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Mechanism of Bacitracin Resistance in Gram-Negative Bacteria That Synthesize Exopolysaccharides

THOMAS J. POLLOCK,* LINDA THORNE, MOTOHIDE YAMAZAKI, MARCIA J. MIKOLAJCZAK, AND RICHARD W. ARMENTROUT

Shin-Etsu Bio, Inc., San Diego, California 92121

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Four representative species from three genera of gram-negative bacteria that secrete exopolysaccharides acquired resistance to the antibiotic bacitracin by stopping synthesis of the exopolysaccharide. *Xanthomonas campestris*, *Sphingomonas* strains S-88 and NW11, and *Escherichia coli* K-12 secrete xanthan gum, sphingans S-88 and NW11, and colanic acid, respectively. The *gumD* gene in *X. campestris* is required to attach glucose-P to C_{55}-isoprenyl phosphate, the first step in the assembly of xanthan. A recombinant plasmid carrying the *gumD* gene of *X. campestris* restored polysaccharide synthesis to bacitracin-resistant exopolysaccharide-negative mutants of *X. campestris* and *Sphingomonas* strains. Similarly, a newly cloned gene (*spsB*) from strain S-88 restored xanthan synthesis to the same *X. campestris* mutants. However, the intergeneric complementation did not extend to mutants of *E. coli* that were both resistant to bacitracin and nonproducers of colanic acid. The genetic results also suggest mechanisms for assembling the sphingans which have commercial potential as gelling and viscosifying agents.

The bacitracins are a mixture of structurally related cyclic polypeptides with clinically useful antibiotic properties and are produced by certain species of *Bacillus* (13). Bacitracins interfere indirectly with the biosynthesis of bacterial cell walls by inhibiting the dephosphorylation of C_{55}-isoprenyl pyrophosphate (IPP) (30). Bacitracin forms a complex with IPP and a divalent cation and prevents phosphatase from acting on IPP (32–34). Bacteria require the C_{55}-isoprenyl phosphates (IP) as carriers during synthesis of the repeat subunits of peptidoglycan. IPP is released when a repeat subunit, a disaccharide-pentapeptide, is transferred to a growing peptidoglycan chain, but the IPP must be dephosphorylated before reuse. Although binding to IPP is thought to be the primary mode of action, bacitracin may also interfere with additional cellular processes: the actions of certain hydrolytic enzymes (19), the formation of ubiquinone precursors (29), the biosynthesis of membrane-derived oligosaccharides (41), and the involvement of membranes in cell division (26).

One mechanism of resistance to bacitracin seems to result from increased de novo synthesis of IP. Cain et al. (3) have recently shown that increased intracellular levels of a lipid kinase, the product of the *bacA* gene of *Escherichia coli*, confers resistance to bacitracin. The kinase appears to increase the level of the carrier IP by phosphorylating isoprenyl alcohol, thereby circumventing the sequestration of IPP by bacitracin. Sutherland (35) had earlier suggested that resistance to bacitracin in *Klebsiella* species might be due to an abundance of isoprenoid lipids; however, actual quantitation of the lipids was incomplete. Fiedler and Rotering (7) described mutants of *E. coli* that display several phenotypes during growth under conditions of low osmolarity: partial resistance to bacitracin, failure to synthesize membrane-derived oligosaccharides, increased production of capsular polysaccharides, reduced motility, and decreased amounts of the OmpF outer membrane protein.

* Corresponding author. Mailing address: Shin-Etsu Bio, Inc., 6650 Lusk Blvd., Suite B106, San Diego, CA 92121. Phone: (619) 455-8500. Fax: (619) 587-2716.

In addition to the essential requirement to synthesize cell walls, many growing bacteria also secrete either cell-associated capsular polysaccharides or unattached exopolysaccharides. The structures of four exopolysaccharides relevant to our study are shown in Fig. 1. Colanic acid is a capsular polysaccharide secreted by certain strains of *E. coli* and other enteric bacteria (20). It confers a mucoid appearance to colonies growing on agar at low temperature. The assembly of colanic acid is initiated by attaching glucose-P to IP (14). Xanthan gum is an exopolysaccharide secreted by *Xanthomonas campestris* and is produced commercially in large quantities for use as an aqueous viscosifier (16). A similar analysis of lipid-linked oligosaccharides for *X. campestris* indicated that the first step in xanthan synthesis is the transfer of glucose-P from UDP-Glc to IP to form Glc-PPI (11, 12). The other two exopolysaccharides, S-88 and NW11, are members of a group of structurally related polysaccharides (22) called sphingans and are secreted by members of the genus *Sphingomonas* (24). The sphingans share the glucose-glucuronic acid-glucose sequence in the backbone of the polymer chain but diverge at the L-mannose (or L-rhamnose) position and have different side chains. The order of attachment of sugars to the IP carrier is unknown for the sphingans.

We previously reported that among bacitracin-resistant (Bac^r) mutants of *X. campestris* the majority lost the ability to secrete xanthan gum (21). We thought that in the absence of xanthan synthesis these mutants might tolerate bacitracin because the remaining unbound IPP would be free to support essential peptidoglycan synthesis. In the present work, we demonstrate that a similar mechanism of Bac^r is present in three different genera of gram-negative bacteria that secrete polysaccharides. We found that many of the mutations conferring Bac^r eliminated synthesis of the corresponding exopolysaccharide. The defects in exopolysaccharide synthesis for *Sphingomonas* strains S-88 and NW11 were overcome by introduction of a normal *gumD* gene of *X. campestris* on a recombinant plasmid. Likewise, Bac^r $GumD^-$ strains of *X. campestris* were complemented by a related gene cloned from *Sphingomonas* strain S-88 (42).

The *gumD* gene is required to form Glc-PPI (39). However,

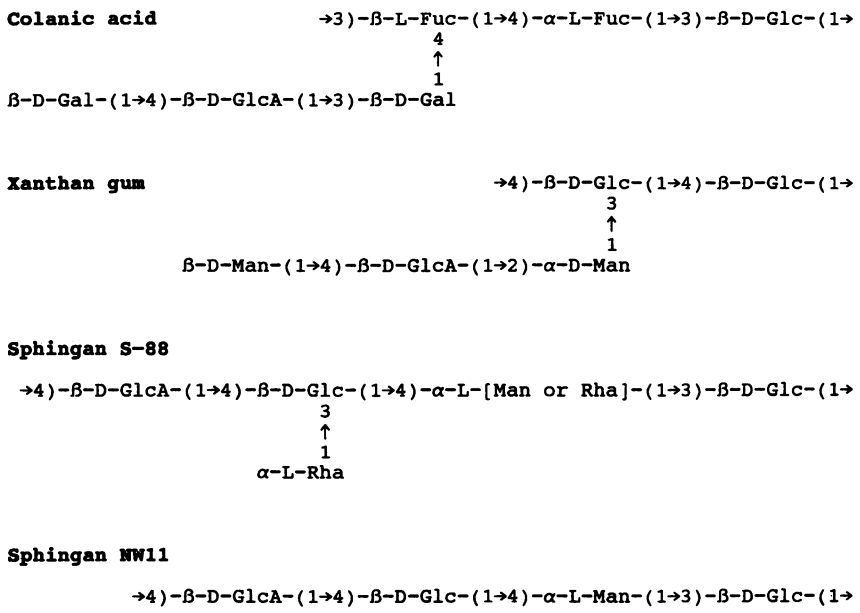

FIG. 1. Primary repeating subunit structures of colanic acid, xanthan gum, and sphingans S-88 and NW11. Abbreviations (all pyranosides): Fuc, fucose; Glc, glucose; Gal, galactose; GlcA, glucuronic acid; Man, mannose; Rha, rhamnose. In one position, sphingan S-88 has either L-mannose or L-rhamnose in equal amounts and is assumed to be a single chain with a random distribution of Man and Rha. Acetyl and pyruvyl groups are not shown. Before polymerization of colanic acid or xanthan gum, each repeat subunit has IPP attached at the reducing end (right).

as yet there is no direct evidence that the product of the *gumD* gene is itself a glucosyl-IP transferase. It is formally possible that *gumD* serves an essential accessory function for the real transferase. The amino acid sequence of the *gumD* open reading frame as deduced from the nucleotide sequence is significantly homologous to members of a family of related genes including *rfbP* of *Salmonella enterica* (23), *exoY* of *Rhizobium meliloti* (25), *pss2* of *Rhizobium leguminosarum* (1), and *cpsD* of *Streptococcus* group B (28). Our working hypothesis is that these related genes actually code for glycosyl-IP transferases, specific for either glucose or galactose.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains, plasmids, and bacteriophage are described and referenced in Table 1.

Culture media. Luria-Bertani (LB) medium contained 10 g of Bacto Tryptone (Difco), 5 g of Bacto Yeast Extract (Difco) and 10 g of NaCl per liter of water. YM medium contained 3 g of Bacto Yeast Extract, 3 g of Bacto Malt Extract, 5 g of Bacto Peptone (Difco), and 10 g of D-glucose (Difco) per liter of water. YT medium contained 8 g of Bacto Tryptone, 5 g of Bacto Yeast Extract, and 5 g of NaCl per liter of water. M9 salts (4×) contained 24 g of Na_2HPO_4, 12 g of KH_2PO_4, 2 g of NaCl, 4 g of NH_4Cl, 250 mg of $MgSO_4$-$7H_2O$, and 14 mg of $CaCl_2$-$2H_2O$ per liter of water. M9CGM medium was 1× M9 salts plus 0.1% (wt/vol) Casamino Acids (Difco), 0.2% D-glucose, and 0.2% D-mannose (Sigma). For certain cultures, individual L-amino acids were added to 40 μg/ml, the Casamino Acids were omitted, and vitamins were added to 1 μg/ml. Solid media contained agar (Difco) at 15 g/liter. Antibiotics from Sigma were added to media as follows: bacitracin, 73 U/mg, as specified in the range 0.01 to 8 mg/ml; rifampin, 50 μg/ml; streptomycin, 25 to 100 μg/ml; kanamycin, 25 μg/ml; tetracycline, 6 to 12 μg/ml; and chloramphenicol, 25 μg/ml.

Gene transfer and mutagenesis. Genetic mapping by Hfr conjugation in *E. coli* was as described by Low (18) with some modifications. Donor and recipient cultures were grown overnight in LB medium plus selective antibiotics and then diluted about 50-fold in LB medium and grown at 37°C to about 2×10^8 cells per ml. Donors (2 ml) and recipients (0.5 ml) were mixed in glass tubes and gently rocked for 20 min at 37°C. Samples were then diluted, vortexed vigorously, and spread at 30°C on M9CGM plates containing tetracycline at 10 μg/ml and streptomycin at 100 μg/ml. The mucoid and nonmucoid colonies among the streptomycin-resistant (Stm^r) tetracycline-resistant (Tet^r) recombinants were counted and tested for kanamycin resistance (Kan^r). As expected, the mucoid recombinants were Kan^s and the nonmucoid colonies were Kan^r.

Plasmid DNA was transferred from *E. coli* to *X. campestris* or *Sphingomonas* strains by triparental conjugation (6). Mixtures of equal numbers of donor, helper, and recipient cells were spotted onto nonselective LB plates and incubated for 6 to 16 h at 30°C. Exconjugants were isolated by spreading a loopful of the mating mixture onto plates containing rifampin (50 μg/ml) to select against the helper and donor cells and tetracycline (6 μg/ml) to select for the plasmid. Polysaccharide-producing exconjugants were identified as visibly mucoid. Plasmid DNA was transferred from *X. campestris* or *Sphingomonas* strains to *E. coli* by transformation of rubidium-treated cells (10) and selection for Tet^r. Construction of the library of genes from *X. campestris* in cosmid vector pRK311 in *E. coli* JM109 was described previously (36).

Insertion mutagenesis was with Tn*10* derivative 103 (mini-Tn*10 kan/Ptac*-ATS transposase) carried by λNK1316 (17), a bacteriophage that fails to replicate in the presence of the *supE44* cellular defect.

Quantitative and qualitative analysis of polysaccharides. Extracellular xanthan from *X. campestris* and polysaccharides S-88 and NW11 from *Sphingomonas* strains were separated from culture media by precipitation with 2 to 3 volumes of isopropyl alcohol. Colanic acid was prepared by growing of *E. coli* TP11 on M9CGM agar plates at 30°C, vortexing of the viscous biomass in 2 to 4 volumes of 150 mM NaCl, centrifu-

TABLE 1. Bacterial strains, plasmids, and bacteriophage

Bacterial strain(s), plasmid, or phage	Genotype or phenotype	Source (reference)[a]
X. campestris		
B1459S-4L	Rif^s Gum^+	NCAUR (2)
X59 (ATCC 55298)	Rif^r Gum^+	These authors (36)
X50 (ATCC 55429)	X59 Gum^+ Bac^r	These authors (21)
m31, m65, m96	X59 Gum^- Bac^r	These authors (36)
m45, m48, m82	X59 Gum^- Bac^s	These authors (36)
s1A, s1B, s1C, s2A, s2B, s2C, s3A, s3B, s3C, s4A, s4B, s4C, s5A, s5B, s5C	X59 Gum^- Bac^r	This work
Sphingomonas strains		
S-88 (ATCC 31554)	Stm^r Eps^+ Bac^s	ATCC (15)
m257, m258, m259, m260, bac8, bac9, bac10	S-88 Eps^- Bac^r	This work
NW11 (ATCC 53272)	Stm^r Eps^+ Bac^s	ATCC (27)
bac3-1, bac3-3	NW11 Eps^- Bac^r	This work
E. coli K-12		
SG20250	F^- *araD139* Δ(*argF-lac*)*U169 flbB5301 deoC1 relA1 rpsL150*(Stm^r)	S. Gottesman (9)
SG20274	F^- *araD139* Δ(*argF-lac*)*U169 flbB5301 deoC1 relA1 rpsL150*(Stm^r) Δ*lon-100 manA274*::MudI	S. Gottesman (38)
TP11	SG20274 Rif^r	This work
TP416–TP419	TP11 Bac^r Cps^-	This work
TP429–TP431	TP11 Kan^r (mini-Tn*10*) Bac^r Cps^-	This work
TP432, TP442, TP452	TP11 Kan^r (mini-Tn*10*) Bac^s Cps^-	This work
BW6163	Hfr (PO45: Hfr KL16) λ^- *relA1 spoT1 thi-1 zed-977*::Tn*10*	CGSC6758 (40)
BW5660	Hfr (PO66: Hfr PK19) λ^- *relA1*? *spoT1*? *thi-1 supE44* Δ(*gpt-lac*)*5 srlC300*::Tn*10*	CGSC6753 (40)
BW5659	Hfr (PO53: Hfr KL98) λ^- *lacY1 mglP1 xyl-7 zdh-57*::Tn*10*	CGSC6752 (40)
BW7261	Hfr (PO2A: Cavalli) *relA1 spoT1 ompF627* Δ(*argF-lac*)*U169 tonA22* $T2^r$ *leu-63*::Tn*10*	CGSC6787 (40)
JM109	*recA1 endA1 gyrA96 thi-1 relA1 supE44 hsdR17*(r_K^- m_K^+) Δ(*lac-proAB*) [F′ *traD36 proAB* $lacI^qZ$ΔM15]	Bethesda Research Laboratories
DH5α	F^- ϕ80d*lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 relA1 supE44 hsdR17*(r_K^- mK^+) Δ(*lacZYA-argF*)*U169*	Bethesda Research Laboratories
SA658	HfrH (PO1: Hayes) *relA1 spoT1 thi-1 galU106*	S. Adhya
CGSC259	HfrH (PO1: Hayes) *relA1 spoT1 thi-1* λ^-	CGSC
Plasmids		
pRK2013	*ori*(ColE1) Kan^r *oriT*(Mob^+) Tra^+	D. Helinski (8)
pRK311	*oriV*(RK2) Tet^r *oriT*(Mob^+) Tra^- λ*cos lacZ*(α)	D. Helinski (5)
pRK311c8	*gumABCDEFGHIJKLMN* (from X59)	These authors (36)
pRK311c8A	*gum′GHIJKLMN* (c8 subclone)	This work
pRK311-1483	*gumDEFG′* (c8 subclone)	This work
pSEB19	*gumDE′* (c8 subclone in pRK311)	This work
pSEB32	*gumABCD*(Cam^r; orientation 1)*EFG* with *ori*(pBR322) and *oriT*	This work
pSEB33	*gumABCD*(Cam^r; orientation 2)*EFG* with *ori*(pBR322) and *oriT*	This work
pRK311-S88c1	*spsB* (within 30-kb S-88 insert)	This work
Bacteriophage λ NK1316	λ *b522*(Δ*attP*) *c*I857 *P*am80 *nin5* mini-Tn*10 kan*/*Ptac*-ATS transposase	N. Kleckner (17)

[a] NCAUR, Midwest Area National Center for Agricultural Utilization Research, U.S. Department of Agriculture, Peoria, Ill.; ATCC, American Type Culture Collection, Rockville, Md.; CGSC, *E. coli* Genetic Stock Center, Department of Biology, Yale University, New Haven, Conn.

gation of the cells, and precipitation of the polysaccharides from the supernatant with 3 volumes of isopropyl alcohol. The precipitates were collected by centrifugation, dried at 80°C, and weighed.

Polysaccharides were hydrolyzed with acid, and monosaccharides were separated by thin-layer chromatography. Dried polysaccharides were resuspended in high-performance liquid chromatography (HPLC) water (Baker) at 5 mg/ml. Anhydrous trifluoroacetic acid (88 μl; Sigma) was mixed with 75 μl of HPLC water (Baker) and 225 μl of polysaccharide (5 mg/ml) in a 0.6-ml snap-cap polypropylene microcentrifuge tube and incubated at 95°C for 16 h. The hydrolysates were dried under vacuum, resuspended in 200 μl of HPLC water, placed in a new microcentrifuge tube, dried again, and resuspended in 45 μl of HPLC water. The samples (25 μg/ml) could be stored frozen. Sugar standards (D-glucose, D-glucuronic acid, D-mannose, L-mannose, L-rhamnose, and L-fucose) were resuspended in HPLC water at 4 mg/ml. Precoated, channelled, silica gel chromatography plates (Kieselgel 60 CF 254, 10 by 20 cm; E. Merck) were soaked overnight in 0.3 M NaH_2PO_4 and dried for 30 min at room temperature and then for 10 min at 95°C. Samples of 1 to 2 μl were spotted, and the chromatogram was exposed to a rising solvent mixture of 40 ml of acetone, 5 ml of butanol, and 5 ml of deionized water for 2.5 to 3 h at room temperature. The plate was dried at 65°C for 3 min and then stained by being dipped in a solution of 25 ml of

TABLE 2. Complementation of Gum$^-$ mutants of *X. campestris* by plasmids

Group[a]	Mutant	Result with[b]: pSEB19	pRK311-1483			pRK311c8			pRK311c8A		
			Expt. no. 1	Expt. no. 2	Expt. no. 3	Expt. no. 1	Expt. no. 2	Expt. no. 3	Expt. no. 1	Expt. no. 2	Expt. no. 3
1	m31	+	+			+			−		
	m65	+	+			+			−		
	m96	+	+			+			−		
	m8	+	+			+			−		
	m45	−	−			+			+		
s1	A	NT	−	−	−	+	+	−	−	−	−
	B	+	+	+	+	+	+	+	−	−	−
	C	NT	+			+			−		
s2	A	NT	+			+			−		
	B	NT	+			+			+		
	C	NT	+	+	+	+	+	+	−	−	+
s3	A	NT	+	+	+	+	+	+	−	+	−
	B	NT	−	−	−	+	+	−	−	−	−
	C	NT	+			+			−		
s4	A	NT	+	−	+	+	+	+	−	−	−
	B	NT	−	−	−	+	+	−	−	−	−
	C	NT	+			+			−		
s5	A	NT	+	−	+	+	+	+	−	−	−
	B	NT	+			+			+		
	C	NT	−	+	+	−	+	+	−	−	−

[a] Each group of mutants was derived from a different parental colony of strain X59 (Rif^r). Although mutants m31, m65, m96, and m8 are not necessarily independent, m8 is phenotypically distinct from the other three (36).

[b] Three independent experiments are indicated by columns labeled 1, 2, and 3. For example, all of the results under the three columns labeled 1 were obtained from the same donor and recipient cultures. Each plasmid was transferred by conjugation to the mutants, and colonies of Rif^r Tet^r exconjugants were judged by eye for mucoidy: +, mucoid; −, nonmucoid; NT, not tested.

acetone, 0.5 ml of aniline, 0.5 g of diphenylamine, and 3.75 ml of phosphoric acid and then dried for 30 min at 95°C.

RESULTS

Isolation and mapping of Bac^r Gum$^-$ mutants of *X. campestris*. We tested previously isolated Gum$^-$ mutants of strain X59 (36) for the Bac^r phenotype. Four Gum$^-$ mutants (m31, m65, m96, and m8) were Bac^r at 100 μg/ml in YM medium. The parent strain, X59, grows very poorly at bacitracin concentrations as low as 20 μg/ml. Complementation tests (Table 2) indicated that xanthan synthesis was restored for these four mutants when they contained a plasmid carrying a cloned copy of the *gumD* gene. Figure 2 shows the genetic maps of the cloned *gum* genes carried by the complementing plasmids. The *gum* genes are arranged and expressed in alphabetical order *A* to *N* (39). The *gumDE'* sequence in plasmid pSEB19 contains 180 bases of intercistronic DNA immediately upstream from *gumD*, all of *gumD*, and only about 10% of *gumE*. The intercistronic region probably contains a promoter for expressing *gumD*, since *gumD* was expressed in the complementation tests irrespective of the orientation of flanking plasmid sequences (results not shown) and since possible transcriptional termination sequences were identified in the intercistronic region (4).

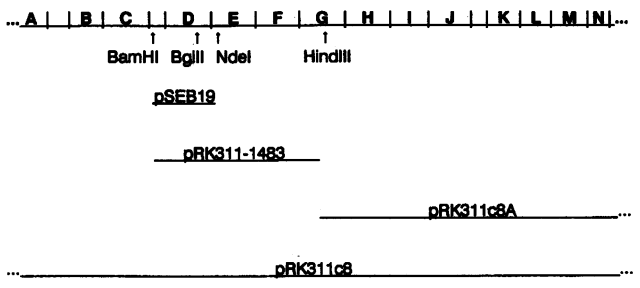

FIG. 2. Plasmids containing *gum* genes of *X. campestris*. Key restriction sites and the extents of cloned DNA are indicated. The nucleotide sequence of the *gum* gene cluster was determined by Capage et al. (4).

By comparison, three Gum$^-$ mutants (m45, *gumK*; m48, *gumHIJ*; and m82, with an unknown gene not in the *gum* cluster) were Bac^s and failed to grow with bacitracin at 100 μg/ml. Mutants m45 and m48 were complemented by pRK311c8 and pRK311c8A, and mutant m82 was complemented by an unlinked cloned segment (pRK311c9) described elsewhere (36). The *gumK* gene codes for transferase IV, which transfers glucuronic acid to Man-Glc-Glc-PPI (39). The *gum HIJ* genes code, respectively, for transferase III, which adds mannose to Glc-Glc-PPI; transferase V, which adds mannose to GlcA-Man-Glc-Glc-PPI; and an unknown function (39).

We then reversed the order of screening by first selecting for Bac^r colonies and then mapped the Gum$^-$ defects by complementation. The starting strain for isolating Bac^r Gum$^-$ mutants of *X. campestris* was X59, a spontaneous rifampin-resistant (Rif^r) derivative of B1459S-4L. As described in an earlier report (21), spontaneous Bac^r mutants of X59 were selected from YT plates containing bacitracin at 500 μg/ml. The frequency of Bac^r colonies was about 10^{-5}, and about three-fourths of the Bac^r mutants of strain X59 were also Gum$^-$. These mutants were nonmucoid and lacked a shiny surface. The other one-fourth were mucoid in appearance, like X59. We do not know whether the relatively high frequency of Bac^r colonies resulted from a high intrinsic mutation rate for *X. campestris* or whether it was due to a growth advantage for the Gum$^-$ cells.

A random sample of 15 Bac^r Gum$^-$ mutants originating from five independent colonies of X59 were mapped by

complementation with DNA segments cloned from the *gum* gene cluster of *X. campestris*. The cloned segments are diagrammed in Fig. 2, and the complementation results are given in Table 2. Ten of the fifteen Bac^r Gum$^-$ mutants (s1B, s1C, s2A, s2C, s3A, s3C, s4A, s4C, s5A, and s5C) were restored to Gum$^+$ mucoidy by plasmids pSEB19, pRK311-1483, and pRK311c8. One of the mutants, s1B, was also restored to mucoidy by plasmid pSEB19, which contains only the *gumD* gene. However, as indicated in Table 2, caution is necessary in interpreting individual complementation tests. A positive result is meaningful unless two nonoverlapping segments both show restoration of mucoidy. For example, two mutants (s2B and s5B) became mucoid after apparently receiving either of the nonoverlapping cloned segments in plasmids pRK311-1483 and pRK311c8A. The mucoidy of these two mutants probably resulted from reversion of the original mutation, since we could not find the intact donor plasmid in the exconjugants. Similarly, single negative results among repeated positive trials are usually due to large deletions arising in the complementing segment. Mutants s1A, s3B, and s4B were complemented by pRK311c8 which carries the entire *gum* gene cluster but not by either of the subfragments. It is possible that these mutations are within the *gumBC* region. The functions of *gumB* and *gumC* are unknown, but they are believed to act at an early step like *gumD* (39). None of the Bac^r Gum$^-$ mutations were complemented solely by pRK311c8A; however, the plasmid did complement a Gum$^-$ Bac^s mutation in *gumK* (m45). We determined that the plasmids were required for the phenotypic changes by isolating Gum$^-$ segregants from the Gum$^+$ exconjugants for pRK311-1483 and testing for the plasmid trait Tet^r. All of the Gum$^-$ segregants were Tet^s.

We confirmed the complementation results and the mapping interpretation by showing that a specific chromosomal mutation in *gumD* caused by insertion of a drug resistance gene simultaneously produced the Bac^r and Gum$^-$ phenotypes. A chloramphenicol resistance (Cam^r) gene originally from pACYC184 was removed from mini-Tn*10* *cam*/*Ptac*-ATS transposase on a *Bam*HI restriction fragment and inserted into the *Bgl*II site located within the *gumD* sequence (Fig. 2) in plasmids pSEB32 and pSEB33. The mutated *gumD* gene was flanked by normal *gumABC* (about 4.5 kb) and *gumEFG* (about 3.5 kb) sequences and was carried on a narrow-host-range matable plasmid (pSEB32 or -33) which carried the origin of replication of plasmid pBR322. Each of the plasmids had a selectable Kan^r gene outside of the *gum* cluster and an *oriT* sequence for conjugation. The plasmids were transferred by triparental mating into *X. campestris* X59 (Gum$^+$), and Cam^r Gum$^-$ or Gum$^+$ exconjugants were isolated. All of the Gum$^+$ exconjugants were Kan^r, as if the entire plasmid was inserted into the chromosome by a single recombination event. Integration into the chromosome of the entire plasmid with the Kan^r gene would create a partial duplication (...ABCD-Cam^r-EFG...plasmid-Kan^r...ABCDEFGHIJKLM...) with one normal and one mutant *gumD* gene. By contrast, all of the Gum$^-$ exconjugants were Kan^s, as expected for a double recombination leading to replacement of the normal chromosomal gene with the mutated plasmid copy (...ABCD-Cam^r-EFGHIJK LM...). The Gum$^-$ exconjugants were Bac^r, while the Gum$^+$ isolates were Bac^s. The most straightforward interpretation is that specific inactivation of the *gumD* gene by insertion of a Cam^r gene can cause Bac^r in *X. campestris*.

Growth yields for *X. campestris* in the presence of bacitracin. If the dual phenotypes were the result of a single mutation, then we expected to also observe restored sensitivity to bacitracin when multiple copies of the wild-type *gumD* gene were introduced into the Bac^r Gum$^-$ mutants. We measured cell densities of cultures grown with different concentrations of bacitracin in YM medium. As shown in Fig. 3, the growth of wild-type strain X59 (Bac^s Gum$^+$) was very sensitive to bacitracin compared with that of the *gumD* mutant X59-s1B (Bac^r Gum$^-$). When plasmid pRK311c8, containing the *gumABCDEFGHIJKLMN* genes, was added to the X59-s1B cells, they became as sensitive to bacitracin as wild-type X59. Nearly identical results were obtained with four other Bac^r Gum$^-$ *gumD* mutants (s5A, s5C, s3A, and s4A) and plasmid pRK311-1483, which carries the *gumDEFG'* genes (data not shown). In each case, addition of pRK311-1483 restored sensitivity to bacitracin and allowed the production of xanthan gum. Figure 3 also shows the resistance of strain X50 to bacitracin. As for the Bac^r Gum$^-$ mutants, strain X50 was derived from X59 by selection on plates containing bacitracin. However, X50 makes even more xanthan gum than X59 (21). This indicates a second mechanism for Bac^r that does not involve *gumD*.

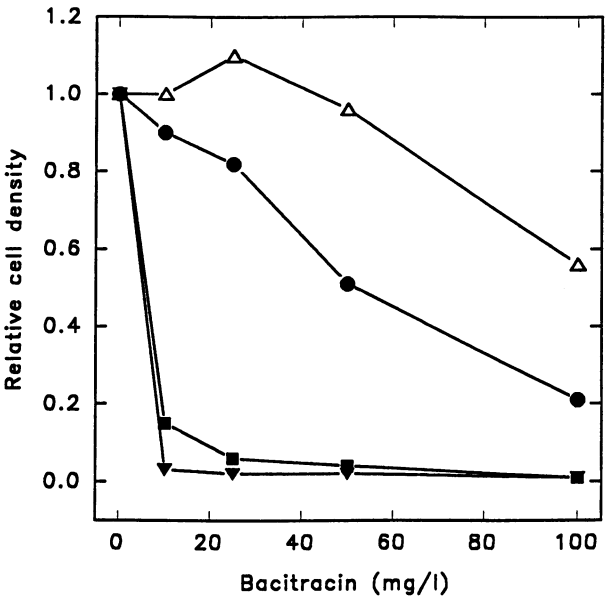

FIG. 3. Effect of bacitracin on cell yield for *X. campestris*. Cell densities were measured (A_{600}) for cultures grown for 24 h at 30°C with shaking in flasks containing YM medium with 2% (wt/vol) glucose. Symbols: ■, parental strain X59 (Bac^s Gum$^+$); ●, X59-s1B (Bac^r Gum$^-$); ▼, X59-s1B-pRK311c8 (Bac^s Gum$^+$); △, X50 (X59 Bac^r Gum$^+$).

Isolation and mapping of Bac^r Cps$^-$ mutants of *E. coli*. The starting strain for isolation of Bac^r Cps$^-$ mutants of *E. coli* K-12 was TP11, which is a Rif^r mutant of SG20274 that arose spontaneously on a nutrient plate containing rifampin (80 μg/ml). Due to the *lon-100* mutation, strain SG20274 is very mucoid when grown on M9CGM agar plates at 30°C. Because of the *manA* mutation, SG20274 cannot grow on D-mannose as the sole carbon source and requires D-mannose for synthesis of colanic acid. We confirmed that the exopolysaccharide was colanic acid by showing that acid hydrolysates of the alcohol-precipitated material contained only glucose, galactose, fucose, and glucuronic acid (chromatogram not shown). TP11 cells were spread on M9CGM plates containing bacitracin at 4 to 5 mg/ml and incubated at 30°C. The spontaneous frequency of Bac^r colonies was about 1 to 10 per 10^6 cells. On the initial selection plates, about 25 to 60% of the Bac^r colonies were nonmucoid for independent samples of TP11. About 5 to 25%

TABLE 3. A_{600} for *E. coli* cultures grown with bacitracin

	Expt and strain	Medium[a]	Temp (°C)	A_{600} with indicated concn of bacitracin (mg/ml)			
				0	2	4	6
1							
	TP11	Glc, Man	30	0.6	0.4	0.1	0.0
			37	0.6	0.4	0.2	0.1
		Glc	30	0.5	0.7	0.4	0.1
			37	0.4	0.6	0.6	0.4
	TP416	Glc, Man	30	0.8	0.7	0.7	0.5
2							
	SG20250 (Lon$^+$)	Glc, Man	30	1.3	1.1	1.1	1.0
	SG20274 (Lon$^-$)			1.1	0.6	0.2	0.1
	CGSC259 (GalU$^+$)	Glc		1.2	1.0	1.1	1.0
	SA658 (GalU$^-$)	Glc		0.9	0.0	0.0	0.0
3							
	TP416	Glc, Man	30	0.7	0.5	0.4	
	TP416(pRK311-1483)[b]			0.5	0.3	0.2	
	TP416(pRK311-1483)			0.5	0.4	0.2	

[a] Supplements were added to 1× M9 minimal salts with 0.1% Casamino Acids as follows: 0.2% D-glucose (Glc); 0.2% D-galactose; 0.2% D-mannose (Man); 1 μg of thiamine per ml in experiment 2. The culture times were 8 h at 30°C or 4 h at 37°C for experiments 1 and 3 and 16 h for experiment 2. Inocula were equal numbers of cells from overnight cultures adapted to M9CGM medium (with thiamine at 1 μg/ml for experiment 2). Absorbance values are averages for two cultures.

[b] Two isolates of TP416(pRK311-1483) were tested.

were copiously mucoid like the parent TP11, and the remainder were slightly mucoid and of many different sizes, but they were not studied further. Independent mutants (TP416-419) were obtained by culturing single colonies of TP11 before selecting a single Bac^r Cps$^-$ mutant originating from each culture. The absence of a colanic acid capsule was verified gravimetrically by scraping of plates seeded with each mutant, removal of cells and cell-bound material by centrifugation, and then precipitation of cell-free polysaccharides with three volumes of isopropyl alcohol. The mutants remained Rif^r Stm^r and filamentous like the parent strain TP11. When we conditionally eliminated the synthesis of GDP-fucose by removing mannose from the selection medium, we still recovered Bac^r Cps$^-$ mutants. Thus, the concurrent synthesis of GDP-fucose in *E. coli* was not required during the selection of Bac^r Cps$^-$ mutants.

Experiment 1 in Table 3 shows that in contrast to its parent, TP11 (Lon$^-$ Cps$^+$), strain TP416 (Bac^r Cps$^-$) grew even in high concentrations of bacitracin. The sensitivity to bacitracin for TP11 was partially reduced by culturing at 37°C or by removal of D-mannose, two conditions that would be expected to reduce colanic acid production (20). Strain TP11 is a Rif^r derivative of SG20274 (Lon$^-$ Cps$^+$), and experiment 2 indicates that the Lon$^-$ mutation in SG20274 confers sensitivity to bacitracin. An essentially isogenic Lon$^+$ strain, SG20250, was Bac^r. Experiment 2 also shows the detrimental effect of a *galU* mutation on growth with bacitracin. The GalU$^-$ strain is unable to convert glucose-P to UDP-glucose and unable to assemble a complete core oligosaccharide attached to lipopolysaccharide. Consequently, the GalU$^-$ strain has a rough colonial appearance when growing on glucose. The GalU$^-$ strain (SA658) was very sensitive to bacitracin, unlike the essentially isogenic GalU$^+$ strain CGSC259. Experiment 3 shows that when a multicopy plasmid carrying the *gumD* gene of *X. campestris* is conjugally transferred to strain TP416 the exconjugants do not become as sensitive to bacitracin as TP11, the parent of TP416. This suggests that the *gumD* gene is not functioning in *E. coli*. As described later, we could also not detect restoration of synthesis of colanic acid in complementation tests.

Analogous Bac^r Cps$^-$ Kan^r mutants were derived from TP11 by mutagenesis with transposon mini-Tn*10 kan*. Kan^r derivatives were spread on M9CGM plates at 30°C to observe mucoidy, and 21 Cps$^-$ mutants were found among about 5,000 colonies. One of these (TP429) was Bac^r and able to grow on M9CGM plates containing bacitracin at 5 mg/ml. An independent colony of TP11 was also mutagenized with mini-Tn*10 kan*, and in this case two Bac^r mutants (TP430 and 431) were obtained from among 14 Kan^r Cps$^-$ colonies. One can reverse the order of selection by spreading a mutagenized TP11 culture on plates containing kanamycin and bacitracin and then observe mucoidy of the Kan^r Bac^r mutants on M9CGM plates. In this case, five of nine Kan^r Bac^r mutants were Cps$^-$. Thus, not all Cps$^-$ mutants are Bac^r and vice versa. The generation of the Bac^r Cps$^-$ Kan^r insertion mutants indicated that a single mutation can lead to the dual phenotype in *E. coli* as in other exopolysaccharide producers.

We located the Bac^r Cps$^-$ mutations on the genetic map of *E. coli*. The results of Hfr mapping with one of the spontaneous Bac^r Cps$^-$ mutants (TP416) and three mini-Tn*10 kan*-induced Bac^r Cps$^-$ mutants (TP429, -430, and -431) were consistent with close linkage to *xed*::Tn*10* and *his*. There are two clusters of genes involved in polysaccharide synthesis that map in this region: *cpsABCDEG* and *rfbABD*. The percentages of Kan^s Cps$^+$ colonies among the Stm^r Tet^r recombinants were 60 to 70% for Hfr strain BW6163 (origin of transfer at 60 min and Tn*10* at 43 min), 40 to 50% for strain BW5659 (origin at 48 min and Tn*10* at 37 min), 30 to 40% for strain BW5660 (origin at 42 min and Tn*10* at 58 min), and 0% for strain BW7261 (origin at 13 min and Tn*10* at 2 min).

Isolation of Bac^r sphingan polysaccharide-negative (Sps$^-$) mutants of *Sphingomonas* strains. The starting strains for isolation of spontaneous Bac^r *Sphingomonas* mutants were S-88 and NW11. After determining a suitable inhibitory concentration of bacitracin, we spread about 6×10^7 S-88 cells on YM plates containing bacitracin at 500 to 800 μg/ml. We obtained 56 Bac^r mutants of S-88, of which 50 were visably Sps$^-$ and produced no S-88 exopolysaccharide in liquid cultures, as seen by an absence of isopropyl alcohol-precipitable material in the cell-free culture broths. Another set of Bac^r mutants of S-88 were initially observed to be Sps$^-$ mutants and then subsequently shown to be Bac^r. The failure to make exopolysaccharides in the S-88 mutants results in a colony appearance that is less translucent when the culture plate is held up to light and viewed from below. The wild-type colonies appear to have a narrow light-refracting halo when viewed from below. However, the wild-type S-88 or wild-type NW11 colonies do not appear as copiously mucoid and shiny as *X. campestris*. For strain NW11, the effective concentration of bacitracin was 20 μg/ml. A representative set of the Sps$^-$ mutants are listed in Table 1.

Intergeneric complementation between *X. campestris* and *Sphingomonas* strains. Introduction of plasmids carrying a normal *gumD* gene of *X. campestris* into Bac^r Sps$^-$ mutants of *Sphingomonas* strain S-88 restored polysaccharide synthesis to the recipient. Of the four recombinant plasmids depicted in Fig. 2, only pRK311c8A failed to complement the Sps$^-$ mutations. The complementation was monitored by colony appearance on solid medium containing glucose or gravimetrically after precipitation of extracellular polysaccharides from liquid cultures. When agar plates were held up to light and

observed from underneath, positively complemented colonies were surrounded by a ring of light and had a more translucent yellow color. Similar results were obtained for strain NW11 (not shown). However, for NW11 the Sps^+ colonies were opaque and the Sps^- colonies were translucent.

We prepared a library of genes from *Sphingomonas* strain S-88 in cosmid pRK311 in *E. coli* and screened the library for individuals which complemented Sps^- mutants of S-88. One recombinant plasmid (pRK311-S88c1) contained 30 kb of cloned DNA and restored mucoidy to several independent mutants of strains S-88 (m257 to m260, bac8, bac9, and bac10) and NW11 (bac3-1 and bac3-3). The region that restored mucoidy by complementation was localized to about 2 kb within a 4.5-kb *Eco*RI fragment (42). The cloned S-88 DNA also restored mucoidy and bacitracin sensitivity to the *gumD* mutants of *X. campestris* m31, m65, and m96. The complemented colonies of *X. campestris* appeared very mucoid and shiny, just like the wild type, indicating restoration of xanthan synthesis. To verify that the mucoid appearance was due to a recipient type of exopolysaccharide, we identified the monosaccharides in acid hydrolysates by thin-layer chromatography. When *X. campestris* was the recipient of the S-88 genes, glucose, mannose, and glucuronic acid were present in amounts like that of xanthan. When strain S-88 was the recipient for the *gumD* gene of *X. campestris*, rhamnose, mannose, glucose, and glucuronic acid were present in amounts similar to those present in exopolysaccharide S-88.

However, the intergeneric complementation did not extend to *E. coli* as the recipient. Recombinant plasmids containing either the *gumD* gene of *X. campestris* (pRK311c8 and pRK311-1483) or the genes from *Sphingomonas* strains in plasmid pRK311-S88c1 were introduced into Bac^r Cps^- spontaneous mutants of *E. coli* (TP416, -417, -418, and -419) by mating and shown to be intact by restriction enzyme analysis. However, almost all of the exconjugants remained nonmucoid. The plasmids in these nonmucoid exconjugants were then returned to Bac^r Gum^- *X. campestris* X59m31 by mating, whereby they restored mucoidy to the mutant. This indicated that the plasmids were still functionally intact. A few mucoid colonies arose in *E. coli*, and they were initially thought to indicate complementation since they also contained the expected intact plasmids. However, they are probably only the result of reversion of the *E. coli* mutations since their mucoid phenotype remained even after the plasmids were lost by growth for many generations in the absence of antibiotic selection.

DISCUSSION

We became interested in the relationship between resistance to bacitracin and polysaccharide synthesis after finding that most Bac^r mutants of *X. campestris* were defective in biosynthesis of xanthan gum (21). Since bacitracin binds to the IPP precursor of the IP carrier for polysaccharide biosynthesis (30), we thought that the Bac^r mutations which blocked xanthan synthesis might lead to more IP carrier being available for essential cell wall synthesis. Genetic complementation tests described here indicated that these mutations were defective in *gumD*, which is necessary to form Glc-PPI (39). There is also a second mechanism of Bac^r in *X. campestris* that leads to increased accumulation of xanthan gum instead of eliminating synthesis of xanthan. Strain X50 is one of these Gum^+ Bac^r mutants (21). Strain X50 might have elevated amounts of the IP carrier, as seen in *E. coli* when the cells have multiple copies of the *bacA* gene (3).

The correspondence between Bac^r and lack of exopolysaccharide synthesis for *X. campestris* appears to be a general phenomenon for polysaccharide-secreting gram-negative bacteria. Three representative species from two other genera, *Sphingomonas* and *Escherichia*, showed similar dual phenotypes. The intergeneric complementation between *X. campestris* and either *Sphingomonas* strain S-88 or strain NW11 suggests that there is a common step in the synthesis of xanthan gum and polysaccharides S-88 and NW11. On the basis of the primary structures of these three polysaccharides (Fig. 1), the only possible common step is the attachment of the initial glucose-P to the carrier IP. The association of Bac^r and the exopolysaccharide-negative phenotypes in each of these species is consistent with the common involvement of carrier IP. The *gumD* gene product of *X. campestris*, which is required to attach glucose-P to IP, was able to act in *Sphingomonas* strains. Similarly, a gene from *Sphingomonas* strain S-88, *spsB*, was able to complement *gumD* mutants in *X. campestris*. The *spsB* gene has recently been shown to be another member of the family of putative glycosyl-IP transferases (42). If we assume that each of these related genes codes for a glycosyl-IP transferase, then the assembly of S-88 and NW11 exopolysaccharides begins with one of the two glucoses in the backbone.

Knowing which sugar-P is attached first to the IP carrier allows one to deduce whether the assembled oligosaccharide subunit is branched or unbranched prior to polymerization of the subunits. For example, with reference to Fig. 1, both colanic acid and xanthan gum are initiated with glucose at the reducing end, which is attached to IPP. Therefore, branching in the completed polysaccharides is a consequence of the polymerization step. The results presented here indicate that glucose is also the initiating sugar for the sphingans S-88 and NW11. If glucose is the first sugar attached to the IP carrier by *Sphingomonas* strain S-88, then the pentasaccharide subunit shown in Fig. 1 must be assembled as a branched structure. The oligosaccharide subunit must also be branched if the other glucose is the initiating sugar. The branch must be created either by the enzyme that adds glucuronic acid or by the enzyme that adds the side chain L-rhamnose. This is quite different from synthesis of xanthan by *X. campestris* (11, 12), colanic acid by *E. coli* (14), or the succinoglycans by *Rhizobium* species (37), in all of which the subunits are unbranched. In these three cases the branching is introduced when the repeat subunits are polymerized.

We also obtained spontaneous Bac^r Cps^- mutants for *E. coli* K-12. By contrast to Bac^r Lon^+ wild-type *E. coli*, a Lon^- Cps^+ $ManA^-$ strain was found to be Bac^s at 30°C when mannose was present. The latter strain became conditionally Bac^r in the absence of mannose or at 37°C. We isolated Bac^r Cps^- derivatives of the Lon^- Cps^+ $ManA^-$ strain. Then we made two observations consistent with the idea that these mutants, like the corresponding mutants of *X. campestris* and *Sphingomonas* strains, were defective in the attachment of glucose-P to IP. First, the selection pressure for Bac^r occurred even for the $ManA^-$ strain in the absence of mannose, a condition under which GDP-fucose is not available for the second step in assembly of colanic acid. Second, a $GalU^-$ strain was found to be very sensitive to bacitracin. We think that the sensitivity reflects an altered cell surface. $GalU^-$ mutants have an incomplete carbohydrate structure (the core) attached to lipopolysaccharide because of the absence of UDP-glucose. This causes $GalU^-$ colonies to have a rough surface appearance. We believe that if the Bac^r Cps^- mutants were defective in a step that preceded the attachment of glucose-P to IP, for example, the formation of UDP-glucose, then they would also

be very sensitive to bacitracin like the surface-altered *galU* strain.

Since glucose-P was previously shown to be the initial sugar attached to IP in the assembly of colanic acid (14), we thought the intergeneric complementation might also extend to *E. coli*. However, we were unable to complement the Bac^r Cps$^-$ mutations of *E. coli* with either the *gumD* gene of *X. campestris* or the homologous *spsB* gene from *Sphingomonas* strain S-88. Nor were we able to detect increased sensitivity to bacitracin when *E. coli* TP416 carried multiple copies of the *gumD* gene of *X. campestris*. One reason might be a lack of expression of the foreign gene in *E. coli*. The average G+C compositions are quite different: 50% for *E. coli* and about 60 to 65% for *X. campestris* and *Sphingomonas* strains. Alternatively, the foreign enzyme might not function properly in *E. coli*. For example, it might require additional compatible accessory proteins.

Mapping of Bac^r Cps$^-$ mutations in *E. coli* to the vicinity of the *cpsABCDE* gene cluster near *his* is consistent with involvement of the corresponding wild-type gene in biosynthesis of colanic acid. As yet we do not know whether any of the existing *cps* complementation groups correspond to our Bac^r mutations. In the related *cps* gene cluster in *S. enterica* LT2, two of the genes have been assigned enzymatic functions: *cpsB* codes for GDP-mannose synthetase, and *cpsG* codes for phosphomannomutase (31). Both of these genes are needed to accumulate the GDP-fucose precursor for colanic acid. However, none of the sugar transferase genes needed for assembling and polymerizing colanic acid have yet been identified. By analogy to our results with Bac^r in *X. campestris* and *Sphingomonas* strains, the mapping results for the Bac^r mutations in *E. coli* suggest that the *cpsABCDE* cluster probably does contain at least one of the transferase genes, the gene responsible for attaching glucose-P to the IP carrier. The mode of action of bacitracin in sequestering IPP and thereby reducing polysaccharide biosynthesis confirms the requirement for carrier IP (14). The general association between Bac^r and exopolysaccharide synthesis demonstrated here might be a useful genetic tool in the study of similar genetic systems in other gram-negative bacteria.

Reciprocal intergeneric complementation between the *gumD* gene of *X. campestris* and the *pss2* gene of *R. leguminosarum* was shown previously by Borthakur et al. (1). However, the earlier study did not limit the essential region to the *gumD* gene itself. Furthermore, there was no determination of exactly what exopolysaccharide was synthesized to restore the mucoid appearance. Although one might expect that the recipient polysaccharide would be synthesized, it would also be possible for novel structures to be polymerized. For the intergeneric complementation described here between *X. campestris* and *Sphingomonas* strains, we isolated the exopolysaccharides from the recipient and determined by hydrolysis and chromatography that they had the sugar composition expected of the recipient.

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