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Translocation of group 1 capsular polysaccharide to the surface of *Escherichia coli* requires a multimeric complex in the outer membrane

Jolyne Drummelsmith and Chris Whitfield[1]

Department of Microbiology, University of Guelph, Guelph, Ontario, Canada N1G 2W1

[1]Corresponding author
e-mail: cwhitfie@uoguelph.ca

Surface expression of the group 1 K30 capsular polysaccharide of *Escherichia coli* strain E69 (O9a:K30) requires Wza_{K30}, a member of the outer membrane auxiliary (OMA) protein family. A mutation in wza_{K30} severely restricts the formation of the K30 capsular structure on the cell surface, but does not interfere with the biosynthesis or polymerization of the K30 repeat unit. Here we show that Wza_{K30} is a surface-exposed outer membrane lipoprotein. Wza_{K30} multimers form ring-like structures in the outer membrane that are reminiscent of the secretins of type II and III protein translocation systems. We propose that Wza_{K30} forms an outer membrane pore through which the K30-capsular antigen is translocated. This is the first evidence of a potential mechanism for translocation of high molecular weight polysaccharide across the outer membrane. The broad distribution of the OMA protein family suggests a similar process for polysaccharide export in diverse Gram-negative bacteria.

Keywords: capsular polysaccharides/outer membrane/secretin/translocation/Wza

Introduction

Bacterial cells are capable of secreting a range of large molecules, including both complex polysaccharides and proteins. Some of these macromolecules are required for cell viability, whereas others are involved in interactions between a bacterial pathogen and its host. Polysaccharides are secreted by many bacteria and occur in two forms: extracellular polysaccharides released by the cell into the surrounding environment, and cell-associated capsular polysaccharides (often referred to as K antigens). Secreted polysaccharides are typically linear or branched glycans with molecular weights in the 10^4–10^6 Da range. These polymers can adopt ordered conformations, often with helical structure (Sutherland, 1988). Cell-surface polysaccharides are synthesized at the inner membrane and must be translocated to the cell surface. However, in Gram-negative bacteria, the outer membrane serves as a selectively permeable protective barrier and presents a significant obstacle to macromolecular translocation. While significant progress has been made recently in understanding the components and processes involved in protein translocation, virtually nothing is known about the translocation of cell surface polysaccharides across the outer membrane.

Isolates of *Escherichia coli* produce over 70 different capsular polysaccharides (Jann and Jann, 1997) and have provided models for the synthesis of capsular and extracellular polysaccharides in other bacteria. *Escherichia coli* capsule structures have been classified into four groups based on their surface organization, the mechanism involved in their assembly, the structure of their biosynthetic gene loci and the regulation of their expression (reviewed in Whitfield and Roberts, 1999). Despite the structural diversity evident in *E.coli* capsules, only two polymerization strategies are known. Group 2 and 3 capsules are polymerized in the cytoplasm by a multienzyme complex including processive membrane-associated glycosyltransferases. The nascent capsule is exported across the inner membrane by a member of the ABC-2 family of ATP-binding cassette transporters (Bliss and Silver, 1996). A similar mechanism is required for synthesis of capsules in a variety of other bacteria, notably the human pathogens *Haemophilus influenzae* and *Neisseria meningitidis.* Polymerization of the *E.coli* group 1 and 4 capsules differs in both mechanism and location. In these capsules, undecaprenol pyrophosphate-linked oligosaccharide repeat units are formed at the cytoplasmic face of the inner membrane, transported across the inner membrane in a Wzx protein-dependent process, and polymerized by a mechanism involving a Wzy protein. A similar pathway is used for capsules and extracellular polysaccharides produced by bacteria that infect or interact with animals and plants, including *Klebsiella pneumoniae*, *Erwinia* spp., *Rhizobium* spp. and *Xanthomonas campestris.*

From the periplasmic face of the inner membrane, capsular polysaccharides are translocated to the cell surface but the underlying processes have not been resolved. Electron microscopy analysis of the insertion of nascent group 1 capsule on the *E.coli* surface shows the sites of insertion are located above regions where the inner and outer membranes are in apposition (Bayer and Thurow, 1977). Biochemical evidence points to a similar spatial organization in the biogenesis of group 2 capsules (Rigg *et al*., 1998). These data are best explained by the presence of a complex that spans the periplasm, linking the biosynthetic proteins in the inner membrane to a translocation system in the outer membrane (Whitfield and Roberts, 1999). However, the mechanism of translocation and the identity of the outer membrane components have yet to be resolved.

The group 1 capsule clusters are characterized by the presence of four genes, *orfX*, *wza*, *wzb* and *wzc* (Drummelsmith and Whitfield, 1999; Rahn *et al*., 1999). The function of the *orfX* gene product is unknown and it has no homolog in the DDBJ/EMBL/Genbank joint databases (Drummelsmith and Whitfield, 1999). The *wza* gene encodes a predicted protein of the OMA (outer-membrane auxiliary) family (Paulsen *et al*., 1997) that

participates in capsule formation (Drummelsmith and Whitfield, 1999). Wzc is a tyrosine autokinase and Wzb is its cognate phosphatase (Grangeasse *et al*., 1998; Vincent *et al*., 1999). The precise roles of these proteins in capsule biogenesis are unclear but, based on mutant phenotypes, we have proposed that Wza and Wzc are involved in surface expression of the capsule (Drummelsmith and Whitfield, 1999). In the absence of Wza and Wzc, assembly of the high-molecular-weight group 1 capsular polysaccharide on the cell surface is impaired, but oligosaccharides containing several repeat units of the polysaccharide are still synthesized and are assembled on the cell surface in a lipopolysaccharide (LPS)-linked form known as K_{LPS}. K_{LPS} is a novel structural feature in *E.coli* strains that form group 1 capsules. Collectively, the available data indicate that: (i) Wza and Wzc are not essential for synthesis or polymerization of the group 1 polysaccharide; (ii) these proteins are specifically involved in surface assembly of the high-molecular-weight capsular forms of group 1 capsular polysaccharides; but (iii) they do not mediate the translocation of the other major cell-surface glycoconjugate in Gram-negative bacteria, LPS.

Here, we describe the characterization of the Wza_{K30} protein from *E.coli* E69 (serotype O9a:K30). We show that Wza_{K30} is an outer membrane lipoprotein with domains exposed at the cell surface. In addition, we show for the first time that Wza_{K30} is present in a multimeric complex that is visible by electron microscopy. The complex resembles the 'secretins' that participate in translocation of proteins across the outer membrane in type II and III secretion pathways.

Results

Outer membrane localization of the Wza_{K30} lipoprotein

Sequence homologies identified Wza_{K30} as a member of the OMA protein family. OMA proteins are found in many polysaccharide gene clusters (Paulsen *et al*., 1997). BLAST searches identified a number of homologs to Wza_{K30}, including both members of the OMA protein family and several other proteins predicted to be involved in polysaccharide biosynthesis (data not shown). Several of these, including two Wza homologs from *E.coli*, one from *K.pneumoniae* and one from *Erwinia amylovora*, are highly conserved with similarity extending throughout the proteins (data not shown). Wza homologs in different *E.coli* capsular K serotypes are virtually identical (Rahn *et al*., 1999).

The predicted amino acid sequences of Wza_{K30} and many other members of the OMA protein family contain consensus motifs for a signal peptidase II cleavage site following a putative signal sequence, indicating that they may be processed lipoproteins. Only two OMA proteins to date, EpsA of *Ralstonia solanacearum* (Huang and Schell, 1995) and CtrA of *N.meningitidis* (Frosch *et al*., 1992), have been shown to be present in cell membranes, and lipid modification has not been addressed in any members of the OMA family. The lipoprotein character of Wza_{K30} was therefore assessed by [^{3}H]palmitate-labeling *E.coli* LE392 (pWQ126) cells overexpressing Wza_{K30} (Figure 1A). After arabinose induction, a ^{3}H-radiolabeled polypeptide with an apparent molecular weight of 40 kDa

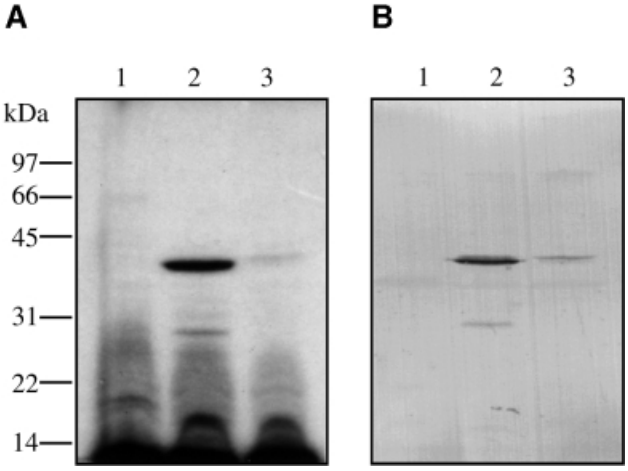

Fig. 1. [^{3}H]palmitate labeling of Wza_{K30} and the inhibition of signal peptidase II cleavage by globomycin. (**A**) Autoradiogram of radiolabeled whole-cell lysates. (**B**) Corresponding Western blot, probed with anti-Wza_{K30} rabbit polyclonal antibody. The analysis was done using *E.coli* LE392 containing pWQ126, with and without arabinose induction. Prior to SDS–PAGE, samples were solubilized in sample buffer at 100°C. Lane 1, uninduced samples; lane 2, induced samples; lane 3, induced samples grown in the presence of globomycin.

was identified in the whole-cell lysate. This size is consistent with that predicted by sequence data for Wza_{K30}. The identity of this polypeptide was confirmed by its reaction in Western blotting with a Wza_{K30}-specific antibody (Figure 1B). The glucose-repressed negative control sample showed no reaction with anti-Wza_{K30} antibody and contained no ^{3}H-labeled polypeptide of the appropriate size. The Western blot shows two poorly separated bands of almost identical size in the induced cells. Presumably, these correspond to processed and unprocessed forms of Wza_{K30}. This was addressed using globomycin, a specific inhibitor of signal peptidase II (Hussain *et al*., 1982). Globomycin-treated cultures showed minimal labeling with [^{3}H]palmitate (Figure 1A), but in the Western blot, only a single polypeptide with the larger size expected of the putative precursor form of Wza_{K30} was evident (Figure 1B). The sequence prediction that Wza_{K30} is a processed lipoprotein is therefore supported by biochemical data.

Cell fractionation was used to determine the cellular location of Wza_{K30}. In this analysis, Wza_{K30} was confined to the membrane fraction; none was detected in the soluble cytoplasm–periplasm fraction (Figure 2). After differential solubilization of the membrane fraction in sarkosyl, which selectively solubilizes the inner membrane (Filip *et al*., 1973), Wza_{K30} was found in both membrane fractions (Figure 2) and could be detected by Western blotting (data not shown). The SDS–PAGE profile of outer membranes isolated from sucrose-density gradients was identical to that of the sarkosyl-insoluble fraction (data not shown), confirming this result. Notably, while the outer membrane fraction of induced cells contains a single Coomassie Blue-stained Wza_{K30} band, SDS–PAGE of the inner membrane reveals two poorly separated polypeptides. The mature form of Wza_{K30} is therefore targeted to the outer membrane. The material seen in the inner membrane reflects the unprocessed precursor detected in Figure 1B and smaller amounts of mature Wza_{K30}. Whether the

Table I. Homologs of Wza_{K30} identified by gapped BLAST

Protein	Organism	Predicted mol. wt (kDa)[a]	Percent identity/percent similarity	Lipoprotein consensus?	Accession No.[b]
Wza_{Kp}[c]	*K.pneumoniae*	41.7	91/96	yes	SP Q48450
Wza_{22min}	*E.coli* K-12	41.8	74/89	yes	SP P75881
AmsH	*E.amylovora*	41.5	67/84	yes	SP Q46629
Wza_{cps}	*E.coli* K-12	41.9	65/81	yes	SP P76388
EpsA	*R.solanacearum*	40.4	36/56	yes	SP Q45407
CtrA	*N.meningitidis*	41.9	27/43	yes	PIR A43845
CpxD	*Actinobacillus pleuropneumoniae*	42.1	27/43	yes	GB U36397
BexD	*H.influenzae*	42.0	27/42	yes	SP P22236
HexD	*Pasteurella multocida*	43.0	26/44	yes	GB AF067175
VexA	*Salmonella typhi*	38.9	22/43	yes	SP Q04976
SpsD	*Sphingomonas* S88	31.5	local	no	GB U51197
GumB	*Synechocystis* sp.	54.1	local	no	GB D90904
OtnA/WbfF	*Vibrio cholerae* O139	100.4	local	yes	GB X90547
GumB	*X.campestris*	–	local	no	PIR S67818
KpsD	*E.coli K1*	60.4	local	no	SP Q03961
ExoF	*Sinorhizobium meliloti*	45.9	local	no	SP Q02728
PssN (C-terminus)	*Rhizobium leguminosarum*	–	local	–	GB AF067140

[a]Of the preprotein.
[b]DDBJ/EMBL/GenBank databases.
[c]Formerly identified as Orf4.

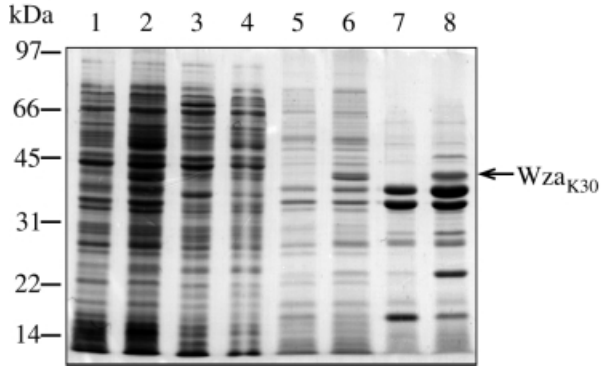

Fig. 2. Subcellular localization of Wza_{K30}. Subcellular fractions of *E.coli* LE392 (pWQ126) were analysed by SDS–PAGE. Prior to SDS–PAGE, samples were solubilized in sample buffer at 100°C. Samples are in pairs, with the first of each pair uninduced, and the second induced by L-arabinose. Lanes 1 and 2, whole-cell lysates; lanes 3 and 4, soluble fractions; lanes 5 and 6, inner membrane fractions; lanes 7 and 8, outer membrane fractions.

appearance of mature Wza_{K30} in the inner membrane is a consequence of overexpression, or is due to residual contaminating outer membrane is not known.

Wza_{K30} has a functional homolog on the E.coli chromosome

One closely related Wza_{K30} homolog identified in BLAST searches was a protein of unknown function encoded by the *yccZ* gene, found at ~22.5 min on the *E.coli* K-12 chromosome (Table I). This locus contains several open reading frames (ORFs), the last three of which are homologous to *wza*, *wzb* and *wzc*. Recently, Ilan *et al*. used PCR to show that wzc_{22min} (which they have named *etk*) was present in all strains of *E.coli* tested (Ilan *et al*., 1999). However, the use of specific antibodies revealed that the protein was only expressed at detectable levels in enteropathogenic (EPEC), enterotoxic (ETEC) and enterohemorrhagic (EHEC) strains of *E.coli*. Using specific primers, the presence of wza_{22min}–wzc_{22min} on the *E.coli* E69 chromosome was confirmed, and sequencing indicated that wza_{22min} from *E.coli* E69 was identical to the same ORF from *E.coli* K-12 (data not shown).

Previous results (Drummelsmith and Whitfield, 1999) showed that a non-polar chromosomal wza_{K30} mutant, *E.coli* CWG307, expressed only trace amounts of capsular polysaccharide when cell lysates were examined by Western blotting with K30-specific antibody (see Figure 3B). In addition, the reduction in capsule resulting from the wza_{K30} mutation unmasked underlying bacteriophage receptors that are inaccessible in a wild-type strain. However, due to the presence of antigenically cross-reactive $K30_{LPS}$ on the cell surface, which is present in both wild-type and mutant strains, we were unable to determine the location of the small amount of high-molecular-weight K30 antigen seen in Western blot analysis of CWG307. This material could accumulate internally due to elimination of all surface-translocation activity, or could reflect a small amount of surface-located polymer that was not sufficient to form an effective masking capsule structure. The identification of wza_{22min} on the *E.coli* E69 chromosome raised the possibility that this protein contributed to K30 capsule assembly and influenced the phenotype of CWG307.

To address these alternatives, a polar spectinomycin-resistance cassette was used to insertionally inactivate the wza_{22min} gene on the chromosomes of *E.coli* strains E69 and CWG307 (wza_{K30}). The resulting mutant phenotypes were assessed by Western blotting (Figure 3) and by sensitivity to bacteriophages specific for the O9a LPS O antigen and for the K30 antigen (Table II). Disruption of the wza_{22min} gene in *E.coli* E69 (resulting in strain CWG258) has no discernible effect on the ability to assemble a capsule that masks the underlying O9a antigen (Table II), although phage titer was reduced in CWG258 relative to the wild type. The basis for these quantitative differences is not known and the profile of the CWG258 cell lysate in Western blot analysis was indistinguishable from that of E69 (data not shown). These techniques

Table II. Assessment of the presence of a K30 capsule based on bacteriophage sensitivities of various mutant strains

Bacterial strain	Titer of bacteriophage recognizing:		K30 capsule assembly
	K30 antigen	O9a antigen	
E69	1.1×10^7	0	+
CWG258 (wza_{22min})	2.6×10^5	0	+
CWG307 (wza_{K30})	2.1×10^6	4.9×10^7	+/–
CWG281 (wza_{22min}, wza_{K30})	0	4.6×10^7	–
CWG281 (pWQ126; wza_{K30})	1.4×10^7	0	+
CWG281 (pWQ127; wza_{22min})	1.9×10^7	4.4×10^6	+/–
CWG281 (pWQ128; *amsH*)	0	4.3×10^7	–

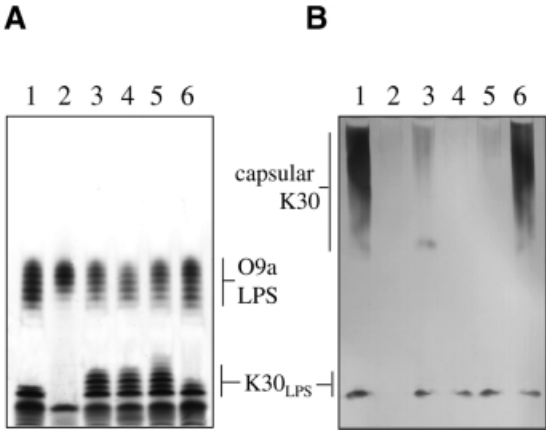

Fig. 3. Analysis of the effects of the Wza_{K30} and Wza_{22min} proteins on the surface polysaccharide profile of *E.coli* E69. (**A**) Silver-stained SDS–PAGE. (**B**) Corresponding Western blot, probed with K30-specific rabbit polyclonal antibody. The position of the O9a-substituted LPS is indicated, and the LPS-linked form of K30 antigen ($K30_{LPS}$) is identified. Note that the capsular antigen is not linked to LPS and, while it can be detected with anti-K30 antibodies, it does not stain with silver. Lane 1, *E.coli* E69 (O9a:K30); lane 2, CWG28 (O9a:K^-); lane 3, CWG307 (wza_{K30}::*aacC1*); lane 4, CWG281 (wza_{K30}::*aacC1*; wza_{22min}::*aadA*); lane 5, CWG281 (pWQ127); lane 6, CWG281 (pWQ126).

provide a qualitative assessment of capsule assembly. The presence of a capsule in CWG258 was expected given the presence of a functional copy of wza_{K30} in this strain. However, when the same mutation was introduced into CWG307 creating strain CWG281, the remaining traces of capsular polysaccharide seen in Western blot analysis of CWG307 were eliminated (Figure 3B). Furthermore, the double mutant was completely resistant to the K30-specific phage but susceptible to the bacteriophage that recognizes O9a LPS (Table II). Strain CWG281 is still able to synthesize and polymerize the K30 antigen because the $K30_{LPS}$ form is still present (Figure 3A), but it does not assemble any high-molecular-weight capsule on the cell surface.

In order to confirm these mutant phenotypes, wza_{22min} was cloned behind an arabinose-inducible promoter in plasmid pWQ127. Plasmids pWQ127 and pWQ126 (wza_{K30}) were independently introduced into the double mutant strain CWG281. The cloned wza_{K30} (plasmid pWQ126) was able to restore wild-type levels of K30 capsule expression in strain CWG281 when examined by Western blotting and phage sensitivities (Figure 3B; Table II). In contrast, wza_{22min} (plasmid pWQ127) restored only low levels of K30 capsule expression under the same conditions, indicating that wza_{22min} functioned only inefficiently in K30 capsule assembly.

The AmsH protein of E.amylovora cannot functionally replace Wza_{K30}

Unlike the other known *wza* homologs, wza_{22min} is not found in a 'complete' gene cluster containing all the activities necessary for polysaccharide synthesis. The 22-min locus does not contain genes encoding Wzx or Wzy proteins or an ABC-2 transporter, proteins necessary for the known major pathways for synthesis and polymerization of bacterial cell-surface polysaccharides (reviewed in Whitfield, 1995; Whitfield and Roberts, 1999). Furthermore, this locus is not associated with the biosynthesis of any known polysaccharide in *E.coli*. As a result, it is conceivable that its poor complementation of CWG281 reflects the accumulation of mutations in wza_{22-min} in the absence of selective pressure for function. Therefore, another member of the OMA protein family with established function was also tested for the ability to complement the wza_{K30} defect. The AmsH protein from *E.amylovora* is closely related to Wza_{K30} (Table I) but could not support cell-surface expression of the K30 capsule (Table II).

One possible explanation for these negative complementation results is that the AmsH and Wza_{22min} proteins were not properly expressed or localized in this heterologous system. In order to discount this possibility, membrane fractions were prepared from *E.coli* CWG281 expressing either Wza_{22min} or AmsH. Sarkosyl-insoluble outer membranes were isolated, and, in both cases, protein bands of ~40 kDa were present in arabinose-induced samples and absent in glucose-repressed samples (data not shown). Upon Western blotting with anti-Wza_{K30} antibodies, both Wza_{22min} and AmsH were detected, although AmsH was only weakly reactive (data not shown).

Wza_{K30} forms a multimeric complex in the outer membrane

Recently, studies on several type II and III protein secretion systems have identified multimeric outer membrane protein complexes, known collectively as 'secretins' and 'ushers', which are required for translocation of secreted proteins across the outer membrane (reviewed in Bitter and Tommassen, 1999). We considered it possible that polysaccharide secretion might use an analogous complex involving Wza_{K30}. To address this question, we first looked for high-molecular-weight forms of Wza_{K30}. Studies invol-

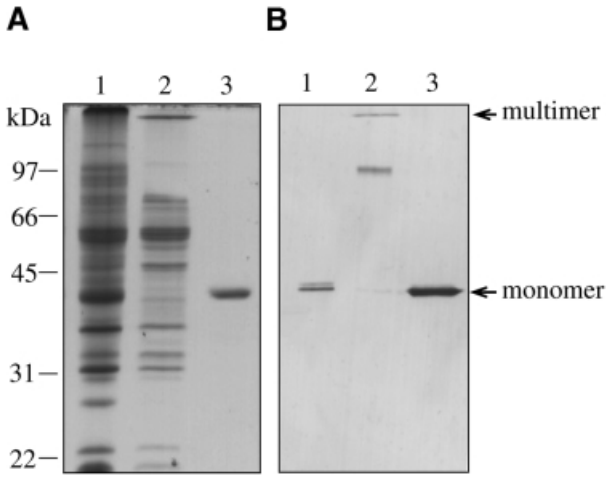

Fig. 4. Multimerization of Wza_{K30}. Membranes from arabinose-induced *E.coli* LE392 (pWQ126) were solubilized in SDS and examined by SDS–PAGE. (**A**) Coomassie Blue-stained SDS–PAGE gel. (**B**) Corresponding Western blot probed with anti-Wza_{K30} rabbit polyclonal antibody. The positions of multimeric and monomeric Wza_{K30} are indicated by arrows. Lane 1, membranes of induced cells solubilized at 100°C; lane 2, membranes of induced cells solubilized at 25°C; lane 3, the band representing high molecular weight multimer was cut from the gel and heated in sample buffer at 100°C, resulting in its dissociation into Wza monomers. In lane 2, small amounts of another form of immunoreactive material were evident in immunoblots but were insufficient to be seen by Coomassie Blue staining.

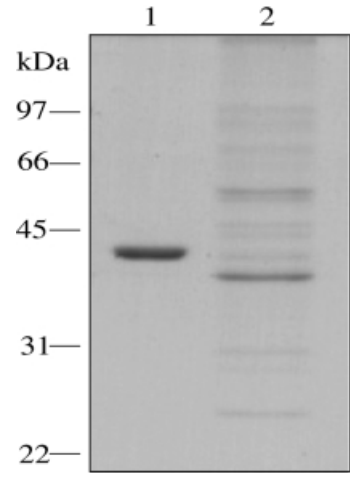

Fig. 5. Purification of Wza_{K30} by solubilization of membranes of *E.coli* LE392 (pWQ126) in 0.4% SDS, 300 mM NaCl and 50 mM Na_2HPO_4 pH 8.0. The insoluble fraction was collected by centrifugation and dissociated in SDS–PAGE sample buffer at 100°C and is enriched in Wza_{K30}. Lane 1, SDS-insoluble fraction; lane 2, SDS-soluble fraction.

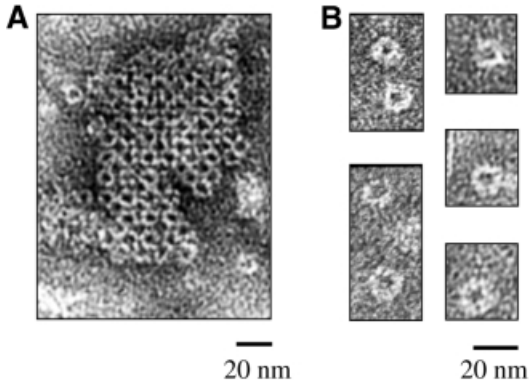

Fig. 6. Electron microscopy of Wza_{K30} multimers. (**A**) Aggregates of Wza_{K30} multimers were prepared as the SDS-insoluble fraction (see Figure 5) and negative staining reveals large, regular arrays. (**B**) This array can be dissociated into component multimers by treatment at pH 9.5. The morphology of the particles shown in (B) was identical in larger fields from different preparations. The scale is indicated by the ruler bar in each panel.

ving protein secretins revealed that these structures are resistant to denaturation by SDS unless heated (Koster *et al*., 1997; Bitter *et al*., 1998; Crago and Koronakis, 1998). As already shown, samples of outer membrane solubilized in SDS–PAGE sample buffer at 100°C show Wza_{K30} as a monomer with a molecular weight of ~40 kDa. However, in membrane samples incubated in sample buffer at room temperature, Wza_{K30} monomers were not detectable. Instead, a novel high-molecular-weight polypeptide (>150 kDa) was detected by Coomassie Blue staining (Figure 4A). This complex reacted in anti-Wza_{K30} antibodies in a Western blot (Figure 4B). Small amounts of an additional form migrating with an apparent molecular weight of ~100 kDa were also detected in the 25°C sample by the anti-Wza_{K30} antibodies, but this material was not readily visible by Coomassie Blue staining. The high-molecular-weight material (indicated by the arrow in Figure 4A) was excised from the gel, solubilized at 100°C, and re-run in SDS–PAGE. After heating, the complex gave a single polypeptide that co-migrated with the Wza_{K30} monomer and reacted with Wza_{K30}-specific antibody. No evidence was found for the presence of polypeptides other than Wza_{K30} in the high molecular weight complex. Only the mature form of Wza_{K30} was evident after disruption of the complex, while the starting membranes contained both processed and unprocessed forms (Figure 4B). Examination of Wza_{22min} showed that it also formed heat-dissociable multimers (data not shown).

Multimeric Wza_{K30} forms structures visible by electron microscopy

The high molecular weight of the Wza_{K30} multimer suggested that the complex might be visible by electron microscopy, and the stability of these multimers in SDS suggested a potential means of isolation. Therefore, the membrane fraction expressing Wza_{K30} was solubilized in a buffer containing 300 mM NaCl and 0.4% SDS. Although such conditions were expected to dissociate outer membranes, a significant proportion of Wza_{K30} remained in the insoluble fraction. SDS–PAGE showed that the insoluble material was preferentially enriched in Wza_{K30}. The only polypeptide evident in this sample showed a molecular weight equivalent to that predicted for Wza_{K30} after heating in SDS sample buffer at 100°C (Figure 5). No contaminating proteins were detected. All of the material co-migrated with Wza_{K30} multimers in unheated samples and reacted with anti-Wza_{K30} antibodies (data not shown). Control samples from uninduced cells gave no equivalent insoluble material (data not shown). A sample of this material was resuspended in water and examined by negative staining and electron microscopy. Large sheets consisting of a regular array of particles were visible in these fractions (Figure 6A), but none were evident in control membranes in which the expression of Wza_{K30} had been repressed by glucose (data not shown). In order

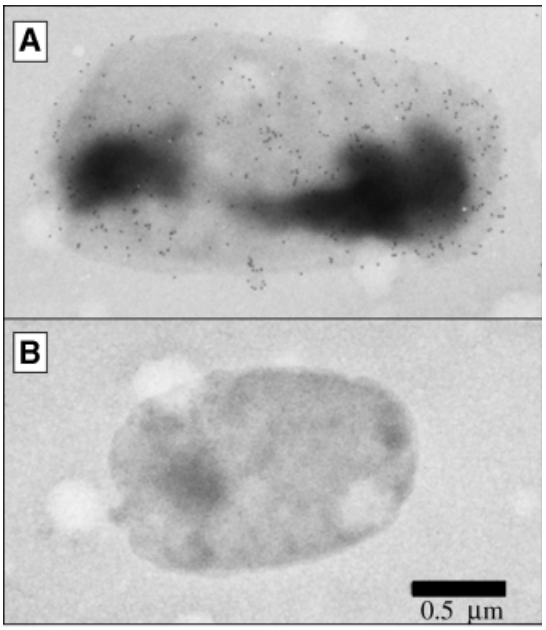

Fig. 7. Immunogold labeling of whole-cell mounts using anti-Wza_{K30} antibodies showing the surface accessibility of Wza_{K30}. (**A**) *Escherichia coli* LE392 (pWQ126) cells induced with 0.002% L-arabinose and expressing Wza_{K30}. A similar extent of labeling was seen on all cells in several fields. (**B**) *Escherichia coli* LE392 (pWQ126) grown in the presence of glucose provides the negative control. All cells in several fields showed no labeling. The size of the gold particles is 10 nm.

to resolve the component multimer of this array, samples were treated with buffers at a range of basic pH, as multimer aggregates of InvG, a type III secretin from *Salmonella* spp., were found to be alkali labile (Crago and Koronakis, 1998). At pH 9.5, the sheets disaggregated, resulting in individual ring-shaped structures (Figure 6B). These structures were stable at pH 10.7; however, in samples treated with 100 mM NaOH, no structures were visible at all, indicating that the multimer may have been dissociated to individual protein units. The Wza_{K30} multimeric rings are ~11 nm in diameter, with a central 'pore' diameter of ~3 nm.

Wza_{K30} is exposed on the cell surface

Some members of the OMA protein family, to which Wza_{K30} belongs, have been predicted to have a secondary structure consisting of amphipathic β-sheets and a β-barrel conformation (Paulsen *et al*., 1997). This structure is common among outer membrane proteins, in particular the porins. In this case, Wza_{K30} might be expected to be accessible at the cell surface. To test this possibility, whole *E.coli* LE392 cells expressing Wza_{K30} were incubated with polyclonal anti-Wza_{K30} antibodies and gold-conjugated goat anti-rabbit antibodies. The cells were then examined by electron microscopy. Significant numbers of randomly distributed gold particles were visible on the cell surface, with many at the cell periphery, indicating that Wza_{K30} is indeed surface exposed (Figure 7A). *Escherichia coli* LE392 cells lacking Wza_{K30} showed no gold labeling under the same conditions (Figure 7B). These data indicate specific labeling of surface molecules, rather than non-specific labeling of surface or intracellular components. The electron microscopy analysis and the membrane localization data presented above are consistent with Wza_{K30} being an integral outer membrane protein with significant regions exposed on the outer face of the outer membrane.

Discussion

The Wza_{K30} protein of *E.coli* (O9a:K30) is a member of the OMA (Paulsen *et al*., 1997) protein family. Proteins of this family are encoded within genetic loci involved in the production of a variety of cell-surface polysaccharides. Mutations have been made in several of these homologs, including AmsH of *E.amylovora* (Bugert and Geider, 1995), EpsA of *R.solanacearum* (Huang and Schell, 1995), VexA of *Salmonella typhi* (Hashimoto *et al*., 1993) and WbfF of *Vibrio cholerae* O139 (Bik *et al*., 1996). In all cases, these mutations have resulted in the abrogation of surface-expressed polysaccharide. However, the function of these OMA proteins has not been resolved. Here, we have shown for the first time that a member of the OMA family, Wza_{K30}, is an outer membrane lipoprotein that forms a multimeric 'secretin-like' structure.

Many members of the OMA family contain the lipoprotein consensus sequence L(AV)-L-A(S)-G(A)-C-X-(S,D) with the invariant cysteine residue residing at or near position 20 of the preprotein (reviewed in Wu, 1996). This cysteine residue is modified with diacylglycerol by prolipoprotein diacylglyceryl transferase, and the leader peptide is then cleaved by signal peptidase II. The second amino acid following the cysteine residue determines the membrane localization of the protein: aspartate in this position directs the protein to the inner membrane, while a serine residue results in an outer membrane lipoprotein. The CtrA protein from *N.meningitidis* (Frosch *et al*., 1992) and EpsA of *R.solanacearum* (Huang and Schell, 1995) have been identified in cell membranes, but Wza_{K30} is the first member of the OMA family for which lipoprotein character and outer membrane location have been shown directly.

The formation of ring-like structures by Wza_{K30} is reminiscent of the secretins of the type II and III protein secretion systems. Recent studies using the filamentous phage export protein pIV, a secretin homolog, have shown that it is a voltage-induced gated aqueous channel (Marciano *et al*., 1999). The PulD secretin of the type II *Klebsiella oxytoca* pullulanase secretion system has also been shown to form small, ion-conducting channels (Nouwen *et al*., 1999), although voltage-induced opening of the pore has not been achieved. Secretins are generally stable in SDS, and in many cases samples must be extensively heated to dissociate the multimer into monomeric subunits. The Wza_{K30} multimer is stable in SDS at room temperature, but is dissociated very easily by heating or freezing. In type II systems, protein secretion takes place in two stages, with a periplasmic intermediate. This intermediate is probably folded before crossing the outer membrane and consequently the secretion pore must be large. The secretins generally consist of 12–14 subunits, and the XcpQ secretin of *Pseudomonas aeruginosa* has a pore diameter of ~9.5 nm (Bitter *et al*., 1998). In the case of capsule export, the polymer is not a large globular structure, but is probably more like a filament. Thus, the internal diameter of the export pore can be smaller. In the

case of Wza_{K30}, the internal diameter of the multimer is ~3 nm.

The PulD and OutD secretins have been found to interact with an outer membrane lipoprotein chaperone, PulS or OutS, respectively (Hardie *et al*., 1996; Shevchik *et al*., 1997). Not all secretins associate with a chaperone, but those that do have an additional, conserved region at the C-terminus of the protein. In the absence of PulS, the C-terminus of PulD is cleaved and the protein is not properly targeted to the outer membrane (Hardie *et al*., 1996). The lack of a helper protein for Wza_{K30} could be explained by the fact that Wza_{K30} itself is a lipoprotein, and encodes its own targeting signals.

Homologs of Wza_{K30} appear to fall into three distinct groups based on their degree of protein similarity. The first group of proteins shows high levels of identity with Wza_{K30}. Interestingly, all of these proteins are associated with polysaccharide polymerization systems that involve Wzx and Wzy homologs. The second group, including such proteins as BexD from *H.influenzae* and CtrA of *N.meningitidis*, show lower levels of similarity to Wza_{K30}. These are all associated with polysaccharides whose biosynthesis occurs through processive glycosyltransferase reactions and whose export across the inner membrane requires an ABC-2 transporter. The third group shows only local regions of homology to Wza_{K30}, and members are generally not predicted lipoproteins. Their functional relationship to other members of the OMA family is unclear. WbfF does contain a lipoprotein consensus sequence, but although it has been classified as an OMA protein (Paulsen *et al*., 1997) it is twice as large as the other OMA proteins. The primary sequence similarity shared by Wza_{K30} and these proteins could be due to their interaction with polysaccharides and is not necessarily a consequence of functional similarity. As a case in point, KpsD, which has low levels of local similarity to OMA proteins (Table I), is a periplasmic protein (Silver *et al*., 1987) that binds polyanions (reviewed in Whitfield and Roberts, 1999).

The *wza* gene products from different *E.coli* group 1 capsule serotypes are identical, even though their corresponding polysaccharide repeat units vary considerably in structure. This indicates that the Wza protein itself is not specific for the capsular structure. Thus, the observation that highly related proteins (AmsH, Wza_{22min}) cannot fully complement a wza_{K30} mutation was somewhat unexpected, particularly since these proteins share enough structural similarity to be cross-reactive with anti-Wza_{K30} antibody and are properly targeted to the outer membrane. One possible interpretation of these results is that the function of this class of proteins is dependent on a specific interaction with another factor. Electron microscopy has shown that nascent group 1 polymer is extruded above membrane junction sites, also known as Bayer's bridges (Bayer and Thurow, 1977; Bayer, 1991). It has been postulated that at these sites the membranes are held in close apposition by the presence of inner and outer membrane proteins that comprise a capsule assembly complex. Wza_{K30} could be the outer membrane component of such a complex.

The presence of the wza_{22min} gene on the *E.coli* chromosome is intriguing, since it is not associated with the export of any specific polysaccharide. Ilan *et al*. have found that the wzc_{22min} gene, which they have called *etk*, is present in all strains of *E.coli* tested, but its putative protein product is only expressed in a subset of pathogenic isolates (Ilan *et al*., 1999). The inability of Wza_{22min} to function inefficiently in K30 capsule secretion suggests its contribution to the formation of the K30 capsule is accidental, and that this protein has a separate, undefined role in *E.coli*.

There is no OMA family member encoded within the *E.coli* group 2 capsule gene clusters. In contrast, the *H.influenzae* and *N.meningitidis* group 2 capsules both require a dedicated OMA homolog for surface expression (BexD and CtrA; Table I). These capsules are all polymerized by the same mechanism, and it would be surprising if the *E.coli* group 2 capsules used a radically different translocation process. Interestingly, group 2 encapsulation in *E.coli* is correlated with the presence of a 40 kDa outer membrane protein (Paakanen *et al*., 1979), which is consistent with the expected size of an OMA family member. Also, studies with conditional expression of *E.coli* group 2 capsules in whole cells identified a 40 kDa outer membrane protein associated with the onset of capsule expression (Whitfield *et al*., 1985). One 40 kDa outer membrane protein identified in these analyses was a porin, protein K (Sutcliffe *et al*., 1983; Whitfield *et al*., 1983), and there is some preliminary evidence suggesting that the surface assembly of group 2 capsules requires porins (reviewed in Bliss and Silver, 1996). Interpretation of these results has been complicated by the question of whether the relatively small channel provided by most *E.coli* porins (1.7–1.8 nm) would allow them to function as a capsular polysaccharide translocation pore. It is entirely possible that an additional co-migrating protein of similar molecular weight (i.e. an OMA protein) could have been overlooked in these early analyses.

The conservation of Wza_{K30} homologs in the assembly systems of many group 1-like polymers indicates that they share a common mechanism of assembly and export. The presence of an OMA family protein in the capsule and exopolysaccharide biosynthesis gene clusters of numerous Gram-negative bacteria implies a common ancestry. This mechanism does not appear to be confined to the export of polysaccharides, as some protein secretion systems clearly exploit a similar strategy to overcome the outer membrane barrier. Secreted preproteins encode signals in either their mRNA or primary amino acid sequence that direct the nascent polypeptide to the secretion apparatus. Polysaccharides cannot carry these signals themselves. The challenge now is to determine (i) how the polymer is directed to the translocation apparatus, (ii) what the components of this apparatus are, and (iii) how they function together to result in the export of capsules and exopolysaccharides.

Materials and methods

Bacterial growth conditions

Bacterial strains were grown and maintained in Luria–Bertani (LB) medium (Miller, 1972) at 37°C unless otherwise indicated. The antibiotics ampicillin (Ap, 100 μg/ml), chloramphenicol (Cm, 34 μg/ml), gentamicin (Gm, 30 μg/ml) and spectinomycin (Sp, 100 μg/ml) were added when appropriate.

Computer analysis

Amino acid homologies were detected using gapped BLAST (Altschul *et al*., 1997), and were calculated using BEAUTY (found at http://

dot.imgen.bcm.tmc.edu:9331/seq-search/alignment.html). Multiple alignments were done using CLUSTAL_W (Thompson *et al*., 1994).

DNA methods

Oligonucleotide primer synthesis and DNA sequencing were carried out at the Guelph Molecular Supercentre (University of Guelph, Ontario, Canada). PCR amplifications were carried out in a Perkin-Elmer GeneAmp PCR System 2400 thermocycler, using conditions optimal for each primer pair and either ExpandLong or *Pwo* polymerase (Roche), depending on the size of the amplified fragment. Geneclean II (Bio101) was used for the purification of PCR and plasmid DNA fragments. Restriction endonuclease digestion and ligation were carried out using standard methods, as recommended by the suppliers.

Overexpression of Wza homologs

The genes encoding the Wza_{K30}, Wza_{22min} and AmsH proteins were cloned into the pBAD24 vector to give plasmids pWQ126, pWQ127 and pWQ128, respectively. The genes were cloned on PCR-amplified fragments in which flanking restriction enzyme sites were incorporated by primer design. The primers were: for wza_{K30}, JD125: 5′-TGA TGA TGT GAA TTC ATG AAG AAA A-3′ and JD126: 5′-TTT CCT CTG CAG ATC ACT AAA ATT-3′; for wza_{22min}, JD139: 5′-TGA CTG ATC AAT TGA AAC CAA TGA AAA AGA-3′ and JD140: 5′-GCT GTC GCA TCT GCA GGG TGT-3′; and for *amsH*, JD143: 5′-CAA ATT ACC AAT TGC GAT GAA CC-3′ and JD144: 5′-CCG ATA CAC ACC ACT AAG CTT GAA TT-3′. The start codon of each gene is underlined. In each case, the sequence of the cloned gene was determined and confirmed to be free of PCR-generated error. The pBAD24 plasmid is a member of a family of expression vectors using the arabinose-inducible and glucose-repressible arabinose promoter (Guzman *et al*., 1995). This particular vector contains an optimal ribosome-binding site upstream of the multiple cloning site. Proteins were overexpressed by growing the cells to an OD_{600} of 0.7–0.8 and inducing by addition of 0.002–0.004% (final concentration) L-arabinose. Negative controls were obtained by repressing the transcription of the *araC* promoter by growth in 0.2% glucose.

[^{3}H]palmitate labeling of Wza_{K30}

Escherichia coli LE392 cells (*supE*44 *supF*58 *hsdR*514 *galK*2 *galT*22 *metB*1 *trpR*55 *lacY*1) carrying the Wza_{K30} expression vector (pWQ126) were induced using 0.004% L-arabinose. At the time of induction 5 μCi/ml of [^{3}H]palmitate (ARC; 60 Ci/mM) was added to the culture. Cells were then grown for a further 2 h, washed three times in phosphate-buffered saline (PBS), and resuspended in SDS–PAGE sample buffer. Samples containing ~10^6 c.p.m. were loaded per lane. Negative controls consisted of *E.coli* LE392 (pWQ126) grown in the presence of 0.2% glucose. To assess the specific inhibition of lipoprotein processing by globomycin (Hussain *et al*., 1982), 100 μg/ml globomycin (generously provided by A.J.Clarke, Department of Microbiology, University of Guelph) was added to cultures 5 min prior to induction and labeling.

Localization of the Wza_{K30} protein

Cell lysates were prepared from induced or uninduced cultures by passing the cells through a French pressure cell. Unbroken cells and large debris were removed by low-speed centrifugation (10 min at 4000 *g*). Cell envelopes were isolated in a pellet following ultracentrifigation of the cell-free lysate for 1 h at 100 000 *g*. In order to determine in which membrane the Wza_{K30} protein resided, cell envelopes were solubilized in 2% sarkosyl for 30 min at room temperature. This anionic detergent selectively solubilizes the inner membrane, while leaving the outer membrane intact (Filip *et al*., 1973). Sarkosyl-insoluble material was isolated by centrifugation for 5 min at 5500 *g* in a benchtop centrifuge.

Chromosomal insertion mutation

In order to inactivate the wza_{22min} gene, a PCR-generated fragment carrying part of the wza_{22min} gene, wzb_{22min}, wzc_{22min} and part of the adjacent *appA* gene from *E.coli* E69 was cloned into the vector pBBR1mcs (Kovach *et al*., 1994). The *aadA* (Sp^r) gene cassette was inserted into the single *Sma*I site in wza_{22min}. This Sp^r gene is flanked by transcriptional terminators and multiple cloning sites and results in polar effects on downstream genes (i.e. *etk*) (Fellay *et al*., 1987). A DNA fragment carrying the mutated wza_{22min} and additional downstream DNA was cloned into the temperature-sensitive suicide delivery vector pMAK705 (Hamilton *et al*., 1989) to produce plasmid pWQ129. Methods for the use of pMAK705 have been described elsewhere (Hamilton *et al*., 1989; Drummelsmith *et al*., 1997). Chromosomal insertions in strains CWG258 (*E.coli* E69 wza_{22min}::*aadA*; Sp^r) and CWG281 (E69 wza_{K30}::*aacC1*, wza_{22min}::*aadA*; Gm^r, Sp^r) were confirmed by analysis of products of PCRs across the insertion using primers flanking the targeted region.

Cell-surface polysaccharide analysis

LPS preparations were made from SDS–proteinase K-treated whole-cell lysates using the method of Hitchcock and Brown (1983). Commercially prepared 10–20% gradient tricine SDS–polyacrylamide gels were obtained from Novex, and PAGE conditions were those recommended by the manufacturer. Silver staining (Tsai and Frasch, 1982) and Western blotting (Towbin *et al*., 1979) procedures were as described elsewhere. The rabbit polyclonal anti-K30 antibody is specific for the K30 antigenic unit, as described previously (Dodgson *et al*., 1996), and recognizes both the $K30_{LPS}$ and capsular K30 antigen. The K30 repeat units in the two forms are chemically identical (C.Whitfield and M.B.Perry, unpublished).

Bacteriophage titration assays

The presence of a capsular structure on the cell surface was assessed using two bacteriophages: bacteriophage ØK30 is specific for the K30 antigenic unit (Whitfield and Lam, 1986), while bacteriophage O9-1 is specific for the O9a antigen resulting from the O9a polysaccharide attached to LPS (McCallum *et al*., 1989). The O9a LPS is masked by the presence of a capsule, rendering the wild-type strain E69 resistant to O9-1 but susceptible to ØK30. Bacteriophage titers were calculated by plating serial dilutions of bacteriophage lysate on lawns of the bacterial strain being tested and counting plaque-forming units.

Protein analysis

Proteins were separated by SDS–PAGE using 12% polyacrylamide gels. Samples were solubilized in sample buffer at either room temperature or 100°C for 15 min. Western blots were carried out using anti-Wza_{K30} antibody. Alkaline phosphatase-conjugated goat anti-rabbit antibodies were used for detection as described by the manufacturer.

Purification of multimeric complexes of Wza_{K30}

Membranes prepared from induced *E.coli* LE392 (pWQ126) were solubilized in 0.4% SDS, 300 mM NaCl and 50 mM Na_2HPO_4 (pH 8.0), for 1 h at 37°C. Samples were centrifuged for 5 min at 5500 *g* in a benchtop centrifuge. The pellet was washed once and resuspended in distilled water. Samples of this insoluble material were incubated in 100 mM Tris–HCl at pH 8.0, 8.5, 9.0, 9.5, 10.0 and 10.7, and with 100 mM NaOH for 5 min at room temperature in order to disaggregate the multimers.

Preparation of anti-Wza_{K30} antibody

New Zealand white rabbits were immunized using multimeric Wza_{K30}, prepared as described above. Aliquots of this material containing 100 μg of Wza_{K30} were emulsified 1:1 in Freund's incomplete adjuvant and used as the immunogen. Serum samples showed a strong anti-Wza_{K30} response, as assessed by Western blot. To obtain Wza_{K30}-specific antibodies, the serum was adsorbed with both whole *E.coli* cells and finally with *E.coli* LE392 whole-cell lysates immobilized on nitrocellulose. For Western blotting experiments, serum diluted 1:2000 was further adsorbed by these membranes in order to completely remove contaminating cross-reacting antibodies.

Negative stain electron microscopy

Multimeric Wza_{K30} complexes were purified as described above and adsorbed onto formvar/carbon-coated copper grids. The samples were negatively stained with 1% uranyl acetate, and visualized in a Philips EM300 electron microscope at 60 KeV.

Immunogold electron microscopy

Escherichia coli LE392 cells carrying the Wza_{K30} expression vector were grown in the presence of arabinose or repressed by glucose (for a negative control) as described above. Bacteria were adsorbed to formvar/carbon-coated nickel grids. The grids were blocked for 20 min in 0.3% skimmed milk in PBS (SM-PBS) and incubated with adsorbed anti-Wza_{K30} antibodies. After 30 min, the grids were washed three times for 2 min in SM-PBS and incubated with a 1:20 dilution of gold-conjugated goat anti-rabbit IgG (Sigma). Finally, the grids were washed three times for 2 min in PBS and three times for 2 min in sterile distilled water. Samples were visualized by electron microscopy as described above but were not negatively stained.

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