JOURNAL OF BACTERIOLOGY, Jan. 2001, p. 139–144 Vol. 183, No. 1
0021-9193/01/$04.00+0 DOI: 10.1128/JB.183.1.139–144.2001

Escherichia coli Strains Blocked in Tat-Dependent Protein Export Exhibit Pleiotropic Defects in the Cell Envelope

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Received 23 August 2000/Accepted 26 September 2000

The Tat system is a recently discovered protein export pathway that serves to translocate folded proteins, often containing redox cofactors, across the bacterial cytoplasmic membrane. Here we report that *tat* strains are associated with a mutant cell septation phenotype, where chains of up to 10 cells are evident. Mutant strains are also hypersensitive to hydrophobic drugs and to lysis by lysozyme in the absence of EDTA, and they leak periplasmic enzymes, characteristics that are consistent with an outer membrane defect. Both phenotypes are similar to those displayed by strains carrying point mutations in the *lpxC* (*envA*) gene. The phenotype was not replicated by mutations affecting synthesis and/or activity of all known or predicted Tat substrates.

Approximately 20% of all proteins synthesized by *Escherichia coli* are predicted to be located outside the cytoplasmic compartment. Most proteins destined for export are synthesized with N-terminal signal sequences that direct translocation by the general secretory (Sec) apparatus (17, 21). Sec-dependent signal sequences lack sequence similarity but have similar overall physical-chemical properties (32).

A subset of periplasmic and periplasmically oriented proteins are exported by a mechanism distinct from the Sec pathway (3, 25, 26, 34). Such proteins are synthesized with, or in some cases associate with partner proteins synthesized with, signal sequences harboring the (S/T)RRxFLK "twin-arginine" motif (2). These distinctive signal sequences target substrate proteins to the twin-arginine signal peptide-dependent protein translocase (Tat translocase) which is structurally and mechanistically related to the ΔpH-dependent protein translocase of plant thylakoid membranes (8, 29). Many Tat-dependent substrate proteins bind redox cofactors and are involved in bacterial energy metabolism. Cofactor acquisition is a cytoplasmic event and is a prerequisite for export, suggesting that proteins are translocated by the Tat apparatus in a folded conformation (22, 25).

Minimally the *E. coli* Tat translocase comprises four probable membrane proteins, encoded by the *tatA*, *tatB*, *tatC*, and *tatE* genes. The *tatA*, *tatB*, and *tatC* genes are cotranscribed with a fourth gene, *tatD*, encoding a soluble protein with no discernible role in Tat-dependent protein export (35). TatA, TatB, and TatE are sequence-related proteins, each of which is predicted to comprise a single N-terminal transmembrane α-helix, immediately followed by an amphipathic α-helix at the cytoplasmic side of the membrane (7, 26). TatA and TatE are more than 50% identical at the amino acid level and have overlapping functions in Tat-dependent protein export. Thus, comutation of both *tatA* and *tatE* is necessary to observe a complete block in export by the Tat pathway (26). TatB, which is more divergently related to TatA/E (approximately 25% amino acid identity), is a distinct and essential component of the Tat apparatus (27). The *tatC* gene encodes a highly hydrophobic protein, predicted to contain six membrane-spanning α-helices, which is critical for Tat function (5). In each case, a block in Tat-dependent protein export results in the mislocalization of Tat-dependent substrate proteins to the cytoplasmic compartment. Tat mutant strains are unable to grow anaerobically with either dimethyl sulfoxide or trimethylamine-*N*-oxide as sole terminal electron acceptor, reflecting the failure to correctly localize the terminal reductases. The mutant strains otherwise display no discernible growth phenotype.

In this paper, we report that strains lacking genes encoding essential Tat components form chains of up to 10 cells, which appear to be defective in cell separation. Electron microscopy reveals that the phenotype is similar to that of an *lpxC* (*envA*) mutant, which has a defect in the synthesis of outer membrane lipid A. Consistent with this, *tat* mutant strains are hypersensitive to hydrophobic drugs and to lysozyme-induced lysis in the absence of EDTA. We show that this phenotype is not due to the mislocalization of any single Tat substrate protein.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains used during this study are listed in Table 1. In-frame deletions of genes *sufI*, *yacK*, *ydhX*, *ydcG*, *wcaM*, *ycdB*, and *yaeI* were constructed based on a strategy described previously (26) and using the method of Hamilton et al. (11). The primer sequences used for construction of these mutations are available on request.

Strains were cultured either aerobically or anaerobically on Luria-Bertani (LB) medium (24), either at 37°C or (for strains carrying Mu insertions) at 30°C. Concentrations of antibiotics were as described previously (26).

Cell fixation and microscopic analysis. Aerobically grown cells at early exponential phase (optical density at 600 nm [OD_{600}] of 0.4) were fixed in a final concentration of 2.5% glutaraldehyde in 10 mM EDTA. The cells were subsequently harvested and suspended in 50 mM Tris-HCl (pH 8.0). The cells were treated for nucleoid analysis using DAPI (4′,6-diamidino-2-phenylindole) staining as described elsewhere (13). The fixed cells were examined using a Zeiss Axioplot microscope under both fluorescent and natural light. Photographs were taken using the microscope internal camera on technical pan film (Kodak).

Electron microscopy. Prior to fixation, the bacteria were embedded in 1% agarose by mixing equal volumes of the *E. coli* liquid cultures with 2% (wt/vol)

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TABLE 1. Strains used

Strain	Genotype	Reference
MC4100	F$^-$ Δ*lacU169 araD139 rpsL150 relA1 ptsF rbs flbB5301*	6
RK4353	*araD139* Δ(*argF-lac*)*U169 deoC1 flhD5301 gyrA219 non-9 ptsF25 relA1 rpsL150*	31
LCB320	*thi-1 thr-1 leu-6 lacY1 supE34 rpsL175*	4
LCB2048	LCB320 *nar25*(*narG-H*) *narU-Z*::Kan^r	4
JCB387	*E. coli* RV Δ*nirB*	9
BØD	MC4100 Δ*tatB*	27
B1LK0	MC4100 Δ*tatC*	5
JARV15	MC4100 Δ*tatAE*	26
HDJ123	Hfr Δ(*gpt-lac*)*5 relA1 spoT1 thi-1* Δ*hya*(Kan^r) Δ*hyb*(Kan^r) Δ*hycB-H*(Cm^r)	28
LP200	LCB2048 *napG-ccmA*::Ery	20
JCB3878	JCB378 Δ*nrfC*	9
RK5221	RK4353 *moa*::Mucts	31
DSS501	MC4100 *torA*::Mu*d*1(Ap^r-*lac*) Δ*dms* (Km^r)	23
JCB20480	LCB2048 *napA*::Ery	20
NRS-3	MC4100 Δ*sufI*	This work
NRS-4	MC4100 Δ*yacK*	This work
NRS-5	MC4100 Δ*yacK* Δ*sufI*	This work
TAN-1	MC4100 Δ*ydhX*	This work
TAN-2	MC4100 Δ*ydcG*	This work
TAN-3	MC4100 Δ*wcaM*	This work
TAN-4	MC4100 Δ*ycdB*	This work
TAN-5	MC4100 Δ*yaeI*	This work

type VII low-gelling-temperature agarose (Sigma) in water at 37°C. These mixtures were plunged onto ice to set the agarose into a firm gel. Then 1-mm^3 pieces of agarose containing the cells were cut out of the blocks and immediately placed in 2.5% (vol/vol) glutaraldehyde in 0.05 M sodium cacodylate (pH 7.3) and left overnight at 4°C to fix the cells.

The fixative was washed out by three successive 10-min washes in 0.05 M sodium cacodylate, and then this wash buffer was replaced with 1% (wt/vol) OsO_4 in 0.05 M sodium cacodylate for 1 h at room temperature. The osmium fixation was followed by three 10-min washes in distilled water before initiation of the ethanol dehydration series. Once into 100% ethanol, the samples were infiltrated with LR White resin (London Resin Company, Reading, United Kingdom) by increasing the ratio of resin to ethanol every hour: 1:1, 2:1, and 3:1 and finally 100% resin. After remaining in resin for 24 h at room temperature, during which time there were two further changes of fresh resin, the samples were transferred into Beem capsules with more fresh LR White and placed at 60°C for 16 h to polymerize the resin.

The material was sectioned with a glass knife using a Reichert ultramicrotome (Leica, Milton Keynes, United Kingdom). Ultrathin sections of approximately 90 nm were picked up on 200-mesh copper grids which had been pyroxylin and carbon coated. The sections were stained with 2% (wt/vol) uranyl acetate for 1 h and 1% (wt/vol) lead citrate for 1 min, washed in water, and air dried. The grids were viewed in a JEOL 1200 EX transmission electron microscope at 80 kV, and photographs were taken on Kodak electron image film.

Sensitivity assays. Antibiotic and detergent sensitivity assays were performed by seeding LB top agar with 50-μl aliquots of stationary-phase cultures of the strains of interest. After solidification, disks containing either 300 μg of erythromycin, 800 μg of rifampin, 25 μg of ampicillin, 10% sodium dodecyl sulfate (SDS) or 20% Triton X-100 were placed on the medium and incubated at 37°C for 16 h. Lysozyme sensitivity assays were performed as described elsewhere (18). Efficiency of plaquing (EOP) assays against bacteriophages P1 and λ were performed with LB medium. Cells grown overnight in LB medium were incubated with the bacteriophage for 5 min at room temperature prior to being seeded in LB top agar and incubated at 37°C.

Qualitative RNase assay. RNase I (ribonucleate 3′-pyrimidino-oligonucleotidohydrolase; EC 3.1.4.22) leakage on agar plates was observed by the method of Weigand and Rothfield (33) except that LB medium was used.

RESULTS

Tat mutant strains show a defect in cell division. Initial light microscopic analysis of exponentially growing cultures of *tat* mutant strains (Fig. 1) indicated that the cells display an apparent defect in cell division. Mutant strains formed chains up to 10 cells long. Chain formation was observed irrespective of whether strains were grown aerobically or anaerobically and was observed for cells cultured at either 30 or 37°C. Strains with deletion mutations in genes *tatAE* (Fig. 1B), *tatC* (Fig. 1C), *tatB* or *tatA* to *E* (data not shown), all of which completely block export of Tat-dependent substrates, displayed the most severe chain-forming phenotype. Consistent with the incomplete block in Tat-dependent protein export observed in a *tatA* mutant (26), this strain showed only a mild chain-forming phenotype (maximum of four to five cells per chain). The *tatE* mutant did not form chains of cells (results not shown). The phenotype is specific to the deletion of the *tat* genes, since introduction of plasmid-encoded wild-type *tat* genes to the mutant strains reverted the morphology of the cells to that of the parental strain, MC4100 (not shown).

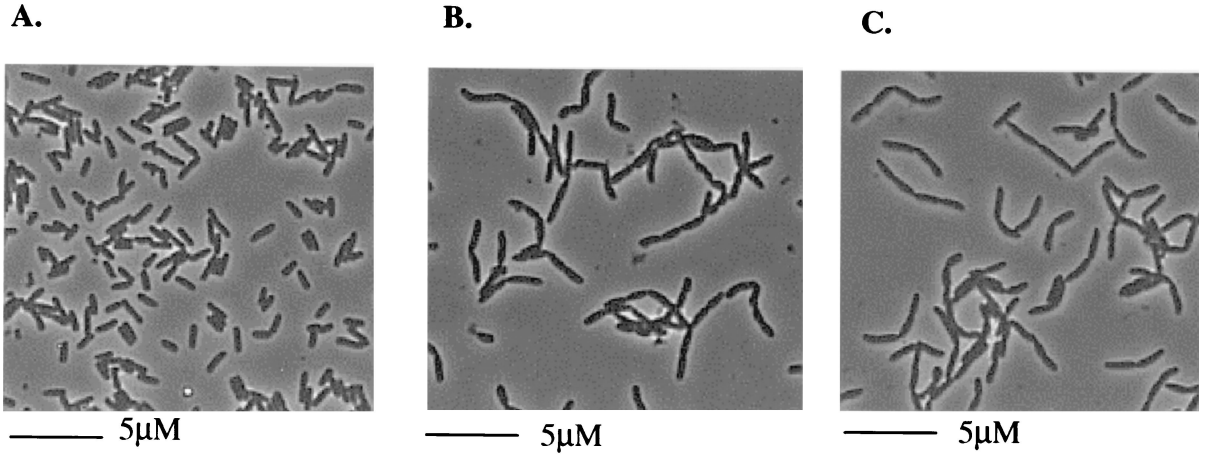

FIG. 1. Light microscope analysis of MC4100 (parental strain) (A), JARV15 (Δ*tatAE*) (B), and B1LK0 (Δ*tatC*) (C). The cells were grown aerobically in LB medium to an OD_{600} of 0.4.

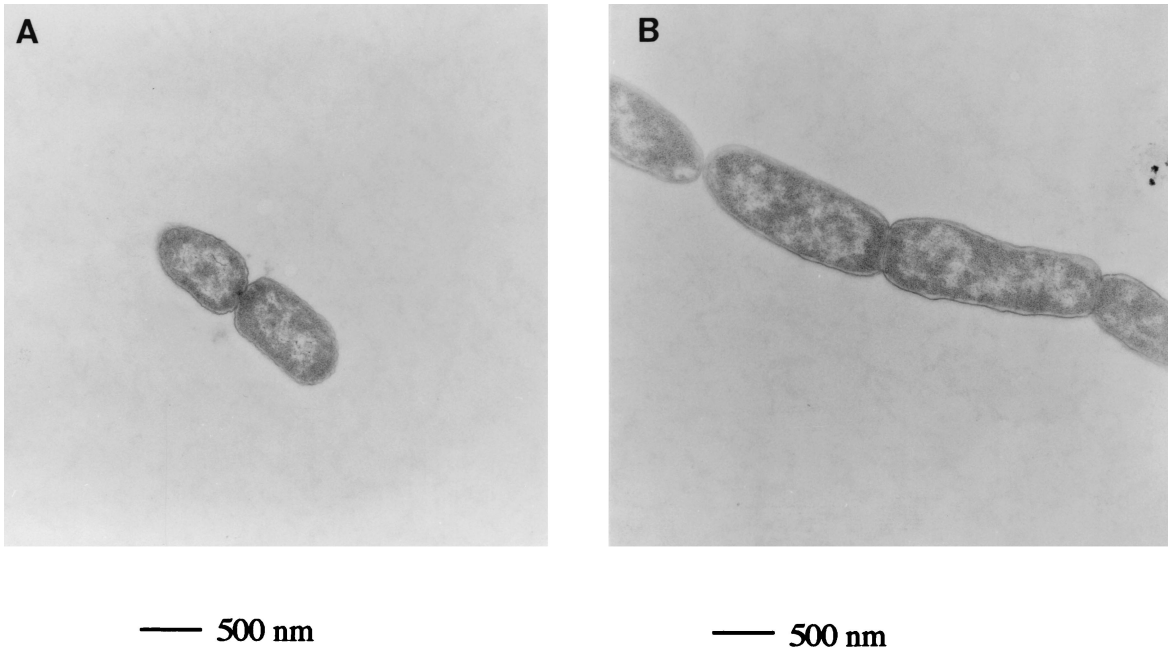

FIG. 2. Transmission electron micrograph of *tat*$^+$ and *tat* deletion *E. coli* strains grown aerobically to an OD_{600} of 0.4 in LB medium. (A) MC4100 (parental strain). The averaged cell length was 0.9 μM, and the averaged breadth was 0.6 μM. (B) B1LK0 (Δ*tatC*). The averaged cell length was 1.8 μM, and the average breadth was 0.6 μM.

Transmission electron microscopy of the parental and Δ*tatC* mutant strains (Fig. 2) shows that the *tat* mutant strain is blocked in a late stage of cell division, since the division septum is clearly visible. This is consistent with light microscopy of DAPI-stained cells, which indicated that nucleoid replication and partitioning, both early events in cell division, were not compromised (results not shown). The *tat* cells are also slightly elongated compared to the parental strain. The overall morphology of the *tatC* strain is strikingly similar to that described for a point mutation in *lpxC* (*envA*), which also forms chains of cells separated by a division septum (18, 19).

Antibiotic and detergent hypersensitivity of *tat* mutant strains. The *lpxC* gene encodes UDP-3-*O*-acyl-*N*-acetylglucosamine deacetylase, which is the second enzyme, and catalyzes the first committed step, of lipid A biosynthesis (30, 38). Although the gene is essential for cell viability (1), a number of point mutations have been described, at least one of which decreases but does not abolish the deacetylase activity (19, 38). A feature of *lpxC* mutants is that they are associated with decreased levels of outer membrane lipid A and are supersensitive to antibiotics. To further explore the similarities between *tat* and *lpxC* strains, we tested the sensitivity of *tat* strains to the antibiotics erythromycin, rifampin, and ampicillin. As shown in Table 2, both the *tatB* and *tatC* mutants were much more sensitive to these antibiotics than the parental strain, suggesting that the permeability barrier of the outer membrane is compromised. This inference is supported by the observation that the *tat* strains are also sensitive to the anionic detergent SDS but not the nonionic surfactant Triton X-100 (Table 2).

***tat* mutants are periplasmically leaky.** A further feature of *lpxC* strains is that they leak periplasmic enzymes into the growth medium (37). We tested whether *tat* mutants also showed periplasmic leakage by looking for the release of periplasmic RNase I into agar plates impregnated with RNA. The *tatB* and *tatC* mutant strains leaked RNase, while the parental strain did not (data not shown).

Lysozyme sensitivity of *tat* mutants. We next tested whether the *tat* strains were sensitive to cell lysis by lysozyme in the absence of EDTA. In wild-type strains, EDTA is required to chelate divalent metal cations that serve to stabilize the structure of the lipopolysaccharide (LPS), in order to allow access of lysozyme to the underlying murein sacculus. This is con-

TABLE 2. Antibiotic sensitivities and EOP conferred by *tat* mutant strains

Relevant genotype (strain)	Zone of inhibition (mm)[a]					EOP[b]	
	E	R	A	S	T	P1	λ
tat$^+$ (MC4100)	3	9	4	0	0	1	1
Δ*tatB* (BØD)	7	14	8	7	0	$<10^{-8}$	0.79
Δ*tatC* (B1LK0)	7	13	9	7	0	$<10^{-8}$	0.73

[a] Measured as diameter on a 13-mm-diameter disk. E, R, A, S, and T disks contained erythromycin (300 μg), rifampin (800 μg), ampicillin (25 μg), SDS (10%), and Triton X-100 (20%), respectively (n = 3 to 4).

[b] The EOP of the parental strain MC4100 was taken as 1, and the mutant EOP values were normalized accordingly.

firmed in Fig. 3A, where significant time-dependent lysis of the parental strain, MC4100, is observed only in the presence of both EDTA and lysozyme. In marked contrast, strains deleted for *tatAE* (JARV15 [Fig. 3B]) and *tatC* (B1LK0 [Fig. 3C]) show very rapid and dramatic lysis upon addition of lysozyme alone. This result is consistent with the observation that the *lpxC* strain D22 is also highly sensitive to lysozyme-induced cell lysis in the absence of EDTA (18) and further supports the contention that *tat* strains have a defect in the outer membrane.

***tat* mutants are highly resistant to infection by P1 phage.** During the course of experiments with *tat* mutants, we noted that they were extremely difficult to transduce with P1 phage (27). A titration of P1 against the parental, *tatB* mutant, and *tatC* mutant strains (Table 2) indicates that the *tat* mutants are at least 10^8 times more resistant to lysis by P1 than the tat^+ strain. Resistance to P1 infection has not been reported for *lpxC* strains, but high levels of resistance are associated with a mutation in the *galU* gene encoding UDP-glucose pyrophosphorylase which adds glucose residues to the LPS core (10). In contrast, the *tat* strains were not significantly resistant to lysis by phage λ, which infects cells by binding to LamB, the maltose receptor, situated in the outer membrane. These observations suggest that *tat* strains specifically have a defect in the LPS component of the outer membrane.

The cell envelope defect in *tat* strains is unlikely to result from mislocalization of any single Tat substrate. One possible explanation for the observed cell envelope defect of *tat* strains is that there is a block in the translocation of a Tat-dependent substrate responsible for outer membrane assembly and/or cell separation. Substrates of the *E. coli* Tat pathway are invariably synthesized as preproteins with distinctive N-terminal twin-arginine signal peptides (2). Tat signal peptides can be readily identified by virtue of both conserved sequence motifs and overall physicochemical properties. In terms of physicochemical properties, Tat signal peptides encompass a positively charged N terminus followed by a hydrophobic region (rich in glycine residues) and are usually punctuated by a short, positively charged C-terminal domain (2, 3). The conserved (S/T)RRxFLK twin-arginine motif is present at the N-terminal/hydrophobic region boundary, and an AxA signal peptidase recognition sequence can usually be identified within the C-terminal region (2). Using these criteria, analysis of the *E. coli* genome sequence reveals 23 open reading frames encoding proteins with plausible twin-arginine signal sequences. Of these, at least 16 bind or are predicted to bind redox cofactors, and many play characterized roles in anaerobic respiration.

In an attempt to ascertain whether any one of these substrates was required for cell separation, we undertook a systematic screen of cellular morphology in either strains with null mutations in genes encoding Tat-dependent proteins or in strains deficient in molybdenum cofactor biosynthesis and therefore in export and function of Tat-dependent molybdoenzymes. Null mutant strains for the molybdoenzymes TorA, DmsA, and NapA as well as the molybdenum cofactor mutant RK5221, which in addition would be expected to have mislocalized BisZ, FdnG, FdoG, YnfE, YnfF, and YagT, appeared wild type when analyzed by light microscopy (results not shown). Further, strains deleted for both uptake hydrogenases, HYD1 and HYD2, the iron sulfur proteins NapG and NrfC,

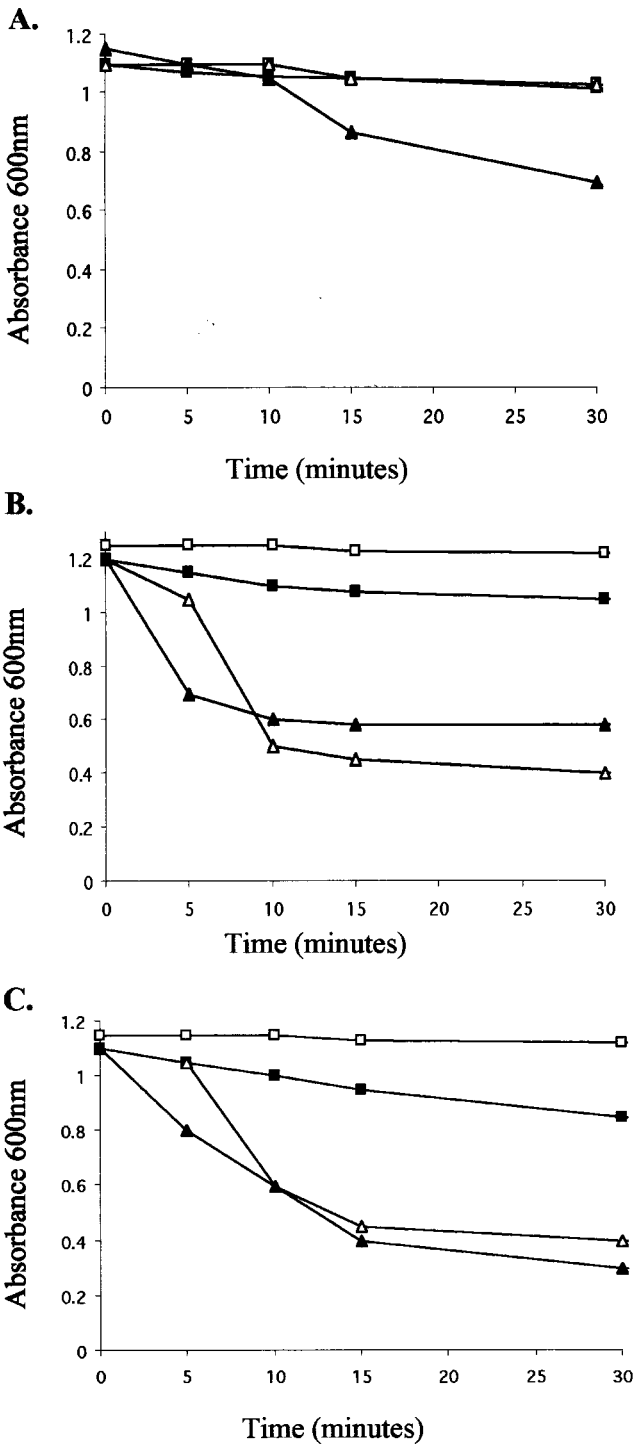

FIG. 3. Lysozyme and EDTA treatment of MC4100 (A), JARV15 (Δ*tatAE*) (B), and B1LK0 (Δ*tatC*) (C). The cells were grown to an OD_{600} of 1.0 to 1.3, harvested, washed in 50 mM Tris-HCl (pH 7.4), and then treated with either no additions (□), 0.25 mM EDTA (pH 8.0) (■), 100 μg of lysozyme/ml (▲), or 0.25 mM EDTA and 100 μg of lysozyme/ml (△). Absorbance of the cells was monitored at 600 nm.

the putative multicopper oxidase YacK and its non-copper binding homologue SufI, and uncharacterized proteins YdhX, WcaM, YdcG, YcdB, and YaeI all displayed wild-type cellular morphology (results not shown). Thus, it is unlikely that the observed phenotype arises from the cellular mislocalization of any one of these proteins alone. We stress that the observed *tat* phenotype may, however, arise from the inability of the cell to properly localize an entire set of proteins. We did not construct a mutation in the gene encoding AmiA since although it has a reasonable twin-arginine signal sequence, pulse-chase analysis indicates that it is not a substrate of the Tat pathway (N. R. Stanley, B. C. Berks, and T. Palmer, unpublished data). It remains a possibility that the phenotype is due to a defect in the translocation of the putative iron-dependent hydrolase YahJ. This has yet to be tested since we have been unable to construct a deletion mutation in the encoding gene.

DISCUSSION

In this paper, we report the observation that *E. coli* strains defective in components of the Tat protein export pathway are impaired in the cell separation stage of cell division. It is likely that this phenotype is linked to a defect in the biosynthesis of the outer membrane, since *tat* mutant strains are supersensitive to killing by hydrophobic drugs and to lysis by lysozyme in the absence of EDTA and are resistant to infection by P1 phage. An obvious explanation to account for the observations is that a protein substrate normally exported by the Tat pathway is required for one of the stages of outer membrane assembly and/or cell separation. In this context, it should be noted that the gene encoding SufI, a previously characterized Tat substrate, was first identified as a multicopy suppressor of the cell division phenotype of an *ftsI* mutant (16). This suggests a role for SufI in the division process. However, strains deleted for genes encoding both SufI and its homologue YacK showed wild-type morphology. Moreover, strains affected in the synthesis and/or assembly of a further 19 putative Tat substrate proteins did not exhibit the chain-forming phenotype. It remains a possibility that the observed phenotype is due to the mislocalization of a hitherto unsuspected Tat substrate protein, a nonprotein substrate, or a combination of several previously characterized protein substrates. It should also be considered that the loss of the Tat translocase itself might have a direct effect on LPS biosynthesis, although a mechanism by which such a situation could arise is unclear.

The cell separation phenotype observed with *tat* mutant strains is strikingly similar to that previously described for a point mutation within the *lpxC* (*envA*) gene. However, it should be noted that although *lpxC* is essential for cell viability, the *tat* strains described here show no growth defect (other than with dimethyl sulfoxide or trimethylamine-*N*-oxide as sole terminal electron acceptor). LpxC is a cytoplasmic metal-containing deacetylase which catalyzes the first committed step of lipid A biosynthesis, one of the major components of the *E. coli* outer membrane (14, 15, 38). In addition to a defect in cell separation, *lpxC* mutations are associated with increased permeability of the outer membrane, reflected in an increased sensitivity to hydrophobic drugs and other compounds. Due to the similar phenotypes shown by these different mutant strains, we investigated whether the LpxC protein was destabilized in *tat* strains by Western blotting. However, cellular levels of LpxC were similar in both the wild-type and *tat* mutant backgrounds (results not shown), indicating that the observed phenotype is probably not due to a direct effect on LpxC.

The *lpxC* mutation is also associated with a sixfold decrease of *N*-acetylmuramyl-L-alanine amidase activity. The amidase enzyme is believed to cleave the division septum and probably accounts for the chain-forming phenotype of *lpxC* strains (35). The genome of *E. coli* codes for three putative periplasmic *N*-acetylmuramyl-L-alanine amidases (13). Of these, the AmiA protein bears a signal sequence that harbors a reasonable twin-arginine motif, differing only in the substitution of a valine at the consensus phenylalanine position. However, pulse-chase experiments indicate that AmiA is not a substrate for the Tat pathway, and therefore it is unlikely that *tat* mutants fail to export enzymes responsible for septal murein cleavage.

In conclusion, *E. coli tat* mutant strains display an unexpected cell separation morphology and cell envelope defect, the reason for which is unclear. It would be interesting to ascertain whether this phenotype is also associated with *tat* mutations in other bacteria.

ACKNOWLEDGMENTS

We thank Katherine Young and Merck Research Laboratories for providing anti-LpxC antiserum. We thank J.-V. Höltje for helpful discussions.

We acknowledge the Norwich Research Park (N.R.S.) and the Royal Society (T.P.) for support.

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