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A Gene (*wbbL*) from *Serratia marcescens* N28b (O4) Complements the *rfb-50* Mutation of *Escherichia coli* K-12 Derivatives

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A cosmid-based genomic library of *Serratia marcescens* N28b was introduced into *Escherichia coli* DH5α, and clones were screened for serum resistance. One clone was found resistant to serum, to bacteriocin 28b, and to bacteriophages TuIa and TuIb. This clone also showed O antigen in its lipopolysaccharide. Subcloning and sequencing experiments showed that a 2,124-bp DNA fragment containing the *rmlD* and *wbbL* genes was responsible for the observed phenotypes. On the basis of amino acid similarity, we suggest that the 288-residue RmlD protein is a dTDP-L-rhamnose synthase. Plasmid pJT102, containing only the *wbbL* gene, was able to induce O16-antigen production and serum resistance in *E. coli* DH5α. These results suggest that the 282-residue WbbL protein is a rhamnosyltransferase able to complement the *rfb-50* mutation in *E. coli* K-12 derivatives, despite the low level of amino acid identity between WbbL and the *E. coli* rhamnosyltransferase (24.80%). *S. marcescens* N28b *rmlD* and *wbbL* mutants were constructed by mobilization of suicide plasmids containing a portion of *rmlD* or *wbbL*. These insertion mutants were unable to produce O antigen; since strain N28b produces O4 antigen, these results suggest that both genes are involved in O4-antigen biosynthesis.

Serratia marcescens N28b is able to produce bacteriocin 28b (6, 35). In studies aimed at identifying a putative bacteriocin 28b immunity system, a gene coding for a 17-kDa outer membrane protein (Omp4) has been previously characterized by us (11). The Omp4 protein was similar to a family of small outer membrane proteins of *Enterobacteriaceae*. Two members of this protein family, Ail from *Yersinia enterocolitica* and Rck from *Salmonella typhimurium*, have been associated with complement resistance, and when the genes encoding these proteins were expressed in *Escherichia coli* HB101, both conferred serum resistance (3, 13). Despite the high level of similarity among Omp4, Ail, and Rck proteins, *E. coli* HB101 or DH5α producing Omp4 protein remained serum sensitive but became bacteriocin 28b resistant (11).

S. marcescens strains have been classified as serum sensitive, delayed serum sensitive, and serum resistant (32). As a general rule in gram-negative bacteria, with many exceptions, smooth strains (O^+) are serum resistant while rough strains (O^-) are serum sensitive (30). *S. marcescens* N28b is a serum-resistant strain, and probably this phenotype is related to O-antigen production. We screened a previously constructed cosmid-based genomic library of this strain (11) introduced into *E. coli* DH5α (which is O^- and serum sensitive) for serum resistance. In this work we report the characterization of a gene from *S. marcescens* N28b that confers the ability to produce *E. coli* O16-antigen lipopolysaccharide (LPS) and serum resistance on *E. coli* DH5α and other K-12-derived strains.

S. marcescens N28b (7) and *E. coli* strains used in this study were grown in Luria-Bertani (LB)-Miller broth and LB-Miller agar (18) supplemented with ampicillin (50 μg/ml), chloramphenicol (50 μg/ml), kanamycin (30 μg/ml), or rifampin (50 μg/ml) when needed. Recombinant clones were selected on LB-Miller agar plates containing the appropriate antibiotics.

Nucleotide sequence accession no. The nucleotide sequences of the *rmlD* and *wbbL* genes from *S. marcescens* N28b have been deposited in GenBank under accession no. U82331 (see Fig. 2).

Cloning of an *S. marcescens* genomic region determining O-antigen production in *E. coli* DH5α. *S. marcescens* N28b is characterized as serum resistant (Table 1), since it can grow in tryptic soy broth medium containing up to 50% serum. In order to determine the basis for this serum resistance, a previously constructed cosmid-based genomic library of *S. marcescens* N28b chromosomal DNA (11) was introduced into *E. coli* DH5α (12) and recombinant clones were selected on LB agar-kanamycin (30 μg/ml). Five serum-resistant clones were isolated by screening the level of serum sensitivity by using a microtiter plate-based assay (36). To check if the serum resistance phenotype was due to *E. coli* DH5α envelope modifications, the recombinant clones were tested for resistance to bacteriocin 28b and bacteriophages TuIa and TuIb by previously described methods (5, 22, 35), and they were found to be resistant to these agents (Table 1). Restriction analysis showed that these recombinant cosmids harbored common DNA fragments, suggesting that they were related. *E. coli* DH5α harboring one of these recombinant cosmids (FGR20) was characterized by analysis of the outer membrane protein and by LPS profile on a sodium dodecyl sulfate (SDS)-polyacrylamide gel. LPS was purified by the method of Westphal and Jann (37). Also, LPS was obtained after proteinase K digestion of whole cells according to the procedure of Darveau and Hancock (4). SDS-polyacrylamide gel electrophoresis (PAGE) was performed by the procedure of Laemmli (15), and LPS bands were detected by the silver-staining method of Tsai and Frasch (33). No differences were found in the pattern of outer membrane protein, but cosmid FGR20 conferred O-antigen production on *E. coli* DH5α (Fig. 1A, lane 3), while no O antigen was detected in *E. coli* DH5α with or without vector Supercos1

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TABLE 1. Resistance phenotype conferred by *wbbL* gene on *E. coli* DH5α

Strain	Plasmid genotype	Serum resistance[a]	Sensitivity to bacteriocin 28b[b]	EOP[c] for:	
				Phage TuIa	Phage TuIb
S. marcescens N28b		100	R	R	R
E. coli strains					
DH5α		0.12	S	1	1
DH5α(Supercos1)		0.13	S	1	1
DH5α(FGR20)	*rmlD wbbL*	98	R	0.015	0.012
DH5α(pBR328)		0.12	S	1	1
DH5α(pJT101)	*rmlD wbbL*	97	R	0.0010	0.013
DH5α(pJT102)	*wbbL*	98	R	0.0013	0.011

[a] Values represent percentages of bacteria surviving in 50% normal pooled serum after 3 h of treatment.

[b] Bacteriocin 28b sensitivity was measured by spotting 10 μl of bacteriocin 28b on an LB agar plate inoculated with 200 μl of 10^9 CFU/ml. R, resistant; S, sensitive.

[c] EOP, efficiency of plating.

(Stratagene) (Fig. 1A, lanes 1 and 2). Cosmid FGR20 conferred on *E. coli* DH5α high levels of serum and bacteriocin 28b resistance, measured quantitatively (Table 1). Cosmid FGR20 was cured from *E. coli* DH5α(FGR20) by serial growth in LB-Miller broth without antibiotics and single-colony isolation on LB-Miller agar, as previously described (10). The cured strain lacked O antigen (Fig. 1A, lane 4) and became sensitive to serum and bacteriocin 28b (Table 1). These results suggested that cosmid FGR20 harbors *S. marcescens* N28b genes involved in O-antigen biosynthesis and responsible for the serum resistance and bacteriocin 28b resistance phenotypes. To localize the genes involved in the O-antigen production and serum resistance phenotype, cosmid FGR20 was partially digested with *Pst*I, and the resulting DNA fragments were ligated to *Pst*I-digested and dephosphorylated plasmid pBR328 DNA and transformed into *E. coli* DH5α by previously described methods (25). Transformants were assayed for serum sensitivity and for O-antigen production. Plasmid pJT101 turned out to be the smallest subclone, about a 2-kb DNA insert, still conferring serum and bacteriocin resistance and O-antigen production (Fig. 1A, lane 6, and Table 1).

Sequencing of the DNA conferring O-antigen production. The nucleotide sequence of the plasmid pJT101 insert was determined in order to identify the *S. marcescens* genes conferring serum resistance and O-antigen production on *E. coli* DH5α. A nucleotide sequence of 2,124 bp was determined in both directions by using oligonucleotides 1240 and 1241 (Bio-Labs) complementary to sequences flanking the *Pst*I restriction site in pBR328. Other sequence-derived oligonucleotides were used to complete the nucleotide sequence. Analysis of the sequence showed two potential open reading frames (ORFs) separated by 174 bp (Fig. 2). The larger ORF1, nucleotides 33 to 896, encoded a protein of 288 amino acid residues with a theoretical molecular mass of 31.10 kDa. The smaller ORF2, nucleotides 1070 to 1915, encoded a putative protein of 282 amino acid residues with a predicted molecular mass of 31.85 kDa. No sequence similar to the *E. coli* promoter consensus sequence was found in the 32 bp between the beginning of the DNA insert and ORF1, suggesting that expression proceeds from a vector promoter. In the DNA region between ORF1 and ORF2, an inverted repeat followed by a run of T's was found, similar to a rho-independent transcription termination sequence. In this intergenic region, no sequences were found similar to the −35 consensus sequence of *E. coli* promoters, but up to four sequences similar to the −10 region were detected: GATAAT (nucleotides 929 to 934), TGAAAT (nucleotides 965 to 970), CCTAAT (nucleotides 1013 to 1018), and GTAAAT (nucleotides 1039 to 1044). These results suggest that a promoter for ORF2 transcription could be located in this region.

Analysis of the ORFs' deduced amino acid sequences. The DNA sequence was translated in all six frames, and all ORFs longer than 100 bp were inspected. Deduced amino acid sequences were compared with those of DNA translated in all six frames from the nonredundant GenBank v. 76 and EMBL v. 34 databases by using the BLAST network service at the National Center of Biotechnology Information (1). This analysis showed amino acid identities among the deduced 288-amino-acid protein encoded by ORF1 and proteins proposed to be dTDP-4-keto-L-rhamnose reductases: RshD from *Sphingomonas* strain S88 (43%) (38), RmlD[RfbD]$_{S.\ typhimurium\ LT2}$ (39%) (14), RmlD[RfbD]$_{E.\ coli}$ (37%) (17), and StrL from *Streptomyces griseus* (36%) (21). This analysis suggests that the ORF1-encoded protein is involved in the synthesis of dTDP-L-rhamnose. Since *S. marcescens* is closely related to *E. coli* and *S. typhimurium*, by analogy we call the *S. marcescens* gene *rmlD*, following the guidelines of a recent proposal for nomenclature of bacterial genes involved in polysaccharide synthesis (24) (protein names given in brackets are those used in the original references). The *S. marcescens* RmlD protein also showed similarities to proteins Oac2 from *Azorhizobium caulinodans* (45%), (9), SpsK from *Bacillus subtilis* (38%) (8), and Rml C [RfbC]$_{Shigella\ flexneri\ 2a}$ (37%) (23).

Comparison of the 282-amino-acid protein encoded by ORF2 (*wbbL*) with amino acid sequences in the GenBank database showed identity and similarity to a reported phosphomannose isomerase (EpsX) from *Acinetobacter calcoaceticus* (47 and 70%, respectively) (GenBank accession no. X81320), a glycosyltransferase from *Sphingomonas* strain S88 (23 and 25%, respectively) (38), and ORF264 from *E. coli* (24 and 26%, respectively) (16). ORF264 has been suggested to be a rhamnosyltransferase (*wbbL* gene) (28). An amino acid alignment of these four proteins is shown in Fig. 3. It has been reported that O-antigen deficiency in *E. coli* K-12 is due to an IS*5* insertion mutation in ORF264 (*rfb-50* mutation) (16). The rhamnosyltransferase defect in the *rfb-50* mutation has been complemented by a gene from *E. coli* EMG2, restoring O16-antigen production (16). Similarly, *S. flexneri rfb* genes also

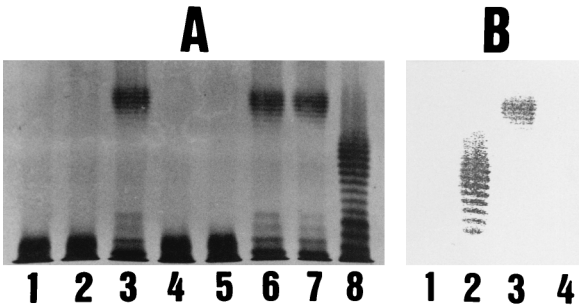

FIG. 1. Silver-stained polyacrylamide gel (A) and Western immunoblot (B) of LPS reacted with *E. coli* O16-specific antiserum. Anti-*E. coli* O16 LPS serum and anti-*S. marcescens* O4 LPS serum were obtained and assayed as described previously for other LPSs (2). LPS samples were prepared from *E. coli* DH5α without cosmid or plasmid (lane A1), harboring Supercos1 (lane A2) or cosmid FGR20 (lane A3), cured from cosmid FGR20 (lane A4), harboring pBR328 (lanes A5 and B1), pJT101 (lane A6), or pJT102 (lanes A7 and B3), or cured from pJT102 (lane B4), and from *E. coli* F11119-41 (O16:K1 [standard O16 strain]) (lanes A8 and B2).

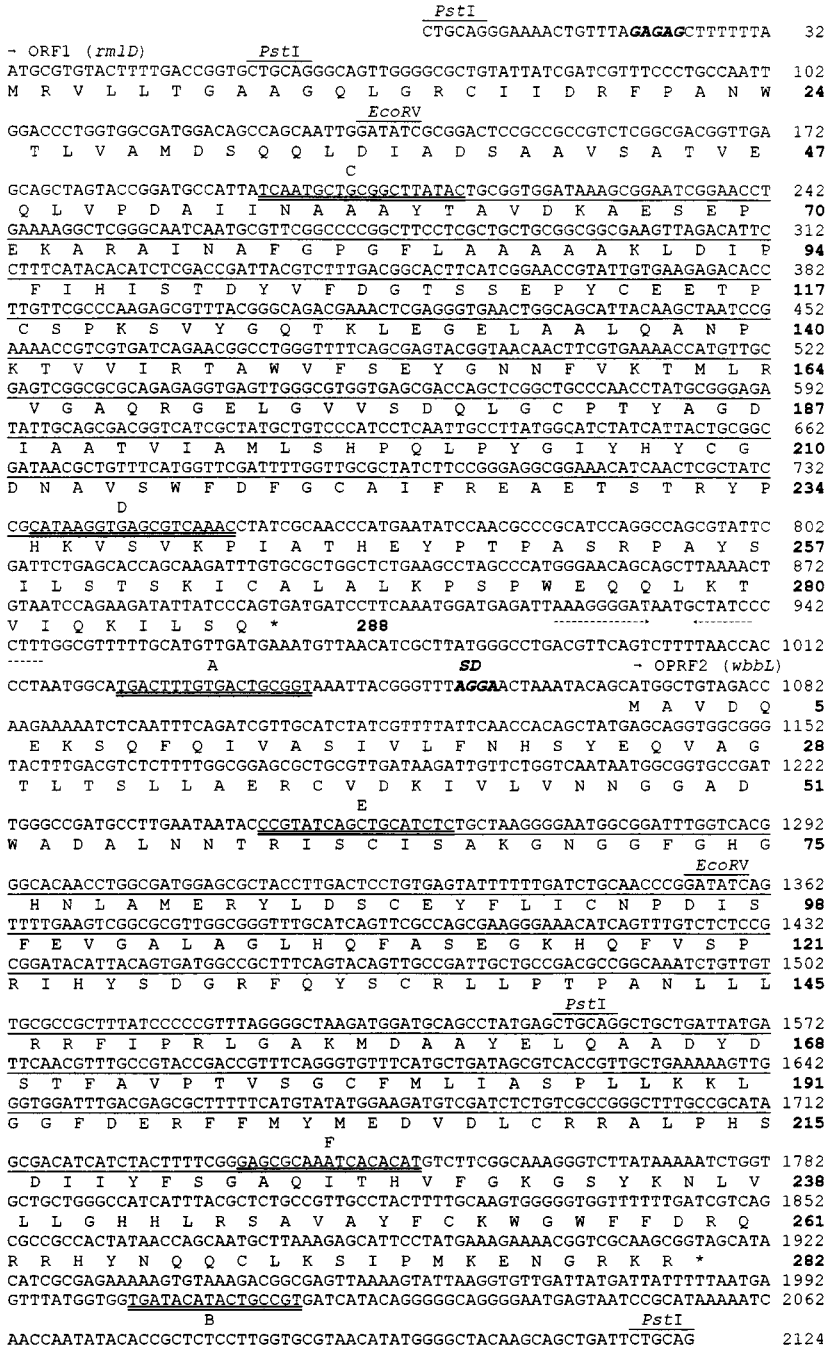

FIG. 2. Nucleotide sequences (GenBank accession no. U82331) and deduced amino acid sequences of the *rmlD* and *wbbL* genes from *S. marcescens* N28b, corresponding to the whole DNA insert in plasmid pJT101. Double-stranded DNA sequencing was performed by using the Sanger dideoxy-chain termination method (26) with the Abi Prism dye terminator cycle sequencing kit (Perkin-Elmer). Primers used for DNA sequencing were purchased from Pharmacia. Oligonucleotides A, C, and E and oligonucleotides B, D, and F (complementary strand) are double underlined. The internal amplified regions of *rmlD* and *wbbL* genes are underlined. Nucleotides defining putative ribosomal binding sites are shown in bold italics.

have been shown to complement the *rfb-50* mutation (39). The above data suggested that *wbbL* from *S. marcescens* N28b could complement the *rfb-50* mutation in the *E. coli* K-12 derivative DH5α and thus should be expected to confer O16-antigen production on *E. coli* DH5α.

The *wbbL* gene complements the *rfb-50* mutation in *E. coli* K-12. To test the above hypothesis, plasmid pJT102, containing only the *wbbL* gene, was constructed. Oligonucleotides A and B (Fig. 2) were used to amplify a 1,069-bp fragment containing the *wbbL* gene. The amplified fragment was ligated to vector pGEM-T (Promega) and transformed into *E. coli* DH5α. LPS extracted from the transformed strain was subjected to SDS-PAGE and was transferred to polyvinylidene fluoride membranes (Millipore Corp.) at 1.3 A for 1 h in the buffer of Towbin et al. (31). The membranes were then incubated sequentially with 1% bovine serum albumin, specific anti-O

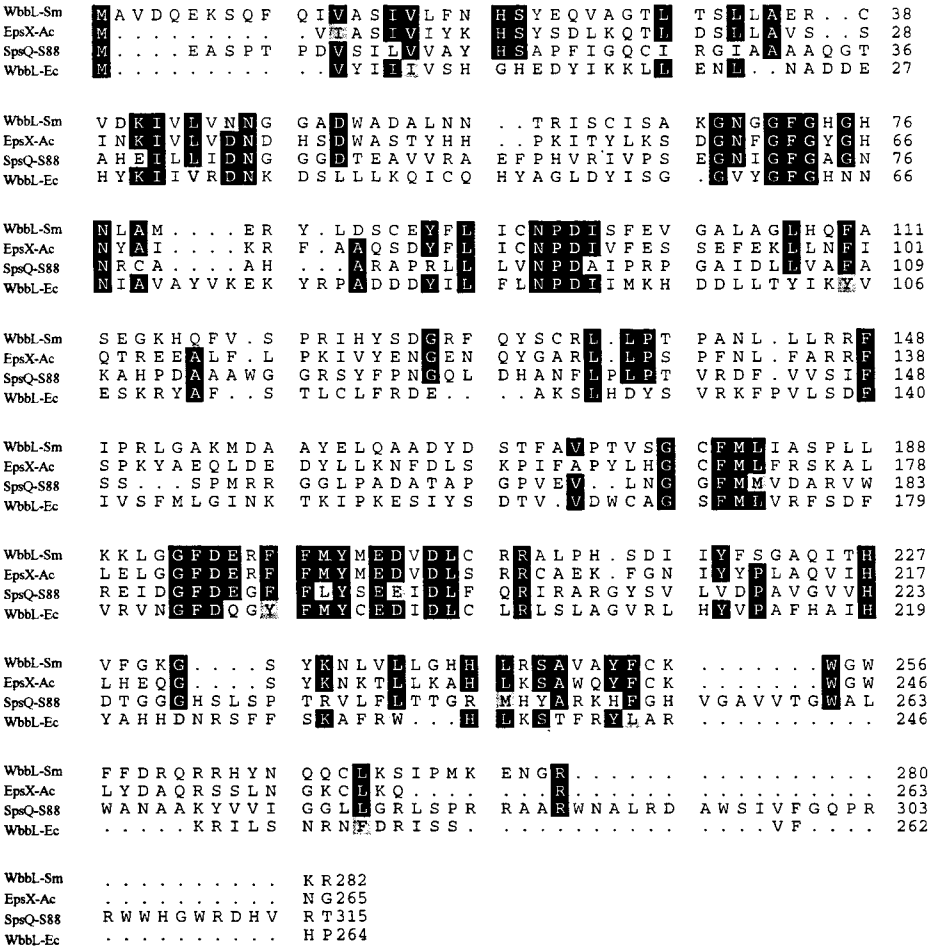

FIG. 3. Amino acid alignment of deduced *wbbL* gene product from *S. marcescens* N28b (WbbL-Sm) with EpsX from *A. calcoaceticus* (GenBank accession no. X81320), SpsQ from *Sphingomonas* strain S88 (38), and WbbL from *E. coli* (WbbL-Ec) (16). Sequences identified by a BLAST (1) search were aligned with the PILEUP program from the Genetics Computer Group package (Madison, Wisconsin) in a VAX 4300. Letters on a solid background represent identical amino acids.

serum (1:500), alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G, and 5-bromo-4-chloro-indolylphosphate disodium-nitroblue tetrazolium. Incubations were carried out for 1 h, and washing steps with 0.05% Tween 20 in phosphate-buffered saline were included after each incubation step. LPS from *E. coli* DH5α harboring pJT102 reacted with *E. coli* O16-specific antiserum (Fig. 1A, lane 7, and Fig. 1B, lane 3) but not with other *E. coli* or *S. marcescens* O-specific antisera (data not shown). The plasmid pJT102 was also able to complement other *E. coli* K-12-derived strains with the *rfb-50* mutation, such as strains C600 or CSH52 (18). The *S. marcescens* N28b O antigen was kindly determined by Hazel M. Aucken and was found to be O4. Comparison of the O-antigen repeating units for *S. marcescens* O4 (20), *E. coli* O16 (28), *S. flexneri* serotypes Y, 1b, 2a, 3a, and 4b (39), and the sphingan S-88 capsule from *Sphingomonas* strain S88 (38) showed that all contain L-rhamnose in the repeating unit, although in both *E. coli* O16 and *S. flexneri* the L-rhamnose is linked by an α1-3 bond to *N*-acetylglucosamine, while in *S. marcescens* O4 the L-rhamnose is linked by an α1-4 bond to D-glucose. Thus, it should be expected that *S. marcescens* N28b would have genes for dTDP-rhamnose synthesis and for rhamnosyltransferase. Complementation of the *rfb-50* mutation in *E. coli* DH5α by the *wbbL* gene suggests that WbbL is a rhamnosyltransferase able to transfer L-rhamnose both to D-glucose via an α1-4 glucosidic bond in *S. marcescens* O4 and to D-*N*-acetylglucosamine via an α1-3 bond when expressed from a multicopy plasmid in *E. coli* DH5α. Alternatively, the WbbL protein from *S. marcescens* could make an α1-4 linkage in *E. coli* DH5α, and the rest of the structure could be completed and polymerized by *E. coli* DH5α *rfb* genes, resulting in a variant of the O16 structure, as previously suggested for *E. coli* K-12 carrying a part of the *E. coli* O4 *rfb* genes (28). Similar results were obtained with low-copy-number plasmids carrying the *wbbL* gene from *S. marcescens* N28b (data not shown).

Three genes from the *S. marcescens* O16 *rfb* cluster have been sequenced, and genes *wzm* and *wzt* (*rfbA* and *rfbB* according to the nomenclature used in reference 29) have been shown to code for ABC-2 type transport system integral membrane protein and ATP-binding protein, respectively (29). The function of the protein encoded by wbbM (*rfbF*) is presently unknown (29). Since *S. marcescens* O16 antigen does not contain L-rhamnose (29), genes homologous to *rmlD* and *wbbL* would probably not be found in the *S. marcescens* O16 *rfb* cluster.

Construction and characterization of *rmlD* and *wbbL* insertion mutations. In enteric bacteria the genes involved in O-antigen biosynthesis are usually found clustered in the so-called *rfb* gene cluster (27); accordingly, the *rmlD* and the *wbbL* genes are probably part of a similar *rfb* cluster involved

in the biosynthesis of the *S. marcescens* O4 antigen. To test this hypothesis, *rmlD* and *wbbL* insertion mutations were constructed in *S. marcescens* N28b by a previously described method (19). Briefly, a 1.5-kb *Sal*I kanamycin resistance (Km^r) cassette obtained from pUC4K (Pharmacia) was inserted into the unique *Sal*I site of pGP704, a *pir*-dependent replication plasmid (19), and transformed into *E. coli* MC1061(λ*pir*) (obtained from J. Barbé) to generate plasmid pFS100. This construction was necessary because *S. marcescens* N28b is resistant to ampicillin (100 μg/ml). Introduction of pFS100 into *S. marcescens* N28b either by transformation or by conjugation yielded no transformants or conjugants, suggesting that this strain does not produce the π protein product of the *pir* gene essential for pGP704 and pFS100 replication. Oligonucleotide pairs C-D and E-F (Fig. 2) were used to amplify internal fragments from the *rmlD* (558 bp) and *wbbL* (505 bp) genes, respectively. Each amplified fragment was ligated to vector pGEM-T (Promega) and transformed into *E. coli* DH5α. Both fragments were recovered by *Sal*I-*Nco*I double digestion and were blunt ended with Klenow fragment; finally, they were ligated to *Eco*RI-digested, blunt-ended, and dephosphorylated pSF100 and transformed into *E. coli* MC1061(λ*pir*) to generate plasmids pJT103 and pJT104. Plasmids pJT103 and pJT104 were isolated, transformed into *E. coli* SM10(λ*pir*) (19), and transferred by conjugation from *E. coli* SM10(λ*pir*) to an *S. marcescens* N28b rifampin-resistant (Rif^r) mutant (from our laboratory collection) as previously described (19). Km^r Rif^r transconjugants arising from pJT103 should contain the mobilized plasmid integrated onto the chromosome by homologous recombination between the *rmlD* gene and the plasmid, leading to two incomplete copies of the *rmlD* gene. Similarly, transconjugants arising from pJT104 should have two incomplete copies of the *wbbL* gene. Chromosomal DNA from 10 transconjugants obtained from each plasmid were analyzed by Southern blot hybridization with appropriate probes to search for true *S. marcescens* N28b Rif^r *rmlD* and *wbbL* insertion mutants (data not shown). Two such mutants, strains N28b-1 (*rmlD*) and N28b-2 (*wbbL*), were found to be unable to produce O antigen when purified LPS was analyzed by SDS-PAGE. These results suggest that the genes *rmlD* and *wbbL* are essential for O4-antigen biosynthesis and that they should be part of an *rfb* region. In *E. coli* the *rfb-50* mutation is found about 9 kb downstream from the *rmlD* gene (27), suggesting that the genetic organization of the two *rfb* clusters is different.

Phenotypic changes conferred by *E. coli* O16-antigen production. *E. coli* O16-antigen production by *E. coli* DH5α harboring the *S. marcescens wbbL* gene either on a multicopy or low-copy-number plasmid confers resistance to serum, bacteriocin 28b, and phages TuIa and TuIb (Table 1). Apparently, all these changes are due to complementation of the *rfb-50* mutation. The WbbL protein is more similar to a reported phosphomannose isomerase from distantly related bacteria, such as *A. calcoaceticus*, than to the putative rhamnosyltransferase of the taxonomically related *E. coli* (Fig. 3), suggesting that rhamnosyltransferases with low overall similarity from different bacteria could complement the *rfb-50* mutation. It is well known that some natural plasmids are able to confer serum resistance on *E. coli* or other gram-negative bacteria, and in some cases the basis for this conversion is not completely known (30). It is tempting to speculate that one possible mechanism for conversion to serum resistance by plasmids could be based on the presence on these plasmids of monosaccharide transferases that are able to complement rough strains (O^-), rendering them O^+, as rhamnosyltransferase is able to complement the *E. coli* K-12 *rfb-50* mutation. The conversion of one rough strain (O^-) into a smooth strain (O^+) may explain the serum resistance phenotype (30).

Bacteriocin 28b, produced by *S. marcescens* strains, is able to kill *E. coli* K-12 and derivative strains (35). We have reported that *E. coli* LPS core and outer membrane proteins OmpA and OmpF are involved in bacteriocin 28b binding (5). *E. coli* K-12 expressing the *S. marcescens wbbL* gene is unable to bind bacteriocin 28b (data not shown). The bacteriocin 28b resistance phenotype of *E. coli* K-12 expressing the *S. marcescens wbbL* gene is probably due to the *E. coli* O16 antigen preventing interaction between the bacteriocin and outer membrane protein receptors. A similar situation may explain the bacteriophage resistance observed on *E. coli* K-12 harboring the *wbbL* gene from *S. marcescens* O4, as has been reported previously for *E. coli* (34).

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