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Differential Effects of Mutations in *tonB1* on Intrinsic Multidrug Resistance and Iron Acquisition in *Pseudomonas aeruginosa*

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Loss of *tonB1* adversely affects iron acquisition and intrinsic multidrug resistance in *Pseudomonas aeruginosa*. Several mutations in *tonB1* compromised the protein's contribution to both processes, although TonB1 derivatives altered in residues C35, Q268, R287, Q292, R300, and R304 were compromised vis-à-vis their contribution to drug resistance only.

Energy-dependent, receptor-mediated ligand uptake across the outer membranes of gram-negative bacteria is dependent upon the function of the product of the *tonB* gene (22). In *Escherichia coli*, this proline-rich protein is anchored to the cytoplasmic membrane via its N terminus and extends into the periplasm (6, 23, 24), where it interacts with receptor proteins in the outer membrane (2, 5, 7, 25, 26). This disposition of the protein reflects its apparent role in coupling the energized state of the cytoplasmic membrane to the outer membrane receptors (17). Such interaction appears to be mediated by the C-terminal region of the TonB protein (10, 12) at or near residue Q160 (2, 7). Indeed, a recent cross-linking study confirmed that the interaction of TonB and the TonB box region of the TonB-dependent vitamin B_{12} receptor, BtuB, is mediated by residues in TonB near Q160 (3).

Two *tonB* genes in *Pseudomonas aeruginosa*, dubbed *tonB1* (21) and *tonB2* (30), have been described. Although TonB1 displays significant homology, e.g., to *E. coli* TonB ($TonB_{Ec}$), this protein is distinguished by the presence of additional sequences at its N terminus, making it larger than all other examples of TonB (21). Still, disruption of TonB1 (but not TonB2) abolishes siderophore-mediated iron uptake (21) and heme uptake (30), consistent with the idea that TonB1 is involved in iron acquisition. Interestingly, *tonB1* (but not *tonB2* [Q. Zhao, unpublished data]) mutants of *P. aeruginosa* are also drug hypersusceptible, apparently owing to a requirement for TonB1 for the operation of multidrug efflux systems in this organism (29). Indeed, the operation of the MexAB-OprM multidrug efflux system, responsible for intrinsic (11, 14, 20, 27) and acquired (9, 15, 16, 20, 32) resistance to several antimicrobials, appears in particular to be compromised in *tonB1* mutants (29). In this report, we assess the functional importance of defined regions of the sequence of TonB1 for intrinsic multidrug resistance in *P. aeruginosa* and note the differential effects of several mutations on resistance versus iron acquisition.

Methods. *P. aeruginosa* PAO6609 (*met9011 amiE200 rpsL pvd9*) (8) is the parent strain of the Δ*tonB1* mutant strain K1040 (29). The chloramphenicol-resistant broad-host-range plasmid pMMB206 (18) was used to clone *tonB1* (29) and its mutated and chimeric variants (31). Construction of chimeras and point mutations involved the use of PCR, and all mutations and constructions were confirmed by nucleotide sequencing (31). The plasmid-borne *tonB1* constructs were introduced into *P. aeruginosa* K1040 via a triparental mating procedure (29). Expression of the various mutant and chimeric TonB1 proteins in *P. aeruginosa* K1040 was confirmed by using Western immunoblotting as described previously, following induction of the cloned genes with IPTG (isopropyl-β-D-thiogalactopyranoside; 20 μM) (31). TonB1 proteins were detected by use of a chicken polyclonal anti-MalE-TonB1 fusion antibody (31) or monoclonal antibody 1C3 to $TonB_{Ec}$ (11a). Susceptibility testing was carried out with L broth with and without IPTG (20 μM) by the twofold serial broth dilution method (13). To alleviate the possible influence of iron limitation on the MIC results (*tonB1* mutants are iron limited in L broth [30]), $FeCl_3$ (200 μM) was included in the growth medium in some experiments (29).

As a measure of mutant-TonB1 function in iron acquisition, the growth of *tonB1* plasmid-carrying *P. aeruginosa* K1040 under iron-restricted conditions upon pyoverdine supplementation was assessed. The K1040 strain lacks *tonB1* and is unable to synthesize pyoverdine. In the presence of a cloned, functional *tonB1* gene, however, addition of exogenous pyoverdine can promote growth under conditions of iron restriction. Thus, *P. aeruginosa* K1040 carrying one of several mutant *tonB1* plasmids was cultured overnight in succinate minimal medium (19) supplemented with methionine (100 μg/ml), $FeCl_3$ (200 μM), and chloramphenicol (16 μg/ml) and used to inoculate an iron-deficient succinate minimal plate containing 180 μg of ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDHA) per ml and 20 μM IPTG (to induce the *tonB1* genes cloned into pMMB206) as described previously (31). Filter disks impregnated with 8 μl of pyoverdine (50 mg/ml of stock) were then placed on the plates. Following incubation at 37°C for ca. 40 h, plates were examined for evidence of growth in the region surrounding the filter disks and the diameter of any zone of growth was measured. Strain K1040 expresses a chromosomal copy of the *tonB2* gene but is not able to grow on the aforementioned EDDHA- and pyoverdine-supplemented medium.

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TABLE 1. Influence of *tonB1* mutations on the antibiotic susceptibility and pyoverdine-stimulated growth of *P. aeruginosa*[a]

Plasmid	TonB or TonB chimera[b]	MIC (μg/ml) of[c]:									Pyoverdine-stimulated growth (%)[d]
		CAR	CFP	CPM	CIP	NOR	NOV	ERY	TET	IPM	
None	Wild type[e]	64	4	2	0.2	1	256	256	8	0.5	ND[f]
pMMB206	None	2	0.12	0.06	0.02	0.12	4	8	2	0.5	0
pQZ-06	Wild type	64	4	2	0.2	1	256	256	8	0.5	100
pQZ-N2	ΔS6–P83	2	0.25	0.06	0.02	0.12	8	8	2	0.5	0
pQZ-CY	C36G	2	0.25	0.06	0.25	0.12	4	8	2	0.5	89
pQZ-T	ΔP82–T111	2	2	0.25	0.02	0.12	4	8	2	0.5	0
pQZ-H	H98G	32	4	1	0.2	1	256	256	8	0.5	96
pQZ-C	ΔD260–A310	2	0.12	0.06	0.12	0.12	4	8	2	0.5	0
pQZ-Y1	Y264A	2	0.12	0.06	0.02	0.12	4	8	2	0.5	0
pQZ-Y2	Y264F	2	0.12	0.06	0.02	0.12	4	8	2	0.5	0
pQZ-P1	P265A	32	4	1	0.2	1	256	256	8	0.5	100
pQZ-P2	P265G	32	4	1	0.2	1	256	256	8	0.5	96
pQZ-Q1	Q269A	2	0.12	0.06	0.02	0.12	4	8	2	0.5	93
pQZ-E1	E274A	2	0.12	0.06	0.02	0.12	4	8	2	0.5	0
pQZ-E3	E274A/D304N	4	0.25	0.12	0.02	0.12	8	16	2	0.5	93
pQZ-E2	E274K	2	0.12	0.06	0.02	0.12	4	8	2	0.5	0
pQZ-K1	K278A	2	0.12	0.06	0.02	0.12	4	8	2	0.5	37
pQZ-K2	K278E	2	0.12	0.06	0.02	0.12	4	8	2	0.5	41
pQZ-R1	R288A	2	0.12	0.06	0.02	0.12	4	8	2	0.5	81
pQZ-R2	R288E	2	0.12	0.06	0.02	0.12	4	8	2	0.5	85
pQZ-Q2	Q293A	2	0.12	0.06	0.02	0.12	4	8	2	0.5	96
pQZ-V1	V294A	8	0.5	0.25	0.1	0.25	64	64	4	0.5	100
pQZ-V2	V294T	32	4	1	0.1	0.5	256	256	8	0.5	100
pQZ-R3	R301A	2	0.12	0.06	0.02	0.02	4	8	2	0.5	93
pQZ-R4	R301E	2	0.12	0.06	0.02	0.02	4	8	2	0.5	97
pQZ-D1	D304A	2	0.12	0.06	0.02	0.02	4	8	2	0.5	0
pQZ-D2	D304K	2	0.12	0.06	0.02	0.02	4	8	2	0.5	0
pQZ-R5	R305A	2	0.12	0.06	0.02	0.02	4	8	2	0.5	93
pQZ-R6	R305E	2	0.12	0.06	0.02	0.02	4	8	2	0.5	100
pQZ-CEc1	TonB1 (1–81) + $TonB_{Ec}$ (1–239)	2	0.12	0.06	0.02	0.02	4	8	2	0.5	37[g]
pQZ-CPa1	$TonB_{Ec}$ (1–152) + TonB1 (254–342)	4	0.25	0.12	0.05	0.02	8	32	4	0.5	96
pQZ-NPa1	TonB1 (1–254) + $TonB_{Ec}$ (154–239)	2	0.12	0.06	0.02	0.02	4	8	2	0.5	0

[a] The antibiotic susceptibility of *P. aeruginosa* K1040 (Δ*tonB1*) expressing the indicated plasmid-encoded mutant TonB1 proteins was assessed as described in the text. Identical results were obtained when assays were carried out in $FeCl_3$-supplemented growth medium.

[b] The mutations present in the TonB1 proteins encoded by the indicated plasmids are listed. The amino acid numbers in parentheses refer to the positions in the protein inclusive of the initiation Met. Results for only three of the TonB1-$TonB_{Ec}$ chimeras are shown. The remaining chimeras yielded results indistinguishable from those with TonB of K1040 harboring pMMB206.

[c] CAR, carbenicillin; CFP, cefoperazone; CPM, cefpirome; CIP, ciprofloxacin; NOR, norfloxacin; NOV, novobiocin; ERY, erythromycin; TET, tetracycline; IPM, imipenem. Comparable results were obtained with or without induction of the cloned *tonB1* genes with IPTG.

[d] *P. aeruginosa* K1040 harboring the indicated plasmids was streaked onto an iron-deficient succinate minimal plate supplemented with EDDHA, and a pyoverdine-impregnated disk was placed on the surface of the plate. The diameter (in millimeters) of any resultant zone of growth was measured and reported relative to (i.e., as a percentage of) the diameter of the zone observed for pQZ-06-carrying K1040. Results are representative of three independent experiments.

[e] Data for the plasmid-free $TonB^+$ parent strain PAO6609 are shown for comparison purposes. The same results were obtained for K201 carrying pMMB206.

[f] ND, not determined.

[g] The observed growth was very weak.

Thus, any growth afforded by the cloned *tonB1* genes is a measure of TonB1 function only.

Influence of mutations at the N terminus on TonB1 function. Recently, several mutations were constructed in *tonB1* in an attempt to assess the functional importance of, e.g., the novel N-terminal extension as well as of the conserved C-terminal region of the TonB1 protein (31). To assess the impact of these mutations on intrinsic resistance compared to their effects on iron acquisition, the antibiotic susceptibility and siderophore-dependent growth capability (under iron-restricted conditions) of *P. aeruginosa* K1040 expressing the mutant TonB1 proteins were determined. Introduction of the wild-type *tonB1* gene (on pQZ-6) into *P. aeruginosa* K1040 fully restored the antibiotic resistance of this mutant strain to the level of the PAO6609 parent strain (Table 1). Similarly, while the mutant is unable to grow on EDDHA-supplemented minimal medium upon pyoverdine supplementation, the plasmid with wild-type *tonB1* permitted pyoverdine-dependent growth of K1040 (Table 1). Since this strain is unable to make its own pyoverdine and lacks *tonB1*, growth under iron-restricted (i.e., EDDHA-supplemented) conditions requires exogenously added pyoverdine as well as a functional TonB1 protein (pyoverdine-mediated iron acquisition is TonB1 dependent [21]). Thus, any growth of *tonB1* plasmid-containing K1040 on EDDHA- and pyoverdine-supplemented minimal medium is an indication of a TonB1 contribution to pyoverdine-mediated iron uptake.

Several mutations in *tonB1* compromised the TonB1 contribution to both intrinsic antimicrobial resistance and pyoverdine-mediated iron acquisition, including one carried by plas-

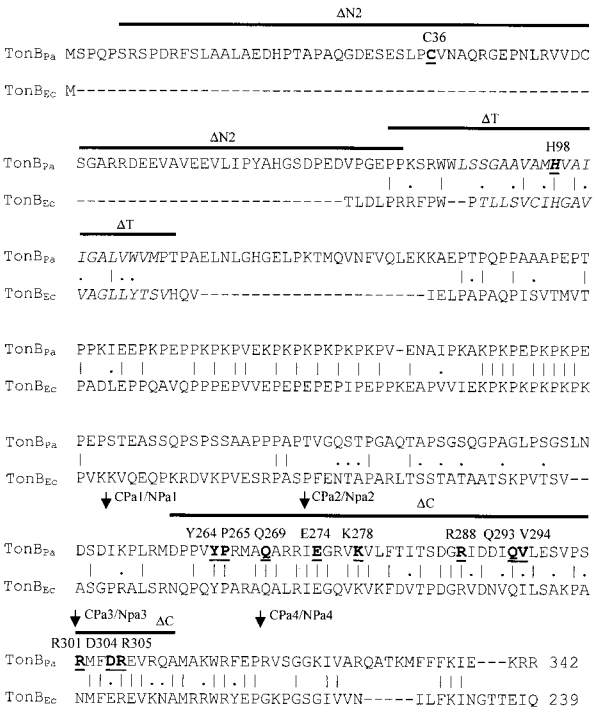

FIG. 1. Alignment of *P. aeruginosa* TonB1 (TonB$_{Pa}$) and TonB$_{Ec}$, and identification of mutations constructed in TonB1. Identical (|) and conserved (.) residues are indicated. The putative transmembrane domain is in italics. Deletions are indicated by a solid bar above the TonB1 sequence. Point mutations are underlined, in bold text, and identified above the TonB1 sequence. The crossover points of TonB1-TonB$_{Ec}$ (NPa series) and TonB$_{Ec}$-TonB1 (CPa series) chimeras are indicated by arrows. Numbers at the right represent the position of the right-most amino acid within the sequence of the respective TonB proteins. Alignment was carried out using the PALIGN program of the PCGene software package (Intelligenetics, Inc.).

mid pQZ-N2 that resulted in a product with a deletion of 78 amino acids (from S6 to P83) (see ΔN2, Fig. 1) from the novel N-terminal extension. Deletion of the predicted transmembrane domain (P82 to T111) (pQZ-T; see ΔT, Fig. 1) or a highly conserved C-terminal region (D260 to A310) (plasmid pQZ-C; see ΔC, Fig. 1) also failed to promote either intrinsic antibiotic resistance or pyoverdine-mediated iron acquisition (Table 1). The lack of activity of the TonB1 derivative lacking the transmembrane domain was expected, given the importance of this region in TonB$_{Ec}$ for proper membrane localization. Still, a conserved His residue (H98 in TonB1) present within the transmembrane domain of several TonB proteins (H20 in TonB$_{Ec}$) (28) and required for TonB function was dispensable for function in *P. aeruginosa* (see the H98G mutation [pQZ-H], Table 1), although mutation of H98 did abolish TonB1 function in *E. coli* (31). The C terminus of TonB$_{Ec}$ is apparently involved in receptor interaction (3, 10, 12) (and possibly TonB$_{Ec}$ dimerization [4]), and the C terminus of TonB1 in *P. aeruginosa* likely plays a similar role. Whether its contribution to intrinsic resistance is related to interaction with, e.g., efflux components remains to be examined.

Influence of mutations at the C terminus on TonB1 function. The highest degree of similarity between TonB1 and other TonB proteins, including that of *E. coli*, occurs within the C termini of these proteins. To assess the functional importance of a number of residues found within the TonB1 C terminus, including residues conserved in other TonB proteins (e.g., TonB$_{Ec}$), several of these were mutated (Fig. 1), with the resulting TonB1 proteins being expressed in *P. aeruginosa* K1040 (Fig. 2B), and the effects on the pyoverdine-dependent growth (i.e., iron acquisition) and antibiotic resistance of this strain were determined. Of 11 residues mutated, 6, including Q269 (pQZ-Q1), R288 (pQZ-R1 and pQZ-R2), Q293 (pQZ-Q2), V294 (pQZ-V1 and pQZ-V2), and R305 (pQZ-R5 and pQZ-R6), were dispensable for TonB1 function as regards pyoverdine-mediated iron acquisition (Table 1). Substitutions at Y264 (pQZ-Y1 and pQZ-Y2), E274 (pQZ-E1 and pQZ-E2), and D304 (pQZ-D1 and pQZ-D2), on the other hand, inactivated TonB1, and K1040 expressing these TonB1 derivatives failed to grow on a pyoverdine-supplemented iron-restricted medium (these were also compromised vis-à-vis antibiotic resistance) (Table 1). Mutations at K278 (pQZ-K1 and pQZ-K2) reduced but did not eliminate the TonB1 contribution to iron acquisition (Table 1). Intriguingly, many of the *tonB1* mutations that had no or only a modest impact on the TonB1 contribution to the pyoverdine-promoted growth of K1040 completely eliminated its contribution to the antibiotic resistance of this strain (Table 1). Indeed, only substitutions P265 and V294 failed to abolish a TonB1 contribution to antibiotic resistance. Thus, substitutions at Q269, R288, Q293, R301, and R305 preferentially compromised the TonB1 contribution to intrinsic antibiotic resistance. A D304N intragenic suppressor of the original E274A mutation (31), which restored the growth of *P. aeruginosa* K1040 in a pyoverdine-supplemented iron-restricted medium (Table 1), had a very modest impact on resistance, providing a slight increase in resistance to only some of the antibiotics tested (Table 1). The specificity of the intragenic suppression with respect to iron acquisition clearly indicates that TonB1 operates differently in these two processes.

Activities of TonB-TonB$_{Ec}$ chimeras. The earlier observation that TonB1 could complement an *E. coli tonB$_{Ec}$* mutation (21) but that TonB$_{Ec}$ could not return the favor in *P. aeruginosa* (Q. Zhao, data not shown) indicated that sequences unique to TonB1 were crucial for TonB function in *P. aeruginosa*. Initially, it was surmised that the novel N-terminal TonB1 extension that is lacking in TonB$_{Ec}$ might be important for this. Still, the addition of the TonB1 extension to the N terminus of TonB$_{Ec}$ (see pQZ-CEc1, Table 1) failed to promote significant pyoverdine-dependent growth or antibiotic resistance (Table 1). It was reasoned, then, that sequences present within the TonB1 C terminus but lacking in TonB$_{Ec}$ might be involved. To test this, a number of TonB1-TonB$_{Ec}$ chimeras were constructed by swapping various portions of the C termini of these proteins (31) and the impact on the iron acquisition (i.e., pyoverdine-dependent growth under conditions of iron restriction) and intrinsic antibiotic resistance of this strain was assessed. Of four TonB derivatives carrying the N terminus of TonB$_{Ec}$ and the C terminus of TonB1 (CPa-1 through CPa-4, Fig. 1), only the chimera encoded by pQZ-CPa1 in which the N-terminal 152 amino acid residues of TonB$_{Ec}$ were fused to the C-terminal 79 amino acids of TonB1 (Fig. 1) promoted both the pyoverdine-dependent growth of K1040 under iron-restricted conditions and antibiotic resistance (Table 1), al-

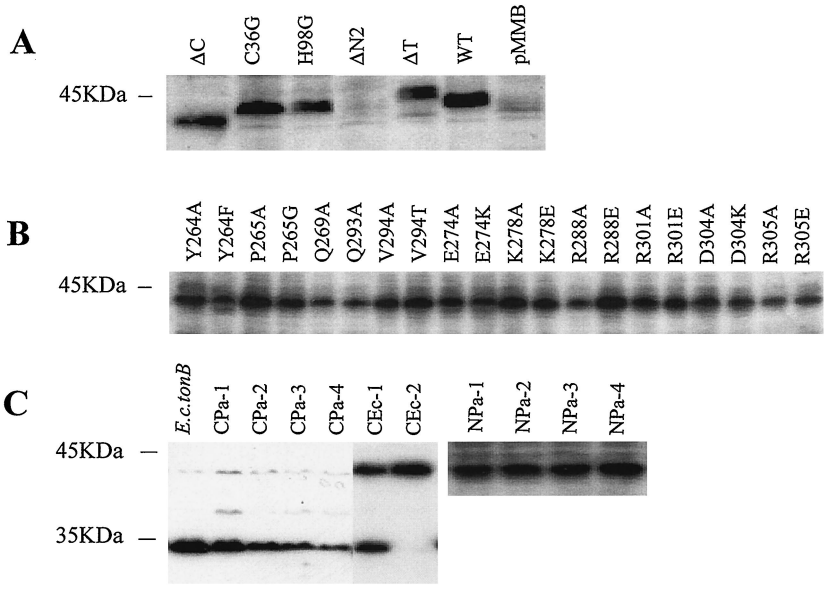

FIG. 2. Expression of plasmid-encoded TonB1 in the *tonB1* deletion strain *P. aeruginosa* K1040. Cell extracts of K1040 harboring various mutant *tonB1* genes (A and B) and *tonB1-tonB*$_{Ec}$ chimeras (C) were immunoblotted and probed with antibodies to TonB1 (with the exception of the CPa and CEc series of chimeras, which were probed with monoclonal anti-TonB$_{Ec}$ antibody 1C3). The nature of the mutation in the sequence of TonB1 is indicated above each lane in panels A and B, with the exceptions of lanes ΔC (deletion of D260 to A310), ΔN2 (deletion of S6 to P83), and ΔT (deletion of P82 to T111). Lanes: WT, wild-type TonB1; pMMB, pMMB206, vector control without a cloned *tonB1* gene. Variations seen in the levels of the various mutant TonB1 proteins in panel B were within the natural variation seen for any given mutant TonB1 as observed over three separate experiments. Chimeras shown in panel C are described in the legend to Fig. 1. *E.c.tonB*, *tonB*$_{Ec}$.

though the impact on resistance was very modest. Chimeras carrying N-terminal sequences of TonB1 and C-terminal sequences of TonB$_{Ec}$ (NPa-1 through NPa-4, Fig. 1) were all inactive as regards antibiotic resistance and pyoverdine-mediated iron acquisition (Table 1). Thus, the C terminus indeed carries a sequence important for the operation of TonB1 in *P. aeruginosa*.

Expression of TonB1 derivatives. A perhaps surprising finding from this study was the differential effects of *tonB1* mutations on the TonB1 contributions to intrinsic resistance and iron acquisition. Most mutations did in fact adversely affect the TonB1 contribution to intrinsic resistance without impacting its contribution to iron acquisition. To rule out lack of mutant TonB1 production as an explanation for these defects in activity, extracts of TonB1-expressing K1040 strains were probed with a TonB1-specific antiserum. As seen in Fig. 2, all TonB1 derivatives, with the exception of the pQZ-N2-encoded N-terminal deletion derivative ΔN2 (Fig. 1), were expressed during growth under conditions used for the MIC assay. No substantial differences were observed in the levels of the various mutant TonB1 proteins and wild-type TonB1, indicating that loss of activity was not attributable to decreased production of the mutant TonB1 constructs. Significantly, detection of the TonB1 derivatives required induction of the plasmid-borne genes with IPTG, although the drug susceptibility and pyoverdine-stimulated growth data were unchanged with or without IPTG (Table 1). Thus, enhanced production of the TonB1 derivatives does not enhance activity or compensate for loss of function, so differences in TonB1 activity vis-à-vis iron acquisition and intrinsic antimicrobial resistance cannot be attributed to, e.g., better expression of TonB1 in the iron-limited minimal medium used in assessing iron acquisition.

The lack of expression of a TonB1 with an N-terminal deletion may reflect the importance of this region for protein stability, which is interesting given that this N-terminal extension is unique to TonB1 among TonB proteins. Within the N-terminal region of TonB1 are two cysteine residues, C36 and C51 (Fig. 1), of which one, C36, was successfully mutated (C36G, pQZ-CY). Such a mutation specifically compromised the TonB1 contribution to intrinsic resistance inasmuch as pQZ-CY-harboring K1040 exhibited resistance levels reminiscent of those in the plasmid-free Δ*tonB1* mutant (Table 1), whereas strain K1040 carrying this vector was capable of near wild-type levels of pyoverdine-dependent growth under iron-restricted conditions (Table 1). The observation that deletion of a substantial portion of the C-terminal region of TonB1 did not destabilize TonB1 in *P. aeruginosa* (Fig. 2A) was interesting, given that a similar C-terminal deletion destabilizes TonB$_{Ec}$ in *E. coli* (1).

Conclusions. In examining the impact of *tonB1* mutations on intrinsic multidrug resistance, it was interesting that susceptibility to agents known not to be substrates for multidrug resistance efflux systems in *P. aeruginosa* (e.g., imipenem) was not affected by such mutations. This finding further supports our earlier suggestion that the enhanced multidrug susceptibility of *tonB1* mutants results from attendant defects in efflux and not in the outer membrane barrier, since defects in the latter would be expected to influence imipenem susceptibility. Most importantly, these results also distinguish the effects of *tonB1* mutations on iron acquisition from their effects on multidrug

resistance and in so doing provide evidence for a direct contribution of this protein to intrinsic resistance.

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