. Characterization of KpsT, the ATP-binding component of the ABC-transporter involved with the export of capsular polysialic acid in *Escherichia coli* K1. J Biol Chem 269: 20149–20158.
39 Pazzani C, C Rosenow, GJ Boulnois, D Bronner, K Jann and IS Roberts. 1993. Molecular analysis of region 1 of the *Escherichia coli* K5 antigen gene cluster: a region encoding proteins involved in cell surface expression of capsular polysaccharide. J Bacteriol 175: 5978–5983.
40 Petter JG and ER Vimr. 1993. Complete nucleotide sequence of the bacteriophage K1F tail gene encoding endo-*N*-acylneuraminidase (endo-*N*) and comparison to an endo-*N* homolog in bacteriophage PK1E. J Bacteriol 175: 4354–4363.
41 Pigeon RP and RP Silver. 1994. Topological and mutational analysis of KpsM, the hydrophobic component of the ABC-transporter involved in the export of polysialic acid in *Escherichia coli* K1. Mol Microbiol 14: 871–881.
42 Poulsen LD, F Lan, CS Kristensen, P Hoabolth, S Molin and KA Krogfelt. 1994. Spatial distribution of *Escherichia coli* in the mouse large intestine inferred from rRNA *in situ* hydridization. Infect Immun 62: 5191–5194.
43 Silver RP and ER Vimr. 1990. Polysialic acid capsule of *Escherichia coli* K1. In: The Bacteria, vol 11, Molecular Basis of Bacterial Pathogenesis (Iglewski B and V Miller, eds), pp 39–60, Academic Press, New York.
44 Silver RP, CW Finn, WF Vann, W Aaronson, R Schneerson, P Kretschmer and CF Garon. 1981. Molecular cloning in the K1 capsular polysaccharide genes of *E. coli*. Nature 289: 696–698.
45 Silver RP, P Annunziato, MS Pavelka, RP Pigeon, LF Wright and DE Wunder. 1993. Genetic and molecular analyses of the polysialic acid gene cluster of *Escherichia coli* K1. In: Polysialic Acid, from Microbes to Man (Roth J, U Rutishauser and FA Troy II, eds), pp 59–71, Birkhaüser, Basel.
46 Springer TA. 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell 76: 301–304.
47 Steenbergen SM and ER Vimr. 1990. Mechanism of polysialic acid chain elongation in *Escherichia coli* K1. Mol Microbiol 4: 603–611.
48 Steenbergen SM, TJ Wrona and ER Vimr. 1992. Functional analysis of the sialyltransferase complexes in *Escherichia coli* K1 and K92. J Bacteriol 174: 1099–1108.
49 Swartley JS and DS Stephens. 1994. Identification of a genetic locus involved in the biosynthesis of *N*-acetyl-D-mannosamine, a precursor of the (α2-8)-linked polysialic acid capsule of serogroup B *Neisseria meningitidis*. J Bacteriol 176: 1530–1534.
50 Troy FA. 1979. The chemistry and biosynthesis of selected bacterial capsular polymers. Annu Rev Microbiol 33: 519–560.
51 Troy FA. 1992. Polysialylation: from bacteria to brains. Glycobiology 2: 5–23.
52 Troy FA, IK Vijay and N Tesche. 1975. Role of undecaprenyl phosphate in synthesis of polymers containing sialic acid in *Escherichia coli*. J Biol Chem 250: 156–163.
53 Troy FA, J-W Cho and J Ye. 1993. Polysialic acid capsule synthesis and chain translocation in neuroinvasive *Escherichia coli* K1: 'activated' intermediate and a possible bifunctional role for undecaprenyl. In: Polysialic Acid, from Microbes to Man (Roth J, U Rutishauser and FA Troy II, eds), pp 93–111, Birkhaüser, Basel.
54 Vimr ER. 1991. Map position and genome organization of the *kps* cluster for polysialic acid biosynthesis in *Escherichia coli* K1. J Bacteriol 173: 1335–1338.
55 Vimr ER. 1992. Selective synthesis and labelling of the polysialic acid capsule in *Escherichia coli* K1 strains with mutations in *nanA* and *neuB*. J Bacteriol 174: 6191–6197.
56 Vimr ER. 1994. Microbial sialidases: does bigger always mean better? Trends Microbiol 2: 271–277.

57 Vimr ER and S Steenbergen. 1993. Mechanisms of polysialic acid assembly in *Escherichia coli* K1: a paradigm for microbes and mammals. In: Polysialic Acid, from Microbes to Man (Roth J, U Rutishauser and FA Troy II, eds), pp 73–91, Birkhaüser, Basel.

58 Vimr ER and FA Troy. 1985. Identification of an inducible catabolic system for sialic acids (*nan*) in *Escherichia coli*. J Bacteriol 164: 845–853.

59 Vimr ER and FA Troy. 1985. Regulation of sialic acid metabolism in *Escherichia coli*: role of *N*-acylneuraminate pyruvate-lyase. J Bacteriol 164: 854–860.

60 Vimr ER, W Aaronson and RP Silver. 1989. Genetic analysis of chromosomal mutations in the polysialic acid gene cluster of *Escherichia coli* K1. J Bacteriol 171: 1106–1117.

61 Vimr ER, R Bergstrom, SM Steenbergen, G Boulnois and I Roberts. 1992. Homology among *Escherichia coli* K1 and K92 polysialyltransferases. J Bacteriol 174: 5127–5131.

61a Vimr ER, RD McCoy, HF Vollger, NC Wilkison and FA Troy. 1984. Use of prokaryotic-derived probes to identify poly(sialic acid) in neonatal membranes. Proc Natl Acad Sci USA 81: 1971–1975.

62 Williams MNV, RI Hollingsworth, S Klein and ER Signer. 1990. The symbiotic defect of *Rhizobium meliloti* exopolysaccharide mutants is suppressed by *lpsZ*$^+$, a gene involved in lipopolysaccharide biosynthesis. J Bacteriol 172: 2611–2632.

63 Wilson IBH, JP Taylor, MC Webberley, NJ Turner and SL Flitsch. 1993. A novel mono-branched lipid phosphate acts as a substrate for dolichyl phosphate mannose synthetase. Biochem J 295: 195–201.

64 Wunder DE, W Aaronson, SF Hayes, JM Bliss and RP Silver. 1994. Nucleotide sequence and mutational analysis of the gene encoding *kpsD*, a periplasmic protein involved in transport of polysialic acid in *Escherichia coli* K1. J Bacteriol 176: 4025–4033.

65 Yamada M, Y Yamada and MH Saier Jr. 1990. Nucleotide sequence and expression of the *gutQ* gene within the glucitol operon of *Escherichia coli*. J DNA Sequencing and Mapping 1: 141–145.

66 Yasuda H, YL Ajiki, J Aoyama and T Yokota. 1994. Interaction between human polymorphonuclear leucocytes and bacteria released from in-vitro bacterial biofilm models. J Med Microbiol 41: 359–367.