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Cell Division Inhibition in *Salmonella typhimurium* Histidine-Constitutive Strains: an *ftsI*-Like Defect in the Presence of Wild-Type Penicillin-Binding Protein 3 Levels

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Histidine-constitutive (His^c) strains of *Salmonella typhimurium* undergo cell division inhibition in the presence of high concentrations of a metabolizable carbon source. Filaments formed by His^c strains show constrictions and contain evenly spaced nucleoids, suggesting a defect in septum formation. Inhibitors of penicillin-binding protein 3 (PBP3) induce a filamentation pattern identical to that of His^c strains. However, the His^c septation defect is caused neither by reduced PBP3 synthesis nor by reduced PBP3 activity. Gross modifications of peptidoglycan composition are also ruled out. D-Cycloserine, an inhibitor of the soluble pathway producing peptidoglycan precursors, causes phenotypic suppression of filamentation, suggesting that the septation defect of His^c strains may be caused by scarcity of PBP3 substrate.

When histidine-constitutive (His^c) mutants of *Salmonella typhimurium* were first isolated, the authors noted that high levels of histidine biosynthetic enzymes caused wrinkled colony morphology on 2% glucose plates (25). Wrinkledness reflects cell filamentation (12, 19), which is triggered by overproduction of *hisH* and *hisF* gene products (5, 9, 19). A similar response has been described for *Escherichia coli* (11). HisH and HisF are subunits of the heterodimeric imidazole-glycerol-phosphate synthase (1, 34), which catalyzes the formation of imidazole-glycerol-phosphate (IGP) with release of the purine precursor AICAR (5-aminoimidazole-4-carboxamide ribonucleotide) (17, 26). However, division inhibition does not require metabolic flow through the histidine biosynthetic pathway, suggesting that HisH and HisF trigger filamentation through an activity unrelated to IGP synthesis (10, 19). The involvement of AICAR has been also ruled out (10, 11). In both *S. typhimurium* and *E. coli*, the cell division defect of His^c strains is unrelated to the SOS response and does not involve the cell division inhibitor SulA (11, 12). We show below that the cell division defect of *S. typhimurium* His^c strains is a block in septum formation, as proposed by Frandsen and D'Ari (11). We also describe the unexpected finding that strains that overproduce IGP synthase contain wild-type levels of active penicillin-binding protein 3 (PBP3). These contradictory data are tentatively reconciled by the ability of D-cycloserine to suppress filamentation in His^c mutants. The latter observation suggests that HisHF overproduction may cause a shortage in PBP3 substrate.

Filament formation by His^c strains. Mid-exponential-phase cultures of strains LT2 (*hisO*$^+$) and TR6753 (*hisO1242* [5, 16]) were observed under the microscope by using Hiraga's fluophase combined method, a procedure that permits the simultaneous observation of nucleoids and cells (15). Nucleoid staining was achieved with DAPI (4′,6-diamino-2-phenylindole). The His^c strain formed long filaments which contained evenly spaced nucleoids, indicating that their division defect is unrelated to DNA synthesis or chromosome partition (Fig. 1). Moreover, the presence of blunt constrictions indicates that the division block lies beyond the stage of FtsZ action (8). The filaments are similar in morphology and length to those formed by *ftsI* and *ftsA* mutants of *E. coli* (8). The *ftsI* gene encodes PBP3, an essential cell division protein involved in septum formation (27). FtsA is a membrane-bound protein that interacts with PBP3 (22, 31). A difference is that *ftsI* and *ftsA* mutants are conditional (thermosensitive) lethals unable to form colonies under restrictive conditions (3, 18), while the filaments produced by His^c strains of *S. typhimurium* in the presence of 2% glucose give rise to colonies which are distinctly wrinkled (9, 19, 25).

Antibiotics that inhibit PBP3, such as aztreonam and azlocillin, produce phenocopies of *ftsI* mutants in *E. coli* (29). Based on this precedent, we investigated whether PBP3 inhibitors were able to reproduce the filamentation phenotype of His^c strains. Addition of aztreonam (1 mg/liter) to a culture of strain LT2 triggered filament formation (Fig. 1C), and the filaments were identical in morphology and length to those formed by the His^c strain TR6753 (Fig. 1A). The same effect was induced by azlocillin (data not shown). At the concentrations used, these antibiotics inhibit specifically PBP3 (21). These observations suggest that His^c strains of *S. typhimurium* behave as *ftsI* mutants.

Additional evidence against an FtsA-like defect was provided by the failure of a plasmid carrying the *E. coli ftsA* gene to relieve cell division inhibition when introduced in strain TR6753. If His^c strains were *ftsA*-like, a plasmid-borne *ftsA* gene should restore the FtsA/FtsZ ratio (6), thereby causing a certain degree of suppression. However, the actual result was that pMFV26, an *ftsA*$^+$ plasmid provided by Miguel Vicente (CIB-CSIC, Madrid, Spain), failed to relieve septation inhibition. The conclusion that His^c strains behave as *ftsI* (rather than *ftsA*) mutants receives further support from the ability of D-cycloserine to suppress filamentation (see below).

HisHF overproduction does not cause reduced synthesis of PBP3. The levels of PBP3 produced by HisO$^+$ and HisO^c strains were compared by using envelope extracts from mid-

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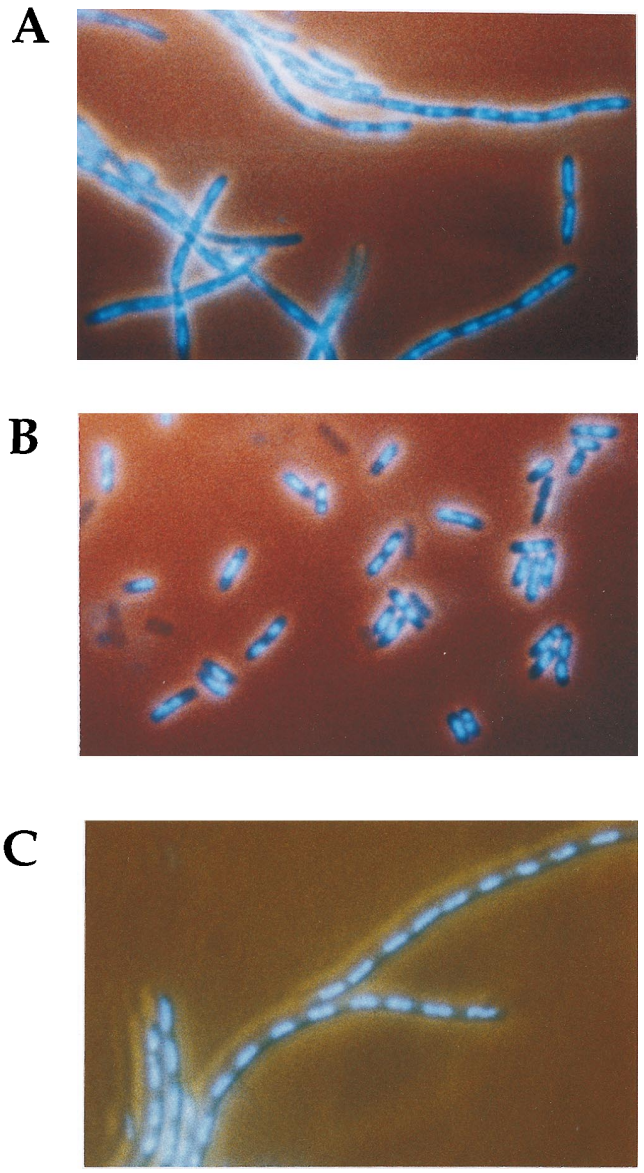

FIG. 1. Microscopic photographs of cells and filaments prepared with Hiraga's fluo-phase combined method (15). (A) Filaments formed by the His^c strain TR6753 grown in E medium containing 2% glucose. (B) An isogenic His$^+$ strain (LT2) grown under the same conditions does not form filaments. (C) Addition of aztreonam to a culture of strain LT2 in E medium induces filaments identical to those formed by a His^c strain in high-glucose medium (compare panels A and C). Bar, 5 μm.

exponential-phase cultures (optical density at 600 nm, 0.5 to 0.6) in E medium containing 2% glucose (33). Envelope proteins were fractionated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (8% acrylamide) and detected by immunoblotting against a polyclonal anti-PBP3 serum (21). The levels of PBP3 protein are similar in HisO$^+$ and HisO^c strains (Fig. 2); densitometric analysis (not shown) confirmed the absence of differences. Thus, the cell division defect associated with IGP synthase overproduction is not caused by reduced synthesis of PBP3. An additional observation is that the electrophoretic mobilities of PBP3 are similar in HisO$^+$ and

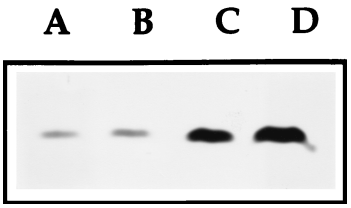

FIG. 2. Immunodetection of PBP3 from the wild type (lanes A and C) and the His^c strain TR6753 (lanes B and D). Lanes A and B contain membrane vesicles corresponding to 80 μg of protein in a final reaction volume of 20 μl. Lanes C and D contain membrane vesicles corresponding to 120 μg of protein in a final volume of 20 μl.

HisO^c strains, thereby eliminating the occurrence of gross structural changes in the protein.

HisHF overproduction does not cause reduced activity of PBP3. As an indicator of the functionality of PBP3 in membrane extracts from HisO$^+$ and HisO^c strains, we analyzed their capacities to bind covalently ^{3}H-labelled benzylpenicillin (2). Cultures were prepared as described above. Protein separation was performed on an SDS-polyacrylamide gel (2). Radioactivity was detected by fluorography (28, 30). Protein quantitation was performed by the method of Bradford (4). No differences in penicillin binding were found (Fig. 3 and densitometric data not shown). A side observation is that the remaining high-molecular-weight PBPs were also unaffected (Fig. 3).

Peptidoglycan composition of His^c strains. To investigate the possibility that His^c strains might synthesize an abnormal cell wall, we prepared peptidoglycan extracts from strains LT2 and TR6753 grown in E medium containing 2% glucose. Concentrated exponential cultures containing approximately 10^{11} bacterial cells were cooled to 4°C, centrifuged at 12,000 × *g* for 15 min, and resuspended in 3 ml of phosphate-buffered saline, pH 7.4. The suspension was mixed 1:1 (vol/vol) with a boiling solution of 8% SDS (24). The SDS-insoluble material was washed in distilled water (13). Peptidoglycan was digested with Cellosyl muramidase (20 μg/ml) (Hoechst, Sommerville, N.J.); this treatment yields muropeptides of low molecular weight (23). The reaction was stopped in a boiling bath for 5 min.

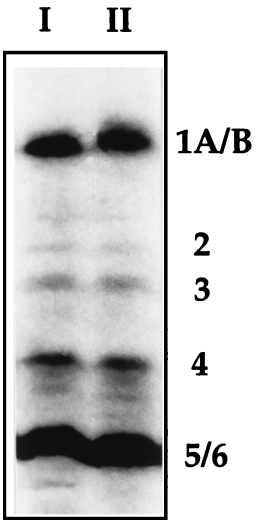

FIG. 3. Binding of ^{3}H-labelled benzylpenicillin to cell envelopes from exponentially growing cells of the His$^+$ strain LT2 (lane I) and the His^c strain TR6753 (lane II). PBPs are numbered on the right by standard nomenclature (27).

TABLE 1. Muropeptide composition of peptidoglycan from His^+ and His^c strains and from a His^+ strain treated with aztreonam

Sample	Relative abundance (mol%)[a]					Cross-linkage (%)
	Monomers	Dimers		Trimers	Lpp	
		D-D	L-D			
LT2 (His^+)	65.2	27.7	2.9	4.12	9.25	38.8
TR6753 (His^c)	66.9	26.4	3.2	3.2	9.0	36.3
LT2 (with aztreonam)	66.0	26.2	3.0	4.6	8.2	38.5

[a] Muropeptides are grouped according to structural similarities (13). D-D are dimeric muropeptides cross-linked by a D-D peptide bridge; L-D are dimeric muropeptides cross-linked by an L-D peptide bridge. Trimers are trimeric muropeptides cross-linked by D-D peptide bridges. Lpp are muropeptides bound to the C-terminal dipeptide of Braun's lipoprotein (13, 14).

Insoluble material was removed by centrifugation (1,000 × *g*, 10 min). Peptidoglycan composition was determined by high-performance liquid chromatography analysis (13, 14). The main conclusions are that (i) abnormal peptidoglycan muropeptides were not found and (ii) gross differences in peptidoglycan composition or organization were not found between $HisO^+$ and $HisO^c$ strains (Table 1). These experiments do not rule out the formation of an abnormal PBP3 substrate but certainly exclude the possibility that any unusual substrate is incorporated into growing peptidoglycan chains. This absence of differences does not eliminate the possibility that His^c strains may have a defect in the reaction catalyzed by PBP3; peptidoglycan from strain LT2 treated with the PBP3 inhibitor aztreonam showed also standard composition (Table 1). In fact, a well-known and surprising feature of cell wall synthesis is that the global peptidoglycan composition remains unaltered under conditions that cause major changes in cell shape (20).

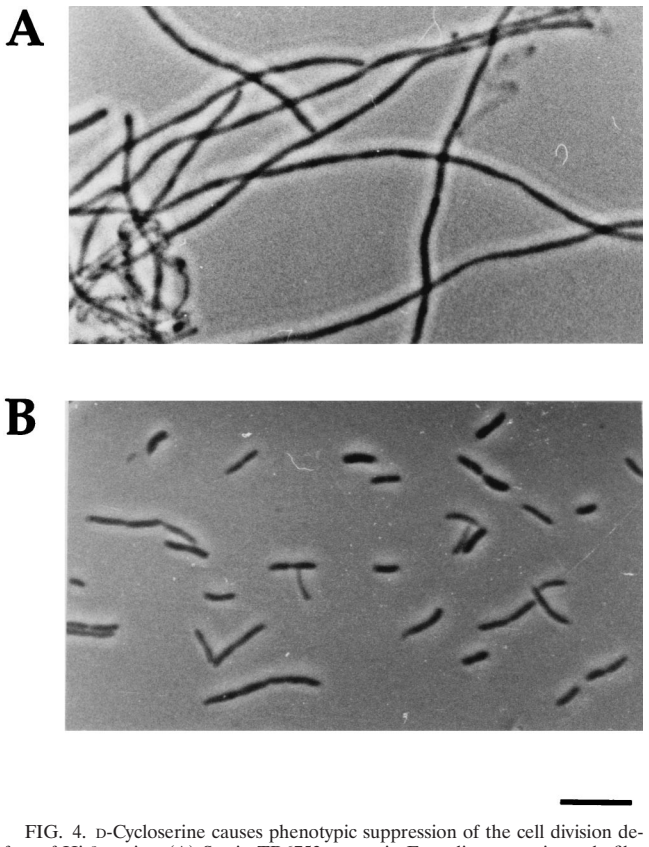

FIG. 4. D-Cycloserine causes phenotypic suppression of the cell division defect of His^c strains. (A) Strain TR6753 grown in E medium contains only filaments. (B) The presence of D-cycloserine causes suppression of filamentation; strain TR6753 forms rod-shaped cells. Bar, 10 μm.

D-Cycloserine causes phenotypic suppression of the cell division defect of His^c strains. To explore the possibility that the septation defect of His^c strains might be caused by lowered levels of PBP3 substrate, we investigated whether D-cycloserine was able to cause phenotypic suppression of filamentation in a His^c mutant. D-Cycloserine reduces the number of pentapeptide side chains in peptidoglycan and increases the number of tripeptides (21), an effect caused by inhibition of D-alanine-D-alanine ligase and alanine racemase (32). The resulting imbalance increases septation at the expense of elongation, giving rise to rounded cells in the wild type and restoring cell division in $FtsI^-$ mutants (3).

His^c strains formed wrinkled colonies on both green plates and E plates containing 2% glucose. In the presence of D-cycloserine (20 mg/liter), the colonies formed by the $HisO^c$ strain TR6753 and those formed by the wild type were identical: smooth and small. For microscopic observation of cultures, strains LT2 and TR6753 were grown in E medium containing 2% glucose and 15 mg of D-cycloserine per liter. Exponential cultures were transferred to agar-coated slides and photographed under phase-contrast optics (7). Strain LT2 formed rounded cells (data not shown). Strain TR6753 underwent nearly complete suppression of filamentation and formed rod-shaped cells (Fig. 4). Because these results strongly resemble those obtained with *ftsI* mutants of *E. coli* (3), we hypothesize that D-cycloserine may suppress the septation defect of His^c strains by increasing PBP3 substrate. If this view is correct, the cell division defect of His^c strains may be tentatively reformulated as a shortage of tripeptide side chains, the proposed substrate of the septal machinery (21).

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