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Colanic Acid Intermediates Prevent *De Novo* Shape Recovery of *Escherichia coli* Spheroplasts, Calling into Question Biological Roles Previously Attributed to Colanic Acid

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ABSTRACT

After losing their protective peptidoglycan, bacterial spheroplasts can resynthesize a cell wall to recreate their normal shape. In *Escherichia coli*, this process requires the Rcs response. In its absence, spheroplasts do not revert to rod shapes but instead form enlarged spheroids and lyse. Here, we investigated the reason for this Rcs requirement. Rcs-deficient spheroids exhibited breaks and bulges in their periplasmic spaces and failed to synthesize a complete peptidoglycan cell wall, indicating that the bacterial envelope was defective. To determine the Rcs-dependent gene(s) required for shape recovery, we tested spheroplasts lacking selected RcsB-regulated genes and found that colanic acid (CA) biosynthesis appeared to be involved. Surprisingly, though, extracellular CA was not required for recovery. Instead, lysis was caused by mutations that interrupted CA biosynthesis downstream of the initial glycosyl transferase, WcaJ. Deleting *wcaJ* prevented lysis of spheroplasts lacking ensuing steps in the pathway, and providing WcaJ in *trans* to a mutant lacking the entire CA operon triggered spheroplast enlargement and lysis. Thus, CA is not required for spheroplast recovery. Instead, CA intermediates accumulate as dead-end products which inhibit recovery of wall-less cells. The results strongly imply that CA may not be required for the survival *E. coli* L-forms. More broadly, these findings mandate that previous conclusions about the role of colanic acid in biofilm formation or virulence must be reevaluated.

IMPORTANCE

Wall-less bacteria can resynthesize their walls and recreate a normal shape, which in *Escherichia coli* requires the Rcs response. While attempting to identify the Rcs-dependent gene required for shape recovery, we found that colanic acid (CA) biosynthesis appeared to be involved. Surprisingly, though, cell death was caused by mutations that interrupted CA biosynthesis downstream of the initial step in the pathway, creating dead-end compounds that inhibited recovery of wall-less cells. When testing for the biological role of CA, most previous experiments used mutants that would accumulate these deadly intermediates, meaning that all prior conclusions must be reexamined to determine if the results were caused by these lethal side effects instead of accurately reflecting the biological purpose of CA itself.

Bacterial cell shape is determined by coordinated and dynamic interactions among cytoskeletal elements, peptidoglycan synthesis, and cell division (as reviewed in references 1 and 2), and the wall and some of its morphological characteristics contribute to cell survival in suboptimal or hostile environments (3). For example, the host immune system elaborates several antibacterial factors, including lysozyme, cationic antimicrobial peptides, and protein complexes, which target the cell envelope and trigger bacterial lysis (4–6). In particular, lysozyme removes the peptidoglycan wall and leads to cell rupture. However, such cells may not lyse if they are immersed in an osmotically protective medium in which they may form wall-less cells of two broad types: those that can grow and divide independently (L-forms) and those that cannot (protoplasts or spheroplasts) (7). These entities can survive and regenerate a functional cell wall (8–13), which may enable them to avoid cell wall-specific host defenses until conditions allow for normal growth (14).

Recent work indicates that both Gram-positive and Gram-negative bacteria that have lost their cell walls can regenerate a defined morphology without the aid of a preexisting peptidoglycan template (12, 13). In particular, lysozyme-induced (LI) spheroplasts of *Escherichia coli* can regenerate their walls and morphology when grown in an osmotically protective medium (12, 15). However, this recovery process is impeded if the original rod-shaped cells lack one of several proteins, including penicillin-binding protein 1b (PBP 1b), LpoB, or Lpp, or if the cells cannot mount an active Rcs stress response (12). These mutants grow as normal rod-shaped cells when the wall is present, indicating that *E. coli* requires these additional proteins or processes to recreate a wild-type sacculus only when a preexisting peptidoglycan template is absent. Why these accessory processes are required is unknown.

L-forms are related to spheroplasts, in that they lack a peptidoglycan cell wall but retain functional membranes (7). The major difference between the two is that L-forms have gained the ability

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to grow and divide as cell wall-deficient forms, thereby perpetuating the strain, whereas spheroplasts may survive for a time but eventually die or revert to their original morphology (7). Though both spheroplasts and L-forms may sometimes regenerate a properly formed cell wall (13), L-forms must be able to divide and survive in the absence of a cell wall, and several genes and proteins are important for this transformation (15–17). One component reported to be required for L-form survival is the production of colanic acid (CA), a product of the Rcs stress response that regulates about 150 genes (15, 16, 18–20). CA is an extracellular capsular polysaccharide common to members of *Enterobacteriaceae* but absent from other bacteria (21), and its putative role in L-form survival is undefined.

Here, we attempted to identify the Rcs-dependent gene or genes required for spheroplast recovery. Of the known Rcs-regulated genes, 19 have RcsB-RcsB or RcsA-RcsB binding sites in or near their promoter regions (www.ecocyc.org), implying that these genes or operons are controlled directly by the Rcs response. We found that only one of these—the operon encoding the CA biosynthetic pathway—affected spheroplast recovery, suggesting that this extracellular capsular compound might be the pertinent Rcs-controlled component.

Surprisingly, we found that spheroplast recovery did not require extracellular colanic acid. Instead, spheroplasts lysed or died when cells carried mutations that interrupted CA biosynthesis downstream of WcaJ, the glycosyl transferase that initiates the pathway. Eliminating WcaJ allowed spheroplasts to survive even though no CA was produced, and spheroplasts enlarged and lysed if they contained WcaJ but no other CA synthetic enzymes. We conclude that CA intermediates are lethal to spheroplasts but that CA itself is not required for the survival of wall-less cells. Importantly, because of the way the CA pathway has been interrupted in previous experiments, it is now unclear whether CA plays a role in several other bacterial processes, including the survival of Gram-negative L-forms, the formation of biofilms, or virulence.

MATERIALS AND METHODS

Bacterial strains and plasmids, DNA manipulation, and media. Bacterial strains and plasmids are listed in Table S1 in the supplemental material. The primers and oligonucleotides used to create each plasmid and bacterial mutant are listed in Table S2 in the supplemental material. Routine cultures were grown in Luria-Bertani (LB) medium, and when appropriate, ampicillin (100 μg/ml) or kanamycin (50 μg/ml) was added. Standard DNA, PCR, and molecular biological techniques were utilized for cloning and plasmid construction (22), and *E. coli* DH5α was used as the intermediate cloning strain. All experiments were performed in the *E. coli* MG1655 background. The correctness of each plasmid was verified by DNA sequencing (UAMS DNA sequencing core).

Plasmid constructions. Plasmids were constructed in the pDEV vector backbone (12). Using primers listed in Table S2 in the supplemental material, the following genes were amplified by PCR from the chromosome of *E. coli* MG1655 and cloned into pDEV to create different plasmids (in parentheses): *ftsAZ* (pFtsAZ), *lolA* (pLolA), *wza* (pWza), *cpsG* (pCpsG), *ugd* (pUgd), and *wcaJ* (pWcaJ). Restriction sites used to clone each gene are underlined in the primer sequences.

Strain constructions. Genes were deleted from *E. coli* MG1655 by moving each mutation from appropriate strains of the Keio collection (23), via P1 transduction. Other mutations were created by λ-red recombination (24), using individual mutagenic oligonucleotides (see Table S2 in the supplemental material). The completely null CA operon mutant (DR38) was constructed as follows. First, the kanamycin cassette from pKD13 was amplified by PCR, using the primers DP263 and DP273 (see Table S2 in the supplemental material). These primers contained 40-nucleotide extensions at their 5′ ends that were homologous to the region upstream of the promoter region of *wza* (DP263) and to the region downstream of *wcaM* (DP273), which are the first and last genes, respectively, of the 20-gene CA operon (see Fig. 2A). To delete the entire CA operon, this kanamycin cassette PCR product was introduced into electrocompetent MG1655 cells that carried the λ-red helper plasmid, which was then induced with L-arabinose (24). Primers from regions flanking the CA operon were used for diagnostic PCR to verify that the entire 22.7-kb segment was deleted, including all 20 genes from *wza* to *wcaM*. Subsequently, the kanamycin cassette from this null mutant was removed by site-specific excision by expressing the FLP recombinase from the helper plasmid pCP20 (24), thereby generating *E. coli* DR38 [*E. coli* MG1655 Δ(*wza-wcaM*)::*frt*]. This strain was unable to synthesize CA, as verified by deleting the *yrfF* gene via P1 transduction (*E. coli* strain BMKM111-3), since this mutation generates prodigiously mucoid colonies only when the CA operon is intact.

Spheroplast recovery, peptidoglycan labeling, and microscopy. The spheroplast recovery assay was performed as described previously (12). Briefly, cells from an exponentially growing LB broth culture were harvested at an optical density at 600 nm (OD_{600}) of 0.2, washed with phosphate-buffered saline (PBS) (pH 8.0), and then plasmolyzed by resuspending them in 0.5 M sucrose plus lysozyme (20 μg/ml) for 10 min at 37°C. A mild osmotic shock was applied by diluting these cells with an equal volume of PBS (no sucrose) containing lysozyme (20 μg/ml) and incubating at 37°C for additional 10 min. To remove lysozyme, cells were centrifuged at 500 × *g* for 15 min and washed with sucrose recovery medium. The resulting cell pellet was transferred onto a sucrose recovery soft agar pad in a chambered slide. These slides were incubated and observed by using a Zeiss Axio imager Z1 microscope enclosed in a 37°C incubation chamber.

Peptidoglycan was labeled by feeding growing cells the fluorescent D-alanine derivative, hydroxy-coumarin-carbonyl-amino–D-alanine (HADA), a gift from Erkin Kuru and Michael S. VanNieuwenhze (25). Spheroplasts from *rcsB* mutant strains were grown for 1 h in spheroplast recovery broth containing HADA (500 μM, final concentration). These spheroplasts were fixed for 15 min in 2.8% formaldehyde and 0.04% glutaraldehyde, washed twice with PBS (pH 7.4), and prepared for microscopy. Spheroplasts and intact cells were visualized by using a wide-field epifluorescent Zeiss Axio imager Z1 microscope fitted with a 100× differential interference contrast objective (1.45 NA). Fluorescent images were captured by using 4′,6-diamidino-2-phenylindole (DAPI) filters (358-nm excitation, 461-nm emission) to image the HADA label. A green fluorescent protein (GFP) filter set (495-nm excitation, 519-nm emission) was used to image superfolder GFP. Images were acquired with a Zeiss Axiocam MRm camera and were processed with Axiovision software.

RESULTS

Rcs-deficient spheroplasts have defective envelopes. If an LI spheroplast from an *E. coli* strain is to recover a rod-shaped morphology, the spheroplast must replace its missing cell wall in a way that regenerates the original cell shape. To do so, the spheroplast must have an intact and functional envelope, including an inner membrane (IM) and outer membrane (OM) with a periplasmic space, and must resynthesize an evenly distributed, rod-shaped layer of peptidoglycan. To determine the status of these components in recovering cells, we visualized the periplasmic space and peptidoglycan synthesis in wild-type and in Rcs-deficient spheroplasts (Fig. 1). (Note that, throughout the text, the term spheroplast refers to an LI spheroplast, that is, a spheroplast induced by lysozyme treatment, as described previously [12].)

The periplasmic space of each cell was visualized by expressing the fluorescent protein DsbA-ss-sfGFP (the DsbA signal sequence fused to the amino terminus of superfolder GFP), which is trans-

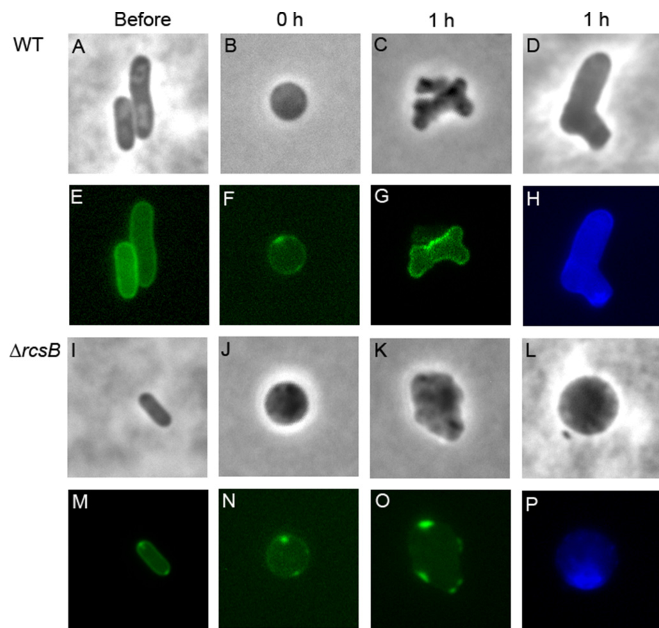

FIG 1 Organization of the periplasm and peptidoglycan in recovering LI spheroplasts. Wild-type *E. coli* strain MG1655 (A to H) and the isogenic Δ*rcsB* mutant DR5 (I to P) were visualized immediately before being transformed into LI spheroplasts (Before; $n = 8$ to 10), immediately after being transformed into LI spheroplasts (0 h; $n = 5$ to 6), and 1 h into the recovery period (1 h; $n = 5$ to 6). Cells expressing the periplasmic fluorescent protein DsbA-ss-sfGFP were visualized by phase (A to C, I to K) and fluorescence (E to G, M to O) microscopy. Newly synthesized peptidoglycan in recovering spheroplasts was labeled with the fluorescent D-alanine derivative HADA (D, H, L, P). The images are representative.

ported to and accumulates within the periplasm (26). Wild-type spheroplasts grew, divided, and eventually returned to their normal wild-type rod shapes (Fig. 1B to D). In contrast, spheroplasts lacking *rcsB* never divided but, instead, enlarged (Fig. 1J to L) until they lysed (not shown), consistent with previous observations (12).

The periplasmic spaces in the two types of spheroplast were quite different. In untreated, wild-type, rod-shaped cells, the periplasm appeared as a continuous, evenly distributed fluorescent line that followed the circumference of each cell (Fig. 1E). The same circumferential distribution of periplasm was observed in each wild-type spheroplast immediately after the wall had been removed and in each recovering cell, no matter how aberrantly shaped these intermediate cells appeared (Fig. 1F and G). Similarly, cells lacking *rcsB* exhibited the same even distribution of periplasm around the cell periphery in untreated rod-shaped cells (Fig. 1M) and in newly formed spheroplasts (Fig. 1N). However, the periplasmic space was clearly aberrant in Rcs-deficient spheroplasts as they expanded, when the periplasm became discontinuous and formed a broken or spotty line around the periphery. In all cases, the periplasmic space was distended or bulged so that the fluorescent marker accumulated in distinctive and brightly lit sacs (Fig. 1M). Thus, the periplasmic space in Rcs-deficient spheroplasts was abnormal, implying that the organization of the cell envelope was defective and that, in the absence of the Rcs response, the envelope could not be reconstructed properly.

Because the periplasm was abnormal in Rcs-deficient spheroplasts, it seemed likely that peptidoglycan synthesis might also be irregular. We therefore visualized newly synthesized peptidoglycan in recovering spheroplasts by labeling cells with the fluorescent D-alanine derivative HADA (12, 25). Spheroplasts were incubated for 40 min in recovery broth, HADA was added for an additional 20 min to label newly synthesized peptidoglycan, and the labeled spheroplasts were visualized by fluorescence microscopy. As reported previously (12), wild-type spheroplasts recovered by forming a series of aberrantly shaped cells (e.g., Fig. 1C and D). HADA staining was distributed evenly in these cells, with a barely discernible thin line around the circumference indicating the presence of an intact and continuous layer of newly synthesized peptidoglycan (Fig. 1H). In contrast, spheroplasts lacking *rcsB* enlarged, but the peptidoglycan in the resulting spheroids was not distributed evenly, appeared to be fibrous, and was concentrated in only a fraction of each cell (e.g., Fig. 1P). Thus, Rcs-deficient spheroplasts failed to synthesize a continuous and normal protective layer of peptidoglycan, consistent with the view that such spheroplasts do not recreate the wild-type cell envelope.

Screening Rcs-regulated genes to identify those required for spheroplast recovery. The Rcs stress response system affects the expression of over 150 genes (18, 19), at least one of which is required for spheroplast recovery (12). As a first step toward identifying the relevant element(s), we scanned databases for genes whose promoter regions contained RcsB binding sites, on the assumption that such genes would be regulated directly by the Rcs response. According to the EcoCyc database (www.ecocyc.org), 13 genes or operons have promoters with RcsB-RcsB binding sites (Table 1), and 5 have promoters with RcsA-RcsB binding sites (Table 2). We first mutated each of the 11 nonessential genes preceded by RcsB-RcsB sites (*osmB*, *bdm*, *osmC*, *gadB*, *safA*, *ydeP*, *rprA*, *hdeA*, *hdeD*, *gadY*, and *gadA*). Spheroplasts lacking any one of these genes grew normally and recovered their wild-type rod shapes (Table 1), indicating that none of these protein products were required for Rcs-dependent recovery. The remaining two

TABLE 1 Spheroplast recovery phenotypes of mutants lacking RcsB-RcsB-regulated genes or operons[a]

Operon	Associated function or characteristic	Gene(s) tested	Strain	Spheroplast recovery[b]
ftsA ftsZ	Cell division	Δ*rcsF* P_{lac}-*ftsAZ*	DR5-PF	−
lolA rarA	Transport lipoprotein to OM	Δ*rcsF* P_{lac}-*lolA*	DR5-PL	−
osmB	Lipoprotein induced by osmotic stress	Δ*osmB*	DR20	+
bdm sra	Biofilm modulation	Δ*bdm*::*kan*	DR21	+
osmC	Induced by osmotic stress	Δ*osmC*::*kan*	DR22	+
gadB gadC	Glutamate decarboxylase	Δ*gadB*::*kan*	DR23	+
safA yedO	Membrane connector for EvgS/EvgA	Δ*safA*::*kan*	DR24	+
ydeP	Oxidoreductase	Δ*ydeP*::*kan*	DR25	+
rprA	Regulatory RNA	Δ*rprA*::*kan*	DR26	+
hdeA hdeB yhiD	Periplasmic acid resistance	Δ*hdeA*::*kan*	DR27	+
hdeD	IM acid resistance	Δ*hdeD*::*kan*	DR28	+
gadY	Regulator of *gadX* and *gadW*	Δ*gadY*::*kan*	DR29	+
gadA gadX	Glutamate decarboxylase	Δ*gadA*::*kan*	DR30	+

[a] *kan*, kanamycin resistance gene; OM, outer membrane; IM, inner membrane.
[b] +, recovered; −, did not recover.

TABLE 2 Spheroplast recovery phenotypes of mutants lacking RcsA-RcsB-regulated genes or operons

Operon	Associated function or characteristic	Gene(s) tested[b]	Strain	Spheroplast recovery[a]
csgD csgE csgF csgG	Curli assembly and secretion	Δ*csgD*::*kan* Δ*rcsF*	DR31	−
flhD flhC	Regulator of flagellum biogenesis	Δ*flhD*::*kan* Δ*rcsF*	DR33	−
rcsA	Auxiliary activator of colanic acid synthesis	Δ*rcsA*::*kan*	DR6	+
yjbE yjbG yjbH	Biofilm formation	Δ*yjbE*::*kan*	DR34	+
wza wzb wzc wcaA wcaB	Colanic acid biosynthesis	Δ*wza*::*kan*	DR35	−

[a] +, recovered; −, did not recover.
[b] *kan*, kanamycin resistance gene.

genes preceded by RcsB-RcsB sites were essential (*ftsAZ* and *lolA*), and it was possible that the Rcs response enhances expression of these genes under spheroplast conditions. To test this, we enhanced gene expression by introducing cloned *ftsAZ* or *lolA* genes into an *rcsF* mutant. Expressing either *ftsAZ* or *lolA* did not rescue these Rcs-deficient spheroplasts (Table 1). Thus, none of the 13 genes preceded by RcsB-RcsB binding sites was required for spheroplast recovery.

Of the 5 genes containing RcsA-RcsB binding sites, the RcsA-RcsB heterodimer negatively regulates *csgD* and *flhD* (27, 28). Thus, an Rcs-deficient mutant may produce higher levels of CsgD or FlhD than the wild type, which might in turn prevent spheroplast recovery. If so, then deleting *csgD* or *flhD* should restore the ability to recover to Rcs-deficient spheroplasts. Instead, Δ*rcsF* spheroplasts lacking *csgD* or *flhD* did not recover (Table 2), indicating that these gene products do not inhibit the process. The remaining 3 genes (*rcsA*, *yjbE*, and *wza*) are positively regulated by RcsA-RcsB (18, 29–31), meaning that deleting one of these from an Rcs-proficient spheroplast might prevent recovery. Spheroplasts lacking either *rcsA* or *yjbE* recovered normally (Table 2), indicating that these two genes did not affect recovery. However, spheroplasts lacking *wza* did not recover their normal rod shapes (Table 2), implying that this gene might encode the pertinent Rcs-dependent factor required for spheroplast recovery.

Spheroplast recovery does not depend on extracellular colanic acid. *wza* is the first gene of the *cps* operon (Fig. 2A), which encodes proteins that synthesize and export CA (Fig. 2B). Triggering the Rcs system induces expression of this operon, leading to the production of copious amounts of this extracellular capsular carbohydrate (21, 32). Because CA was reported to be essential for creating L-forms (15, 16, 20), it seemed possible that Rcs-deficient spheroplasts would not recover, because they could not synthesize this compound. Consistent with this expectation, spheroplasts lacking *wza* did not revert to a normal rod shape but instead expanded to become giant spheroids that lysed (Fig. 3A). (Note, though, that there was no detrimental effect on the growth or morphology of the *wza* mutant as long as the strain did not pass through a spheroplast stage.) Expressing *wza* in *trans* rescued the mutant so that spheroplasts divided and recovered (Fig. 3B), indicating that genes downstream of *wza* were expressed normally. Wza is the outer membrane component of the CA export channel, and the absence of Wza blocks extracellular CA secretion (33, 34). Thus, the inability of Wza-deficient spheroplasts to revert to a rod shape suggested either that extracellular CA was required for spheroplast recovery or that the accumulation of CA intermediates was deleterious to such cells.

To determine if spheroplasts required CA to recover a normal morphology, we deleted 22.7 kb of chromosomal DNA to remove all 20 genes of the *cps* operon, from *wza* to *wcaM* (Fig. 2A), thereby creating a strain lacking the entire CA biosynthetic pathway (Fig. 2B). We confirmed the absence of these genes by diagnostic PCR and by the inability of the strain to produce CA (see Materials and Methods). To our surprise, spheroplasts derived from this strain divided and recovered normally (Fig. 3C), despite the fact that no CA could be synthesized, polymerized, or transported. Thus, the production of extracellular CA was not essential for the recovery process, nor did the absence of CA lead to spheroplast enlargement and lysis.

Colanic acid intermediates inhibit spheroplast recovery. Though CA was not required for spheroplast recovery, a *wza* mutant prevented recovery. Thus, it seemed likely that, in the absence of the CA transporter Wza, intermediate periplasmic or cytoplasmic compounds might accumulate and inhibit recovery. To determine which intermediates were responsible, we interrupted CA assembly by working backward through the steps of CA synthesis, transport, and polymerization, which are illustrated schematically in Fig. 2B. In brief, assembly of the CA repeat unit is initiated by WcaJ, a glycosyl transferase that adds glucose (from UDP-Glu) to undecaprenyl phosphate (Und-P) at the cytoplasmic face of the inner membrane. Subsequently, fucose (from GDP-Fuc), galactose (from GDP-Gal), and glucosamine (from GDP-GlcA) are added to create the basic glycosyl repeat unit. This repeat unit is believed to be flipped across the inner membrane into the periplasm by the action of WzxC (29, 35). WcaD polymerizes these units, after which the CA polymer is transported across the outer membrane via the Wza-Wzc complex (29, 35).

Spheroplasts lacking *wcaD* enlarged and lysed (Fig. 4A), as did spheroplasts lacking *wxzC* (Fig. 4B). Thus, the putative inhibitory intermediate(s) does not have to be periplasmic, because, in the absence of WxzC, such an intermediate would remain in the cytoplasm. We then attempted to determine which parts of the repeat unit were needed to create an inhibitory cytoplasmic intermediate. UDP-glucose (UDP-Glu) dehydrogenase (encoded by *ugd*) produces UDP-glucaronic acid (UDP-GlcA), which supplies glucaronic acid as the next-to-last carbohydrate in the CA repeat unit (Fig. 2B) (29). The Rcs system regulates the expression of *ugd* in *Salmonella enterica*, and an identical promoter sequence precedes *ugd* in *E. coli* (36), suggesting that the gene in *E. coli* is also controlled by the Rcs system, even though it was absent from the list of such genes in the EcoCyc database. *E. coli* spheroplasts lacking *ugd* did not recover but enlarged and lysed (Fig. 4C). The *ugd* phenotype was complemented by providing the gene in *trans* (Fig. 4D), indicating that the mutation had no polar effects on genes in its operon. The second carbohydrate added to the CA repeat unit is fucose (Fig. 2B), which is synthesized by gene products encoded by *cpsB*, *cpsG*, *gmd*, *wcaG*, and *wcaH* (Fig. 2A and B) (29, 37).

Spheroplasts lacking either *cpsB* or *cpsG* failed to recover and instead enlarged and lysed (Fig. 4E and F). The *cpsG* phenotype was complemented by supplying *cpsG* in *trans* (Fig. 4G), again indicating the absence of polar expression effects. These results indicated that the minimally relevant inhibitory compound did

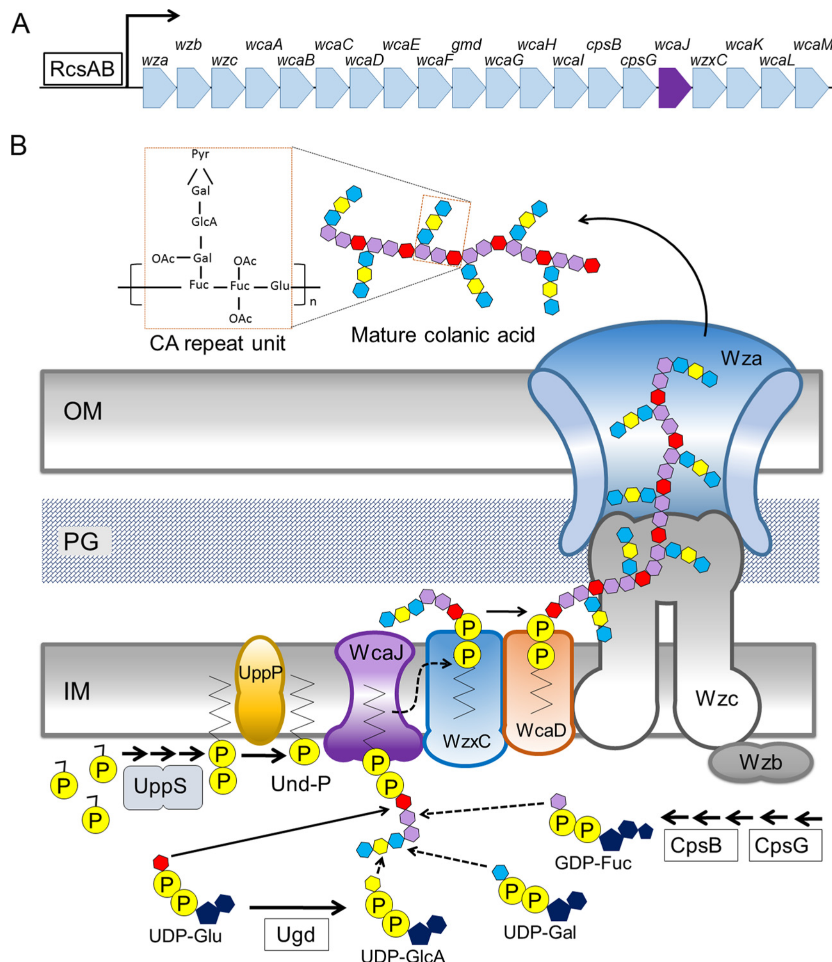

FIG 2 Colanic acid genes and the CA biosynthetic pathway. (A) Genes of the CA operon. The promoter region contains a binding sequence that induces gene expression when occupied by the RcsAB heterodimer. (B) Selected substrates and enzymes in the CA biosynthesis and transport pathway (29, 39, 58). (Adapted from reference 34 with permission of the publisher.) Enzymes are devoted to precursor synthesis (CpsG, CpsG, Gmd, WcaG, and WcaH) or to CA assembly and transport (WcaJ, WcaA, WcaC, WcaE, WcaI, WcaL, WcaB, WcaF, WzxC, WcaD, Wzc, Wzb, and Wza). Undecaprenyl pyrophosphate synthase (UppS) catalyzes the condensation reaction of isopentenyl diphosphate and farnesyl diphosphate to generate undecaprenyl pyrophosphate (Und-PP). Undecaprenyl pyrophosphate phosphatase (UppP) converts Und-PP to undecaprenyl phosphate (Und-P). WcaJ, a glycosyl transferase, initiates the first dedicated step of CA assembly by adding glucose-1-phosphate from UDP-glucose (UDP-Glu) to Und-P, generating Und-PP-Glu. Additional sugar residues are added stepwise to Und-PP-Glu: GDP-fucose (GDP-Fuc), UDP-galactose (UDP-Gal), and UDP-glucaronic acid (UDP-GlcA). WzxC, a polysaccharide transporter, flips the lipid-linked CA repeat unit to the periplasmic face of the inner membrane (IM). WcaD polymerizes the repeat units to create CA polymers, and the Wzc-Wza complex transports mature CA through the outer membrane (OM) and out of the cell. OAc, *O*-acetyl; PG, peptidoglycan; Pyr, pyruvate.

not contain any carbohydrate residues beyond the initial glucose residue.

A WcaJ-dependent intermediate prevents spheroplast recovery and triggers lysis. The foregoing experiments strongly suggested that the initial step in making the CA repeat unit was responsible for creating an inhibitory compound. WcaJ catalyzes this reaction by moving glucose-1-phosphate from UDP-glucose (UDP-Glu) onto the lipid carrier undecaprenyl phosphate (Und-P), thereby generating Und-PP-Glu (Fig. 2B) (38, 39). In fact, spheroplasts lacking *wcaJ* did divide and recover just as well as wild-type cells (Fig. 5A), indicating that the WcaJ-bound Und-P-P-Glu subunit was the minimum-sized inhibitor.

A *wcaJ* mutant not only abolishes the production of extracellular CA (39) but also eliminates the generation of subsequent CA intermediates (Fig. 2B). Thus, removing WcaJ would be expected to eliminate the deleterious effects observed in mutants lacking downstream components of the CA pathway. Consistent with this expectation, spheroplasts lacking both *wcaJ* and *wzxC* divided and recovered normally (Fig. 5B), thus reversing the lethal effects observed in spheroplasts lacking only *wzxC* (Fig. 4B). Similarly, when *wcaJ* was deleted from cells lacking either *cpsG* or *ugd*, the resulting spheroplasts divided and recovered normally (Fig. 5C and D), once again reversing the lethal effects observed in spheroplasts lacking only one of the latter genes (Fig. 4C and F). These results substantially strengthened the idea that any of several CA intermediates were inhibitory.

Finally, we determined whether the presence of WcaJ inhibited recovery in the absence of all other enzymes in the CA biosynthetic pathway. To that end, we overexpressed *wcaJ* in *E. coli* DR38, which lacks the entire CA pathway. Overexpressing *wcaJ* in spheroplasts derived from wild-type *E. coli* did not inhibit recovery (Fig. 5E), and, as described above, spheroplasts derived from

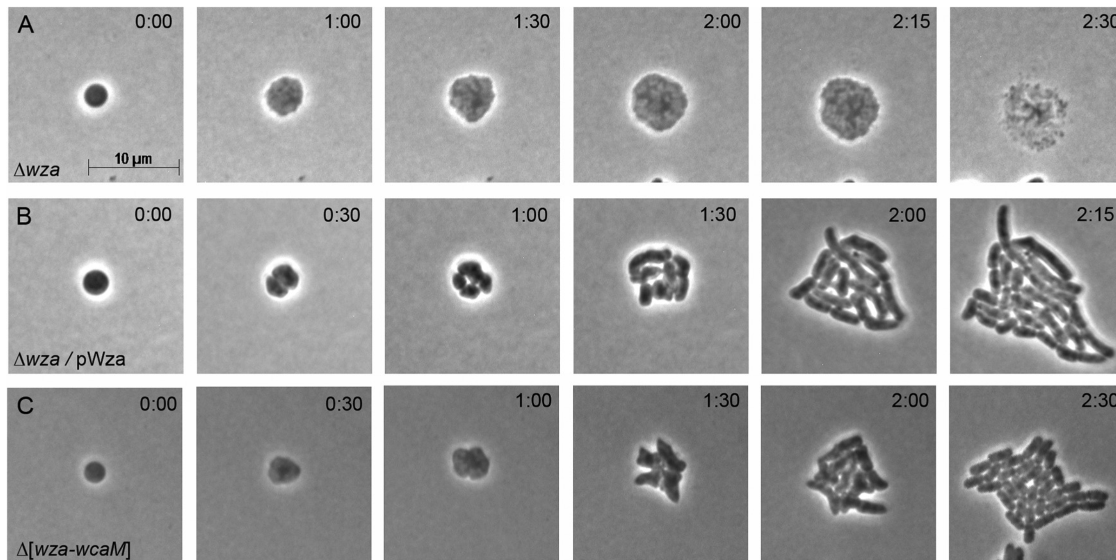

FIG 3 Extracellular colanic acid is not required for spheroplast recovery. Spheroplasts lacking Wza were incubated on recovery medium, and the recovery process was monitored by time-lapse phase-contrast microscopy. Time after plating (hour:minute) is displayed in each panel. The images are representative. (A) *E. coli* DR36 Δ*wza* ($n = 16$); (B) *E. coli* DR37 Δ*wza*, complemented with the pWza plasmid ($n = 9$); (C) *E. coli* DR38 Δ(*wza* to *wcaM*) ($n = 16$).

the CA-negative *E. coli* strain DR38 recovered normally in the absence of additional WcaJ (Fig. 3C). However, when WcaJ was overproduced in CA-negative cells, the resulting spheroplasts did not recover but instead enlarged and lysed (Fig. 5F). Thus, active WcaJ prevented spheroplast recovery without the participation of downstream components of the pathway.

In short, the presence of WcaJ prevented spheroplast recovery in strains unable to complete and export a mature CA or in strains that could not synthesize a complete CA repeat unit. The results also further confirmed that the production of extracellular CA was not required for spheroplast recovery.

WcaJ does not prevent spheroplast recovery by sequestering Und-P. The preceding results suggested the existence of deleterious intermediate products in the CA biosynthetic pathway, products that would not normally accumulate because they would be utilized and removed by later steps. The simplest explanation was that, in the presence of WcaJ and in the absence of a downstream CA pathway component, undecaprenyl-phosphate (Und-P)-linked compounds were accumulating as dead-end products. Spheroplast recovery might be inhibited if these intermediates reduced the pool of Und-P available for other, essential pathways (most notably, that portion of the pool required to synthesize peptidoglycan). Just such a competition for Und-P contributes to the creation of aberrantly shaped cells in mutants that accumulate dead-end Und-P-linked intermediates in the pathway that synthesizes enterobacterial common antigen (40) and in *Bacillus subtilis* mutants that accumulate teichoic acid intermediates (41).

If the above-described scenario was correct, then increasing the cellular pool of Und-P should suppress the effects of WcaJ, as it does in the enterobacterial common antigen (ECA) and teichoic acid cases (40, 41). To test this possibility, we increased the Und-P pool by overexpressing genes encoding enzymes that synthesize Und-P. However, overexpressing *uppS* (*ispU*), the undecaprenyl diphosphate synthase, did not restore the ability to recover for spheroplasts lacking *wza* or *ugd* (see Fig. S1A and B in the supplemental material). Similarly, overexpressing *uppP* (*bacA*), an undecaprenyl pyrophosphate phosphatase involved in recycling Und-P, did not restore recovery to cells lacking *wza* or *ugd* (see Fig. S1C and D in the supplemental material). As a complementary approach, we deleted *uppP* (*bacA*) to see if reducing the Und-P pool by this means might prevent spheroplast recovery, since UppP (BacA) accounts for ~75% of the undecaprenyl pyrophosphate phosphatase activity in a cell (42). However, the resulting Δ*uppP* spheroplasts divided and recovered normally (see Fig. S1E in the supplemental material), suggesting either that the amount of Und-P had no effect on spheroplast recovery or that the *uppP* mutation did not reduce the pool of Und-P to a sufficient degree. In short, WcaJ-derived CA intermediates appeared to inhibit spheroplast recovery by a mechanism other than by sequestering the pool of Und-P.

The Rcs requirement for spheroplast recovery is independent of colanic acid and its intermediates. The above results proved that extracellular CA was not required for spheroplast recovery (e.g., Fig. 3C); instead, CA intermediates were toxic to these cells. However, an active Rcs response is definitely required for spheroplast recovery. Therefore, it was formally possible that an intact Rcs response prevented the accumulation of toxic CA intermediates (e.g., if CA biosynthesis was interrupted for some reason). To investigate this possibility, we tested the recovery of spheroplasts lacking both *rcsB* and the entire CA operon [Δ(*wza-wcaM*)]. If the death of *rcsB* spheroplasts was mediated by CA or its intermediates, then the complete absence of these compounds should allow this mutant to recover normally. Instead, these doubly mutated spheroplasts failed to divide but grew into large spheroids and lysed (Fig. 5G) in exactly the same manner as *rcsB* mutants containing a wild-type CA gene cluster (Fig. 1) (12). This result indicates that the Rcs response is required for spheroplast recovery because it controls one or more processes unrelated to CA biosynthesis. At the moment, this Rcs-dependent mechanism remains unknown.

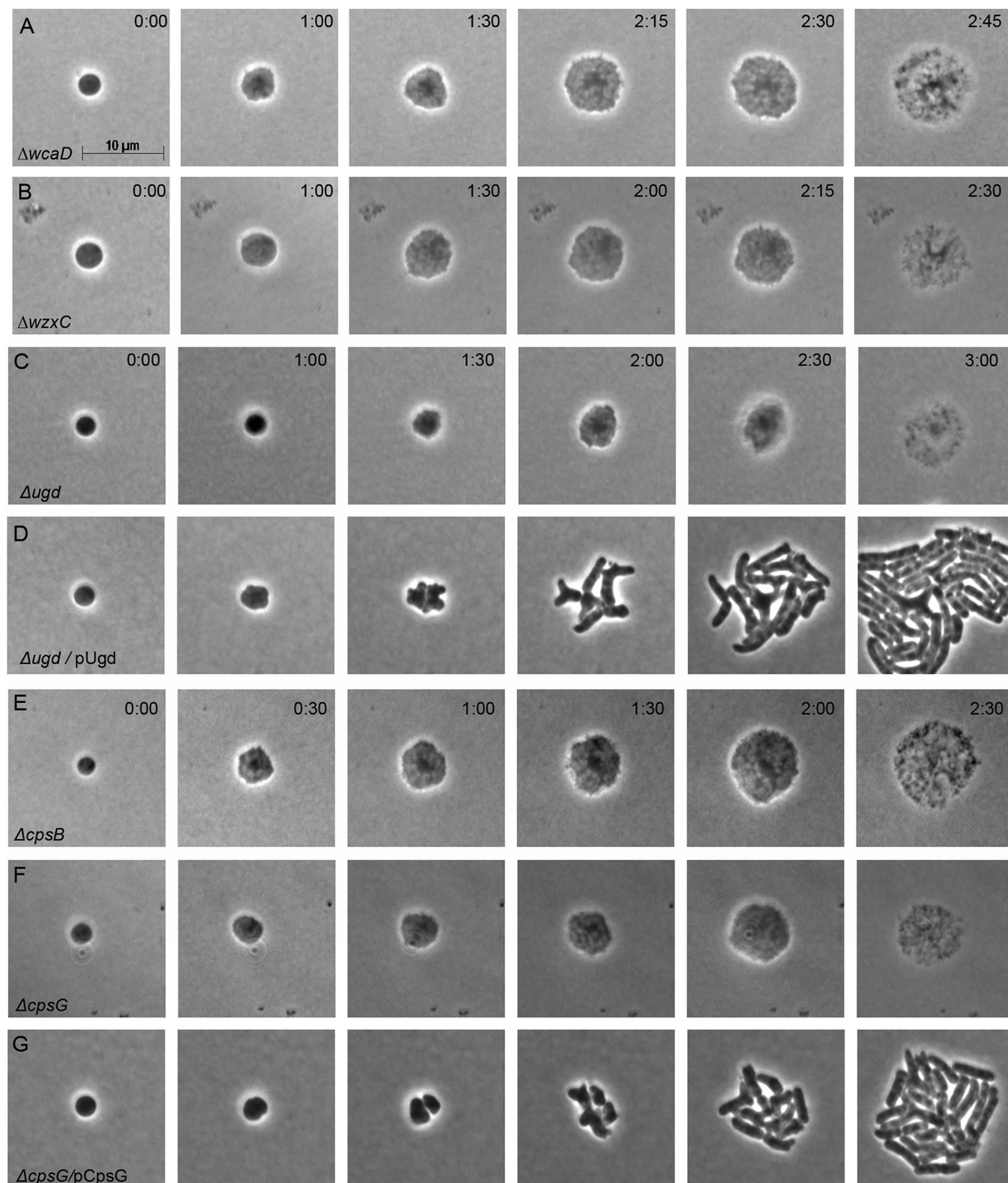

FIG 4 Blocking colanic acid transport inhibits spheroplast recovery. Spheroplasts unable to assemble or transport CA were grown on osmotically protected sucrose recovery medium, and the recovery process was monitored by time-lapse phase-contrast microscopy. Time after plating (hour:minute) is displayed in each panel. The images are representative. (A) *E. coli* DR39 Δ*wcaD* ($n = 9$); (B) *E. coli* DR40 Δ*wzxC* ($n = 22$); (C) *E. coli* DR44 Δ*ugd* ($n = 5$); (D) *E. coli* DR45, in which *ugd* is complemented with the pUgd plasmid ($n = 4$); (E) *E. coli* DR41 Δ*cpsB* ($n = 7$); (F) *E. coli* DR42 Δ*cpsG* ($n = 4$); (G) *E. coli* DR43, in which Δ*cpsG* is complemented with the pCpsG plasmid ($n = 8$).

DISCUSSION

The role of colanic acid in wall-less *E. coli* and L-forms. CA has been deemed to be essential for the continued growth and long-term survival of wall-less *E. coli* cells (variously described as L-forms or L-form-like cells) (15, 16, 20). However, we find that, in the complete absence of the entire CA biosynthetic pathway, LI spheroplasts survive, grow, and revert to a wild-type rod shape. In contrast, spheroplasts enlarge and lyse if they carry mutations that interrupt CA synthesis at any point after the first step catalyzed by WcaJ. Because all L-forms of *E. coli*, by definition, must survive the loss of the cell wall, previous experiments arguing that CA is essential for this process must be reevaluated in light of the present results.

Joseleau-Petit et al. first reported that CA was absolutely required for propagating *E. coli* without its cell wall (20). However, in these experiments, CA production was blocked by inactivating the Rcs stress response or by inserting a *cpsE*::Tn*10* mutation (20). Here, though, we show that the Rcs response seems to be required

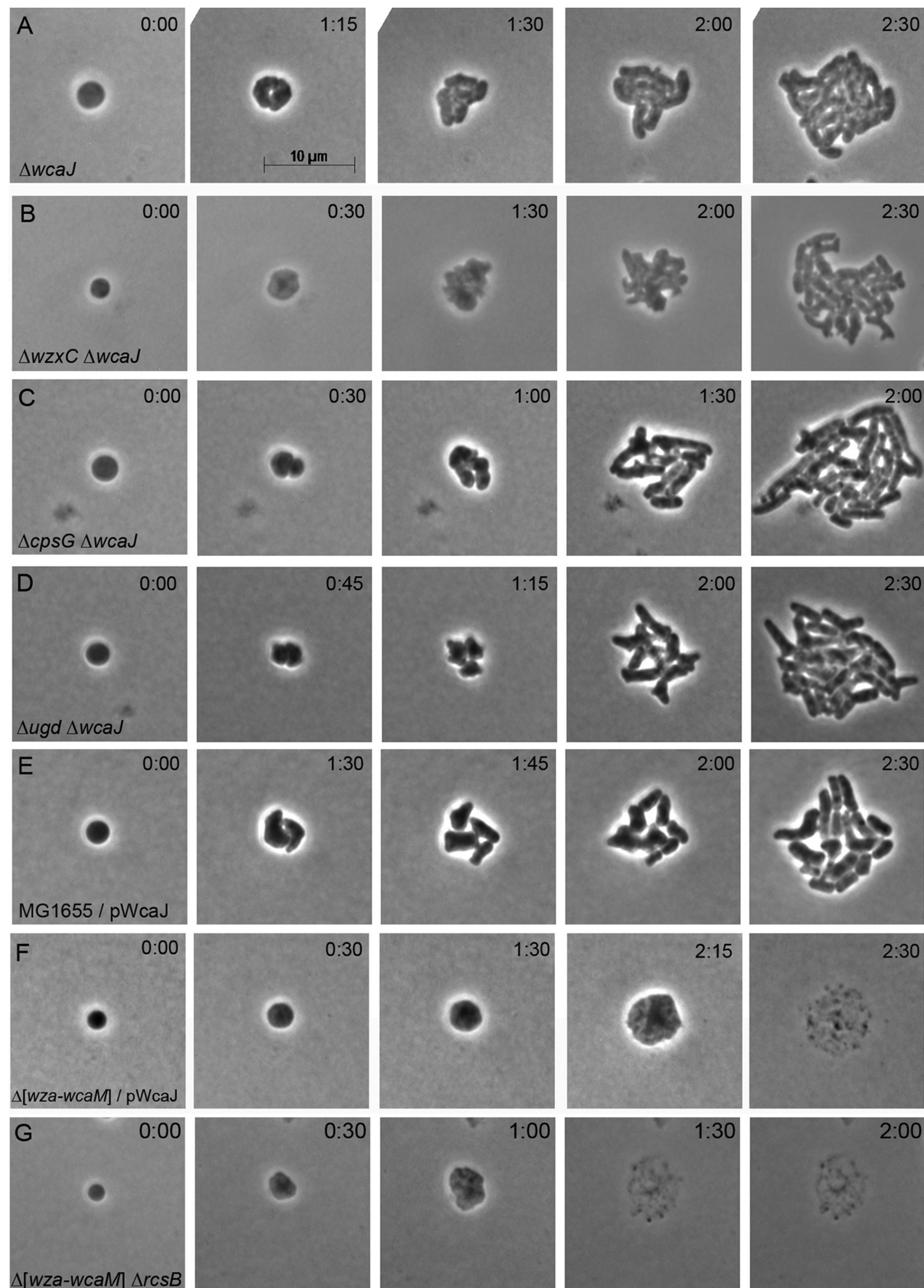

FIG 5 Deleting *wcaJ* restores recovery to spheroplasts lacking components of the colanic acid biosynthesis pathway but not to spheroplasts lacking *rcsB*. Spheroplasts from the indicated mutants were grown on osmotically protected sucrose recovery medium, and the recovery process was monitored by time-lapse phase-contrast microscopy. Time after plating (hour:minute) is displayed in each panel. The images are representative. (A) *E. coli* DR46 Δ*wcaJ* ($n = 13$); (B) *E. coli* DR47 Δ*wzxC* Δ*wcaJ* ($n = 15$); (C) *E. coli* DR48 Δ*cpsG* Δ*wcaJ* ($n = 14$); (D) *E. coli* DR49 Δ*ugd* Δ*wcaJ* ($n = 18$); (E) *E. coli* MG1655 carrying the pWcaJ plasmid ($n = 9$); (F) *E. coli* DR51 Δ(*wza* to *wcaM*) carrying the pWcaJ plasmid ($n = 15$); (G) *E. coli* DR57 Δ(*wza* to *wcaM*) Δ*rcsB* ($n = 12$).

for reasons that are unrelated to the production of CA, meaning that Rcs-negative mutants should not be used to prove that CA is required for a phenotype. As for the *cpsE*::Tn*10* mutation, this allele was originally unmapped (43) but actually inactivates the *wza* gene (44; D. K. Ranjit and K. D. Young, unpublished data). Since a *wza* (≡*cpsE*) mutation impedes CA biosynthesis downstream of WcaJ and prevents spheroplast recovery, the *cpsE*::Tn*10* allele cannot be used to infer that CA is necessary for L-form growth.

Glover et al. also concluded that CA was "essential for L-form colony formation" (16). These authors created unstable L-forms by growing *E. coli* in the presence of penicillin G and found that the Rcs stress response was induced 5- to 53-fold and that genes involved in CA synthesis were induced from 10- to 34-fold (16).

Evidence supporting the importance of CA was that, of 42 mutants unable to grow as unstable L-forms, 3 inactivated the Rcs response and 10 inactivated CA production (including mutations in *cpsB*, *wcaA*, *wzxC*, and related genes). However, as argued above, Rcs-negative mutations have effects beyond the failure to synthesize CA. More to the point, though, is that all CA mutants that were isolated inhibited synthesis after the first WcaJ-initiated step in the pathway. In fact, Glover et al. reported that a *wcaJ* mutant did survive and formed unstable L-forms (16), a result that should not occur if CA was itself required for L-form survival. Instead, the failure of CA mutants to produce unstable L-forms, and the ability of a *wcaJ* mutant to do so, can now be explained more easily by the presence or absence of deleterious Und-P-linked CA intermediates.

Cambré et al. recently created wall-less *E. coli* by treating cells with cefsulodin, an antibiotic that inhibits penicillin-binding proteins 1a and 1b (PBPs 1a and 1b) (15). The resulting cells are technically not L-forms but are instead β-lactam-induced spheroplasts, similar to the LI spheroplasts that we discuss here. However, because antibiotic pressure can be maintained, these β-lactam-induced L-form-like cells grow and produce colonies. By using a transposon mutagenesis screen, Cambré et al. isolated 14 mutants that could not grow as L-forms, and each mutation perturbed either the Rcs stress response or CA synthesis; 5 mutations were in one of two genes of the Rcs pathway (*rcsC*, *rcsD*), while the remaining 9 were in genes encoding steps in CA biosynthesis (*wzc*, *wcaD*, *wcaE*, *wcaK*, *wcaL*, *galE*, or *ugd*) (15). These results led to the conclusion that CA was "crucial . . . for L-form growth" and that "all . . . requirements for L-form growth and multiplication can be attributed to the formation of the capsular polysaccharide colanic acid" (15). Indeed, this is exactly what we believed at first. The results do confirm and reinforce our previous observation that the Rcs system must be active if this type of spheroplast is to survive (12). However, again, Rcs involvement does not mean that CA itself is required. Also, every nongrowing CA mutant isolated by Cambré et al. interrupted CA synthesis downstream of WcaJ (15). The simplest explanation, then, is that these mutants failed to survive the loss of their cell wall because lethal Und-P-linked intermediates accumulated, which is the same reason that LI spheroplast CA mutants lysed in the present work. The fact that Cambré et al. recovered no mutations in *wcaJ* is consistent with this interpretation, because a *wcaJ* mutant would produce L-form-like colonies and would therefore escape notice in their genetic screen (15).

Therefore, reinterpreting the available evidence strongly suggests that CA itself is not required for the survival of wall-less *E. coli*. On the other hand, although an active Rcs stress response is essential for such survival, the identity of this Rcs-dependent factor remains unknown.

Role of colanic acid in virulence. Beyond its supposed role in sustaining Gram-negative L-forms, CA has been implicated as important for several other bacterial traits and activities, including those related to virulence. For example, uropathogenic *E. coli* (UPEC) strains with mutations in either the *wcaM* or *wcaL* gene of the CA pathway are only 11% as effective in colonizing mouse spleens, suggesting that CA makes a significant contribution to UPEC virulence (45). Similarly, a *wcaE* mutant of an avian pathogenic *E. coli* strain survives very poorly in an *in vivo* chicken septicemia model (46). The mutant was especially deficient in colonizing the heart tissue, the lung, and the spleen, leading the authors to infer that this was "direct evidence for an association of colanic acid with virulence and fitness" (46). CA also reportedly imparts serum resistance to extraintestinal pathogenic *E. coli* (ExPEC) (45, 47–49). For example, only 3% of an ExPEC Δ*wcaDE* mutant and <20% of an *rcsB* mutant survived exposure to human serum (48), and a strain lacking any of three CA genes (*wcaF*, *wcaH*, or *wcaI*) was more sensitive to serum killing (47). Finally, *wcaD* and *wcaE* mutants of enterohemorrhagic *E. coli* (EHEC) strain O157:H7 grow less well under acidic conditions, at high temperatures, or in simulated stomach acid, suggesting that CA protects EHEC against these conditions (50, 51).

Unfortunately (in retrospect), all of the above-described experiments tested mutants that impaired CA biosynthesis at stages downstream of WcaJ (Fig. 2). Therefore, the observed phenotypes may have arisen either because CA was absent or because the mutants accumulated metabolic intermediates that impeded growth so that the cells were less able to survive the test conditions. To our knowledge, only one virulence experiment has used a mutant lacking the entire CA operon, in *Salmonella enterica* serovar Typhimurium (52). In this case, the loss of CA caused no difference at all in virulence (52), supporting the idea that CA itself may play no role in these situations. In any event, CA cannot be said to contribute to virulence unless appropriate mutants are created and retested.

The role of colanic acid in biofilms and other phenotypes. One of the earliest functions proposed for CA was that it contributed to the formation of *E. coli* biofilms, in both *E. coli* K-12 and *E. coli* O157:H7 (49, 53, 54). CA-deficient *E. coli* strains adhere to surfaces just as well as wild-type cells, but they fail to mature into the normal three-dimensional biofilm architecture (53), and CA-deficient *E. coli* O157:H7 adheres less well to alfalfa sprouts (54). Similarly, CA is reported to be required for biofilm formation in *Salmonella* species (55). Once again, these conclusions were based on the behavior of strains carrying either *wcaF* or *wcaD* mutations (53, 54) or on the behavior of strains carrying mutations in *wcaM*, *wcaA*, or *wza* (55), all of which interrupt CA biosynthesis downstream of WcaJ (Fig. 2). Because biofilm formation requires cell growth, the accumulation of toxic CA intermediates may have simply impeded the survival of *E. coli* or *Salmonella* in these multicellular environments, making CA only appear to be important for biofilm formation. To our knowledge, only one *E. coli* biofilm experiment has tested a strain lacking the entire CA operon, and this CA mutation reduced biofilm formation by only ~40% (56). In short, mature CA may contribute only moderately to biofilm formation.

Finally, CA-negative *cpsB* mutants of *E. coli*, *Acinetobacter calcoaceticus*, and *Erwinia stewartii* are much less resistant to desiccation, as is the *cpsE* mutant (57), leading to the speculation that CA increases survival in the wake of desiccation or during osmotic or acid stress (50, 57). Because the *cpsB* and *cpsE* (≡*wza*) mutations interrupt CA synthesis downstream of WcaJ, these conclusions should be revisited to be certain that CA is relevant.

Possible mechanisms. In theory, CA intermediates could inhibit spheroplast recovery because these dead-end metabolic compounds compete for and sequester part of the pool of undecaprenyl-phosphate that is also required for peptidoglycan synthesis. We recently showed that just such a sequestration accounts for the inhibitory effects of similar intermediates in the pathway that synthesizes enterobacterial common antigen (40). However, increasing the pool of Und-P by overproducing UppS (IspU) did

not restore the ability of spheroplasts to recover a normal shape, suggesting that this simple form of the sequestration model was not the explanation. The remaining alternative is that CA intermediates are toxic in and of themselves by a mechanism that remains unknown.

What is clear is that some property or process controlled by the Rcs stress response is required for the survival and morphological conversion of *E. coli* LI spheroplasts (12). Although we tried to identify the relevant Rcs-driven activity by mutating genes known to be controlled directly by RcsB, this approach has been unsuccessful so far. One would have thought that such a gene might have been represented in the 5,760 mutants described by Cambré et al., who screened for undirected mutants unable to grow as wall-less cells (15). The fact that no such mutation was isolated suggests either that more than one Rcs-dependent gene product is required for spheroplast survival or that too few mutants have been screened by us and by others.

Summary. Colanic acid is not required for the survival or morphological reversion of wall-less *E. coli* cells, and it is almost certainly not required for the survival of the Gram-negative L-forms tested so far. In fact, all conclusions regarding the biological roles for CA must now be considered uncertain until the underlying experiments eliminate CA by interrupting its biosynthesis at the first committed step performed by WcaJ. Unfortunately, virtually all previous experiments pertaining to the function of CA examined the behavior of mutants in which CA biosynthesis was blocked downstream of WcaJ, which we now posit leads to the accumulation of deleterious UndP-linked intermediates. These intermediates may seriously obstruct bacterial growth under particular experimental conditions, a result that could then be falsely ascribed to a lack of CA. CA might be important for some of the phenotypes listed above, but the relevant experiments must be repeated with appropriate mutants before drawing such conclusions.

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