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Loss ofO-antigen increases cell shape abnormalities in penicillin-binding protein mutants of *Escherichia coli*

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Abstract

Escherichia coli mutants lacking multiple penicillin-binding proteins (PBPs) produce aberrantly shaped cells. However, most of these experiments have been performed in *E. coli* K12 strains, which do not attach a complete O-antigen to their outer membrane lipopolysaccharide. We constructed mutants in different genetic backgrounds and found that the frequency of morphological deformities was higher in strains lacking the O-antigen. Also, complementing O-negative mutants with a heterologous O-antigen from *Klebsiella* returned a substantial fraction of misshapen cells to a normal morphology. Thus, the O-antigen contributes to cell shape in *E. coli*, perhaps by reducing the number of ectopic poles, which may be the proximal cause of shape abnormalities.

Keywords

Escherichia coli; penicillin-binding proteins; O-antigen; cell shape

Introduction

Although it is clear that bacterial morphology is determined by the shape of the rigid peptidoglycan cell wall, the molecular details of how the wall adopts a specific shape are just beginning to become clear (Carballido-Lopez & Errington, 2003; Young, 2003; Cabeen & Jacobs-Wagner, 2005). The most favored current hypothesis is that peptidoglycan synthesis is directed by enzymes associated with one or more shape-directing, polymeric, cytoplasmic scaffolds composed of homologues of actin (e.g. MreB) or tubulin (e.g. FtsZ) (Carballido-Lopez & Errington, 2003; Figge *et al.*, 2004). According to this view, the dimensions and overall shape of the peptidoglycan sacculus are determined by controlling the spatial insertion of monomeric precursors into the existing cell wall.

In Gram-negative cells, other factors may also contribute to bacterial shape, including a possible role for the outer membrane (OM) and its components, which might act collectively as a type of external scaffold (Young, 2003). For example, such a mechanism could, in theory, influence cell length (Cooper, 1991). More tangible experimental evidence for the idea includes the fact that some OM mutants are misshapen. An *Escherichia coli* strain lacking the OmpA and Lpp proteins is spherical instead of rod shaped when grown in the

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presence of magnesium ions (Sonntag *et al.*, 1978; Hiemstra *et al.*, 1987), as are certain *E. coli* mutants lacking the Lpp lipoprotein by itself (Hiemstra et al., 1987). Ellipsoidal-shaped cells are produced by overexpressing the NlpI OM lipoprotein (Ohara *et al.*, 1999), and removal of the OmpA protein creates irregular and flattened cells (Belaaouaj *et al.*, 2000). It appears, therefore, that at least some of the protein components of the OM affect the general shape of *E. coli*.

Previously, we reported that deleting certain low-molecular-weight penicillin-binding proteins (LMW PBPs) from *E. coli*, yields cells with aberrant morphologies (Nelson & Young, 2000, 2001). However, these experiments were performed in an *E. coli* K12 background. This is a potentially important consideration because K12 strains express a truncated form of lipopolysaccharide (LPS), a major constituent of the outer leaflet of the OM in Gram-negative bacteria (Liu & Reeves, 1994). Such strains are described as 'rough' – that is, they lack the O-antigen characteristic of wild-type lipopolysaccharide. The reason is that most K12 strains carry an IS5 insertion in the *rfb* gene cluster that encodes the proteins that synthesize the lipopolysaccharide component (Liu & Reeves, 1994).

To test whether the O-antigen might affect morphology in *E. coli*, we examined the shapes of PBP mutants constructed in a strain of *E. coli* that produces an intact O-antigen. The frequency of cell shape abnormalities in these strains was compared with isogenic mutants in which the rfb gene cluster was inactivated and with *rfb* mutants complemented with O-antigen from another genus. The results indicate that the presence of O-antigen reduces PBP-related deformities, indicating that the lipid composition of the OM also plays a role in the generation or maintenance of bacterial cell shape.

Materials and methods

Bacteria, plasmids and general techniques

The bacterial strains used in this study are listed in Table 1. Plasmid pBMM1 (Meberg *et al.*, 2004) carries a *res-npt-res* kanamycin-resistant cassette from pCK155 (Kristensen *et al.*, 1995). Plasmid pWQ5 [pBluescript KS(+) vector, Amp^R] carries the *rfb* genes that encode the pathway for synthesizing the *Klebsiella* O1-antigen (Bronner *et al.*, 1994), and was provided by C. Whitfield. Plasmid pSH55 [pBluescript KS(+) vector, Amp^R] carries the *E. coli slyD* and *fkpA* genes cloned into the *Eco*RI site (S. Horne and K. Young, unpublished).

Bacteria were grown in Luria–Bertani (LB) broth, on LB agar plates, or in M9 minimal glucose medium supplemented with 40 μg mL^{-1} each of L-threonine, L-leucine, L-arginine, L-lysine and L-histidine (Miller, 1992). Where appropriate, antibiotics were added to the following final concentrations: ampicillin, 100 μg mL^{-1}; chloramphenicol, 20 μg mL^{-1}; kanamycin, 50 μg mL^{-1}; and tetracycline, 25 μg mL^{-1}. Unless otherwise specified, chemicals and reagents were from Sigma Chemical Co. (St Louis, MO). Microscopy was performed as described previously (Ghosh & Young, 2005).

Strain constructions

PBPs were deleted from *E. coli* 2443 by introducing a kanamycin resistance gene cassette flanked with *res* sites into individually targeted PBP genes, as described previously (Denome *et al.*, 1999; Nilsen *et al.*, 2004). Gene deletions were confirmed by labeling the PBPs with ^{125}I-penicillin X, followed by autoradiography (Henderson *et al.*, 1994; Denome *et al.*, 1999). For creating multiple mutants, the kanamycin resistance cassette was removed by expressing the RP4ParA resolvase (Kristensen *et al.*, 1995; Denome *et al.*, 1999; Nilsen *et al.*, 2004), and the procedures were repeated to remove successive genes.

The *rfb*$_{O8}$ genes in *E. coli* 2443 were replaced by the inactive *rfb*$_{K12}$ gene segment from *E. coli* K12 by P1 cotransduction with a *hisI*::Kan marker, as described previously (Ghosh & Young, 2005). Cells that had lost the O8-antigen formed rough colonies, and the *rfb* gene replacement was confirmed by diagnostic PCR and the inability to bind concanavalin A-conjugated Alexa-Fluor 488 (Ghosh & Young, 2005). Lipopolysaccharide was extracted and the absence of the O-antigen was confirmed by gel electrophoresis and lipopolysaccharide detection (Ghosh & Young, 2005).

Results and discussion

PBP mutants expressing O8-antigen have fewer morphological deformities

Mutants lacking certain low molecular weight penicillin-binding proteins (LMW PBPs) have extensive morphological malformations (Denome *et al.*, 1999; Nelson & Young, 2000, 2001). However, these strains were derived from *E. coli* K12, which does not express O-antigen (Liu & Reeves, 1994). To test the proposition that components of the OM might contribute to the control of cell shape, we deleted multiple PBP genes from *E. coli* 2443, which expresses the O8 antigen (Sandlin *et al.*, 1996), and characterized the cells as normal, abnormal or branched (Fig. 1). In the *E. coli* K12 strain CS315-1, approximately half the population exhibited shape abnormalities. However, deleting the same PBPs from the O8-positive *E. coli* 2443 background yielded a population in which only 23% of the population was abnormal (a decrease of 54%) (Table 2, strain AG375-3). A similar tendency was observed in strains lacking four PBPs. Approximately half of the cells of the *E. coli* K12 strain CS446-1 displayed deformities, whereas similarly aberrant cell shapes were observed in 36% of the cells of the O8-positive strain, AG456-1 (a decrease of 27%) (Table 2). Thus, the presence of the O8-antigen appeared to reduce the number and extent of shape abnormalities in *E. coli*.

If the presence of the O-antigen minimizes the number of cellular deformities in PBP mutants, then its removal should increase the percentage of deformed cells. Therefore, we deleted the *rfb*$_{O8}$ gene cluster from *E. coli* 2443 strains and replaced it with the corresponding cluster from *E. coli* KM32, a K12 strain. The O8-antigen polysaccharide ladder was confirmed to be absent from 2443-derived mutants in which the *rfb*$_{O8}$ genes had been replaced (e.g. see strain AG456-1* in Fig. 2, lane 6).

When the morphology of four strains was examined in four independent experiments, in 15 of 16 cases it was clear that removing the O8-antigen increased the proportion of cells that exhibited deformities (Table 3). The single exception (Table 3, AG70C-12* Exp #1) was a strain that displayed substantial increases in deformities in three other experiments. In the O-negative mutant AG456-1* (lacking four PBPs), the percentage of the population exhibiting deformities increased to a level similar to that of PBP mutants constructed in the naturally occurring O-negative *E. coli* K12 strain CS446-1 (Table 2). However, although removing the O-antigen from AG375-3 increased the proportion of abnormal cells to 29% (Table 2, AG375-3*), this was not quite as high as seen in the K12 strain CS315-1 (Table 2). It should be noted that the absolute values of deformed cells varied from day to day for unknown reasons. Nonetheless, O8-negative strains exhibited greater morphological variation than did their paired O8-positive parents in all but one case (Tables 2 and 3). In addition, three other PBP mutants lacking five, six or seven PBPs exhibited the same increase in deformities upon deletion of the *rfb*$_{O8}$ genes (data not shown). Overall, therefore, the results support the idea that the O-antigen helps maintain normal cellular morphology in *E. coli*.

A heterologous O-antigen reduces the frequency of shape deformities

The heterologous *rfb* genes from *Klebsiella* (Vinés *et al.*, 2005), cloned in a pBluescript vector, were introduced into two *E. coli* K12 mutants to determine whether a non-*E. coli* O-antigen would complement the misshapen phenotype. In both CS446-1 (which lacks four PBPs) and CS703-1 (which lacks seven PBPs), introduction of the pBluescript vector by itself increased the proportion of the population with abnormal shapes. The percentage of abnormal cells for CS446-1 increased from 60% of the population to 82% in cells carrying the vector, and for CS703-1 the fraction of abnormal cells increased from 73% of the population to 87% (Table 4). The reason for this effect is unknown. Nonetheless, using these vector-containing cells as a baseline, it was clear that expressing a heterologous O-antigen complemented the phenotype in the PBP mutants (Table 4). Adding the *Klebsiella* O1-antigen increased the fraction of normally shaped cells from 9% to 22% in CS446-1 and from 9% to 28% in CS703-1 (Table 4, pWQ5-containing cells). To eliminate the possibility that cloning just any gene into the pBluescript vector would reverse these morphological effects, plasmid pSH55, which contains unrelated *E. coli* genes, was introduced into the mutants. The presence of this plasmid did not reduce the number of abnormal cells in either mutant (data not shown), indicating that O-antigen complementation was not an artifact of cloning into the vector. Therefore, the frequency of misshapen cells in PBP mutants of *E. coli* was reduced when these strains expressed an O-antigen from a related genus.

Possible mechanisms for OM effects on cell shape

At first, it seems odd that the state of the OM helps shape bacteria since the peptidoglycan sacculus and the enzymes that construct it are located internal to this structure. However, altering the OM could affect bacterial shape either directly, by disrupting an external mechanical scaffold, or indirectly, by interfering with the activities of enzymes that synthesize the peptidoglycan sacculus. The idea of the OM as a physical scaffold has been discussed (Cooper, 1991), but little experimental evidence exists for the idea. The main support for such a view is that OM proteins (Braun's lipoprotein, Pal or OmpA) bind peptidoglycan by covalent and noncovalent interactions (Braun & Rehn, 1969; Sonntag *et al.*, 1978; Wang, 2002; Parsons *et al.*, 2006). These intimate connections may impose physical stresses on peptidoglycan that affect how the wall is constructed.

The likelihood of an indirect relationship between the OM and cell wall has more support. Defects in OM synthesis or structure might affect the sacculus either by altering the supply of new peptidoglycan precursors or by changing the way they are incorporated into the pre-existing wall. With regard to precursor supply, it seems reasonable that synthesis of O-antigen and peptidoglycan should be coordinated so that the surface area of the OM and murein sacculus will increase in tandem, to avoid stretching or buckling one or the other (Ehlert & Höltje, 1996). Because the precursors for synthesizing O-antigen and murein are translocated to the periplasm by the same phospholipid carrier (Höltje, 1998), disrupting O-antigen synthesis may alter the sites or rate at which peptidoglycan precursors are delivered to the periplasm. Such an imbalance might, in turn, affect cell shape. This possibility is made more plausible because insertion of O-antigen into the OM has a spatial component (Ghosh & Young, 2005). As for incorporation into the pre-existing wall, previously created peptidoglycan bonds may be held in a particular conformation by virtue of numerous OM protein-to-peptidoglycan interconnections. Should these be disrupted, improper insertion of new material by the penicillin-binding proteins might lead to aberrant shapes in mutants lacking specific DD-carboxypeptidases or endopeptidases (Nelson & Young, 2000, 2001).

Regardless of whether the OM affects cell wall synthesis by mechanical or enzymatic means, it seems clear that neither mechanism affects cell shape by changing the general characteristics of the poles. Whether or not the O-antigen is present, pole-specific proteins

localize to natural and ectopic poles in malformed cells (Nilsen *et al.*, 2004) and a subset of OM proteins are retained for long periods at these same sites (Nilsen *et al.*, 2004; Ghosh & Young, 2005). Instead, the present results show that in the absence of O-antigen, the number of ectopic poles increases, and it is these structures that provoke the increase in cellular deformities (de Pedro *et al.*, 2003). Therefore, on the one hand, the OM may provide a mechanical stability that restricts peptidoglycan-synthesis so that the placement of new cell poles is constrained. Alterations in the OM might weaken this mechanical restraint and allow the production of poles at unusual sites. On the other hand, if the OM affects cell shape indirectly by regulating peptidoglycan-synthesizing enzymes, then altering the membrane composition may interfere with an inhibitory function, thus allowing the creation of multiple, misplaced polar zones.

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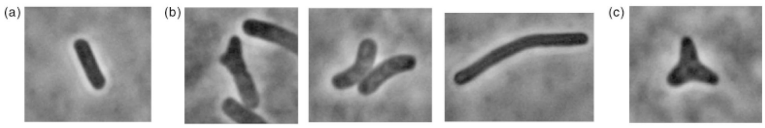

Fig. 1.

Morphological scoring for bacterial shape. (a) ‘Normal’ cells were those with a uniform rod shape. (b) ‘Abnormal’ cells were those exhibiting significant curvatures, bulges or other deformities. (c) ‘Branched’ bacteria were those having three or more arms, each of which comprised a significant portion of the cell volume.

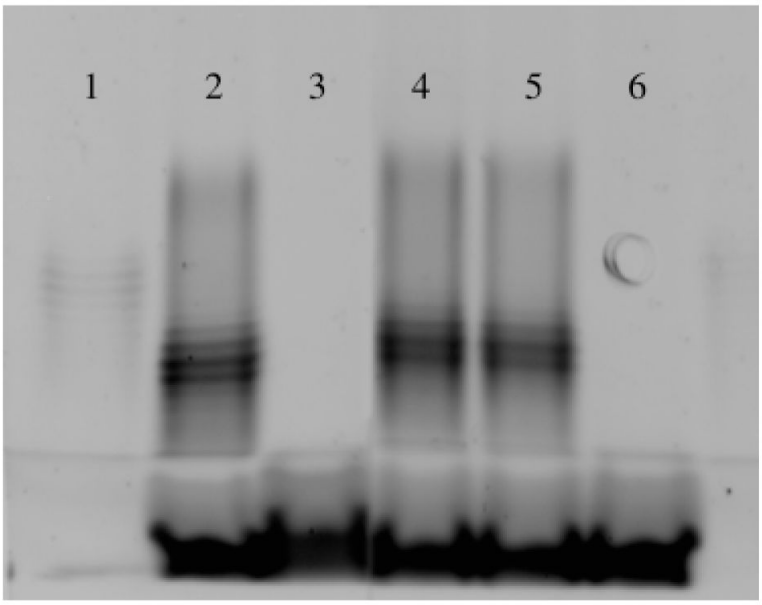

Fig. 2.

Lipopolysaccharide profiles of *Escherichia coli* strains with and without the O-antigen. Lipopolysaccharide from *E. coli* was separated by SDS-PAGE and visualized with Pro-Q Emerald LPS stain. Lipopolysaccharide was prepared from the following strains. Lane 1, *E. coli* serotype O55:B5; Lane 2, *E. coli* 2443T (O8-antigen); Lane 3, *E. coli* CS109 (O-negative K-12 strain); Lane 4, AG375-3; Lane 5, AG456-1; Lane 6, AG456-1*. The banding patterns represent O-antigen side chains present in *E. coli* 2443T and its derivatives (lanes 2, 4 and 5) but absent in the control K12 strain *E. coli* CS109 (lane 3) and in strain AG456* (lane 6), where the O8-antigen was replaced with that of CS109.

Table 1

Escherichia coli strains

Strain	Genotype	O-antigen	Source
2443	*thr-1 leuB6* Δ(*gpt-proA*)*66 argE3 thi-1 rfb*$_{O8}$ *lacY1 ara-14 galK2 xyl-5 mtl-1 mgl51 rpsL31 kdgK51 supE44*	O8	A.T. Maurelli (Rick *et al.*, 1994; Sandlin *et al.*, 1996)
2443T	2443 *zba-3000::Tn10* (Tet^R) (by P1 transduction from KL743)	O8	Ghosh & Young (2005)
CS109	W1485 *rpoS rph*	None	C. Schnaitman
CS315-1	CS109 ΔdacA ΔdacB ΔpbpG	None	Meberg *et al.* (2001)
CS446-1	CS109 Δ*dacA* Δ*dacB* Δ*dacC* Δ*pbpG*	None	Meberg *et al.* (2001)
KL743	MG1655 *zba-3000::Tn10* LAM− *rph*−	None	M. Goldberg (CGSC 6213)
KM32	*leuB6 proA2 thr-1 argE3 lacY1 galK2 ara-14 xyl-5 thi-1 rpsL31 mtl-1 tsx-33 supE44* Δ(*recC ptr recB recD*):: P_{lac}-*bet exo*(Cam^R)	None	K.C. Murphy (Murphy, 1998)
KM32I-9	KM32 *hisI::npt* (Kan^R) (by λ Red recombination)	None	Ghosh & Young (2005)
AGTO2-1K	2443T (Tet^R) *hisI::npt* (Kan^R) *rfb*$_{K12}$	None	Ghosh & Young (2005)
AG375-3	2443 *zba-3000::Tn10* (Tet^R) Δ*dacA* Δ*dacB* Δ*pbpG*	O8	This work
AG375-3*	AG375(Tet^R) *hisI::npt* (Kan^R) *rfb*$_{K12}$	None	This work
AG430-2K	2443 *hisI::npt* (Kan^R) *rfb*$_{K12}$	None	Ghosh & Young (2005)
AG456-1	2443 *zba-3000::Tn10* (Tet^R) Δ*dacA* Δ*dacB* Δ*dacC* Δ*pbpG*	O8	Nilsen *et al.* (2004)
AG456-1*	AG456-1 *hisI::npt* (Kan^R) *rfb*$_{K12}$	None	This work
AG60B-1	AG456-1Δ*mrcB* Δ*dacD*	O8	Nilsen *et al.* (2004)
AG60B-1*	AG60B-1 *hisI::npt* (Kan^R) *rfb*$_{K12}$	None	This work
AG70A-6	AG456-1Δ*mrcA*Δ*dacD* Δ*ampC*	O8	Nilsen *et al.* (2004)
AG70A-6*	AG70A-6 *hisI::npt* (Kan^R) *rfb*$_{K12}$	None	This work
AG70C-12	AG60B-1 Δ*ampC*	O8	Nilsen *et al.* (2004)
AG70C-12*	AG70C-12 *hisI::npt* (Kan^R) *rfb*$_{K12}$	None	This work

Table 2

Effect of O-antigen on cellular deformities

Strains*	PBPs deleted	O8-antigen	Deformities (% population)	Total cells
CS315-1	4 7 5	−	50	529
AG375-3	4 7 5	+	23	707
AG375-3*	4 7 5	−	29	750
CS446-1	4 7 5 6	−	49	408
AG456-1	4 7 5 6	+	36	414
AG456-1*	4 7 5 6	−	49	432

*Strains designated with the ‘CS’ prefix were derived from *Escherichia coli* K-12, which lacks the O-antigen (Liu & Reeves, 1994). Strains designated with the prefix ‘AG’ was derived from *E. coli* 2443, which expresses the O8-antigen (Rick *et al.*, 1994). AG names followed by an asterisk (*) designate strains from which the O8-antigen genetic locus was deleted and replaced by mutated genes from *E. coli* K12.

Table 3

Effect of removing O8-antigen on shape deformities in *Escherichia coli*

Strain	O8-antigen	Exp #1			Exp #2			Exp #3			Exp #4			Cumulative		
		Total # cells	Abnormal (%)	Increase (%)	Total # cells	Abnormal (%)	Increase (%)	Total # cells	Abnormal (%)	Increase (%)	Total # cells	Abnormal (%)	Increase (%)	Total # cells	Abnormal (%)	Increase (%)
AG60B-1	+	358	24	−	386	33	−	65	23	−	57	40	−	866	29	−
AG60B-1*	−	240	51	113	741	38	15	90	52	126	71	52	30	1142	43	48
AG70A-6	+	815	38	−	371	47	−	77	28	−	81	17	−	1344	39	−
AG70A-6*	−	521	50	32	346	62	32	286	30	7	329	30	76	1482	44.6	14
AG70C-12	+	177	42	−	203	51	−	219	26	−	175	32	-	774	38	−
AG70C-12*	−	960	42	0	194	67	31	139	48	85	128	55	72	1421	47	24
AG456-1	+	298	64	−	263	57	−	136	24	−	126	36	−	823	51	−
AG456-1*	−	287	77	20	407	66	16	424	48	100	519	49	36	1637	58	14

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Table 4

Complementation of shape defects by heterologous O-antigen

Strain	Plasmid	O-antigen	Cells (Total #)	Normal (%)	Abnormal (%)	Branched (%)
CS446-1	None	−	1008	29	60	11
	pBluescript	−	594	9	82	9
	pWQ5	*Klebsiella* O1	952	22	76	2
CS703-1	None	−	1313	23	73	4
	pBluescript	−	498	9	87	4
	pWQ5	*Klebsiella* O1	359	28	69	3

CS446-1 and CS703-1 are *Escherichia coli* K12 strains lacking four and seven pencillin-binding proteins, respectively.

The O-antigen was introduced into these strains on plasmid pWQ5 (Vinés *et al.*, 2005), and cells categorized as in Fig. 1.