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Lack of Stage-Specific Proteins in Coccoid *Helicobacter pylori* Cells

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Helicobacter pylori **exists in two distinct forms, rod shaped or coccoid, in stomachs of infected patients. Based on in vitro proteome comparisons, there are no detectable coccoid-specific proteins, which argues against the specific adaptation of coccoid *Helicobacter* to distinct biological functions, such as enhanced persistence or transmission to other hosts.**

The gram-negative bacterium *Helicobacter pylori* is an important human pathogen that infects half of the world's population and can cause gastritis, gastric and duodenal ulcers, and gastric cancer (26). Electron micrographs of gastric biopsy samples reveal *Helicobacter* cells with elongated rod-shaped or coccoid morphologies in variable proportions (4, 17). The functional relevance of this dimorphism is unclear, but the predominance of rods in exponentially growing in vitro cultures suggest that this form represents proliferating *Helicobacter* cells.

Coccoid forms dominate aging poststationary cultures and are difficult to recultivate, which could suggest that this morphology merely represents degrading dead *Helicobacter* cells. Indeed, various physiological parameters and lack of infectivity support the passive decay of coccoid cells (6, 11, 23). Moreover, similar changes in morphology also occur in *Helicobacter* cells after killing by bacteriophage ϕX174 protein E-mediated lysis (18). On the other hand, coccoid *Helicobacter* cells could also represent a viable-but-not-culturable state that is more resistant to environmental stresses than actively proliferating cells, and could thereby facilitate transmission to new hosts, or might mediate relapsing infection after incomplete eradication (21, 27). Data on various cellular activities seem to support this view (5, 13–15). Interestingly, mutations in *cdrA*, a cell division-related gene, prevent the transition of rods to coccoid cells, potentially suggesting an active process (24). In addition, proteome studies previously demonstrated the appearance of several distinct protein species in coccoid *Helicobacter* total lysates (7) as well as in extracts of surface-associated proteins (16), but these coccoid-specific protein species have not yet been identified. Recent reports about the heterogeneity of coccoid *Helicobacter* with a viable A form and a nonviable B form might partially resolve this long controversy (19, 20). However, even if some coccoid *Helicobacter* cells remain viable and potentially infectious, it is unclear if such cells have distinct properties that could be of relevance for specific aspects of infection or transmission. In the well-characterized dimorphic bacterium *Bacillus anthracis* (12), transition between forms with distinct functions (i.e., proliferation versus long-term survival) is accompanied by extensive changes in protein composition with de novo synthesis of many stage-specific proteins. Whether such dramatic protein expression changes also occur during the transition between rod-shaped and coccoid forms of *Helicobacter* cells remained largely unclear.

To address this issue on a global scale, we compared the protein compositions of rod-shaped *Helicobacter pylori* strain 26695 cells (25) from exponentially growing liquid cultures (Fig. 1A) and coccoid cells arising after 7 days of continuous liquid culture (Fig. 1B). Based on scanning electron microscopy data and the assumption of simple spherical or cylindrical shapes, coccoid *Helicobacter* had, on average, a 40% lower cell volume than rod-shaped *Helicobacter* in fresh cultures (0.23 versus 0.39 μm^3). Transmission electron microscopy revealed that both A (Fig. 1C) and B forms (Fig. 1D) of coccoid *Helicobacter* (19, 20) were present in our long-term cultures. Two-dimensional gel electrophoresis (9, 10) of rod-shaped and coccoid cells revealed some 1,500 protein species that are reproducibly present under both culture conditions but also a few reproducibly stage-specific protein species (Fig. 2) (the pattern for fresh cultures is freely available at http://www.mpiib-berlin.mpg.de/2D-PAGE/) in agreement with previous low-resolution data (7). Peptide mass fingerprinting (9, 10) allowed the identification of all 16 detectable rod-shape-specific proteins and 11 of the 14 coccoid-specific proteins (Table 1).

Two rod-specific protein species, vacuolating toxin VacA (spot r_4) and the serine protease HtrA (spot r_8), had high staining intensities in fresh cultures. The important virulence factor VacA has previously been shown to be absent in coccoid *Helicobacter* (16) despite the presence of *vacA* mRNA (25). The outer membrane protein VacA is known to be prone to autoproteolysis resulting in at least two released fragments (2), which could explain its disappearance in coccoid cells. HtrA is secreted by *Helicobacter* (2), and this might contribute to its disappearance. In addition, destruction of this protease by autoproteolysis might also occur. All other protein species that were exclusively present in rod-shaped *Helicobacter* had a low abundance even at this stage. Among these weak spots there were three minor degradation products of the important virulence factor CagA (spots r_1, r_2, and r_7). However, the major full-length CagA species was preserved in coccoid *Hel-*

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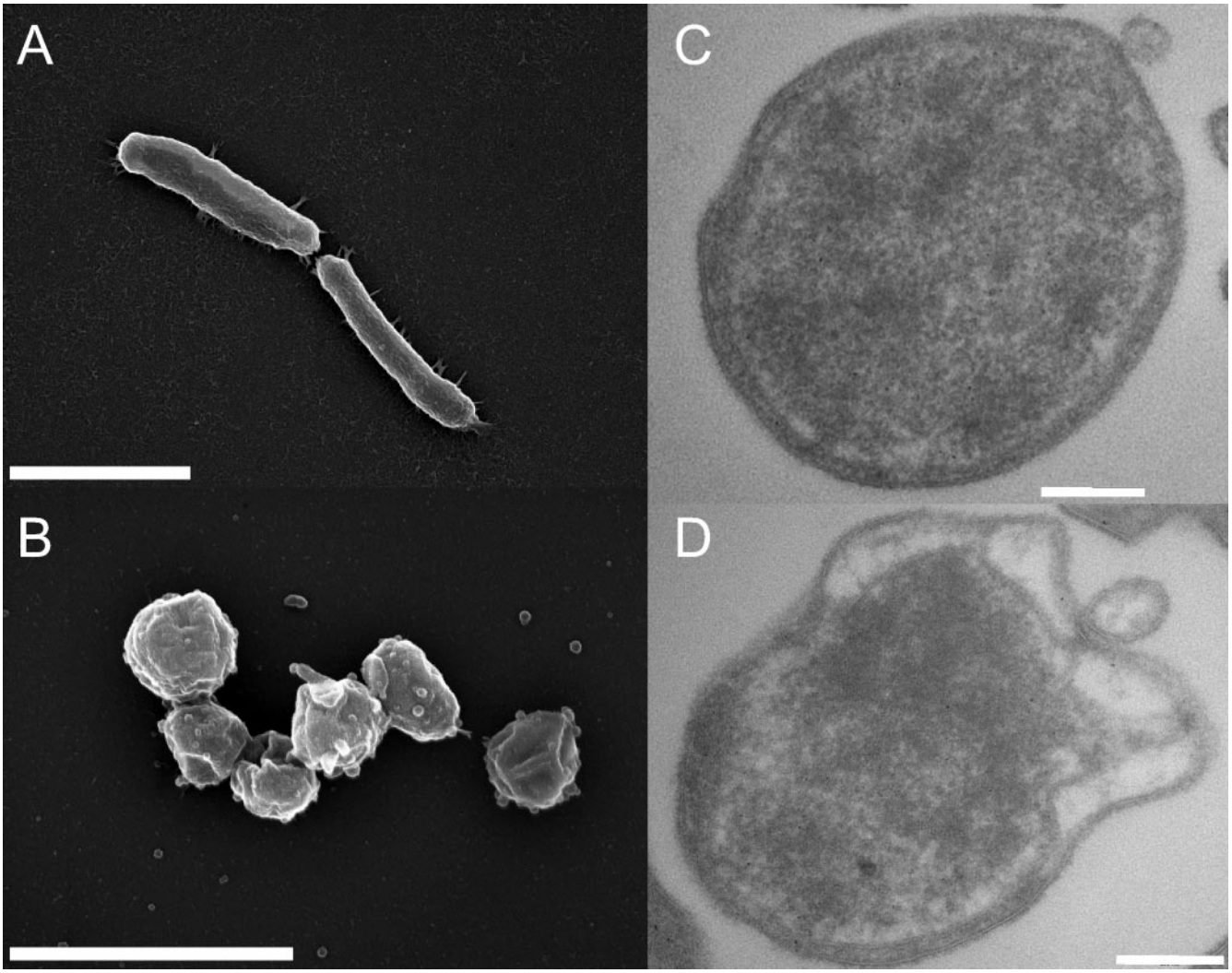

FIG. 1. Scanning (A, B) and transmission (C, D) electron microscopy of rod-shaped (A) and coccoid (B, C, D) *Helicobacter* cells. Aging cultures contained both apparently intact form A (C) and substantially damaged form B (D) coccoid cells. The scale bars represent 2 (A, B) or 200 nm (C, D).

icobacter in agreement with previous studies (3, 22). Other minor rod-shape-specific protein species included various enzymes and proteins of unknown function (Table 1).

Coccoid-specific protein species were all moderately expressed and belong to diverse functional classes, including chaperones, enzymes, a ribosomal protein, and the important virulence factor urease. However, although the identified protein species were coccoid stage specific, they all were merely minor variants of previously identified proteins (9, 10) that are highly abundant in both rod-shaped and coccoid *Helicobacter* cells (Fig. 2), indicating that posttranslational modification, but not de novo synthesis during the transition to coccoid cells, resulted in coccoid-specific protein species. The majority of coccoid-specific protein species seem to have resulted from partial proteolysis to smaller fragments as indicated in Fig. 2. On the other hand, both ureases A and B (spot c_1) and Hsp60 (spots c_2 and c_3) formed high-molecular-weight complexes that were stable under the denaturing electrophoresis conditions, suggesting the formation of covalent bonds between different monomers in initially reversibly assembled tetradecamers of Hsp60 (1) or heterododecamers of ureases A and B (8), respectively. Finally, some protein species with shifted pI values indicate removal or introduction of charges, and the lower electrophoretic mobility of Rsp6 might suggest an altered three-dimensional conformation.

The apparent absence of stage-specific protein expression argues against major adaptive changes in coccoid *Helicobacter*, but the detected posttranslational modifications still might result in altered properties and functions that could have some relevance for *Helicobacter* infection biology. On the other hand, all detected modifications accounted for only tiny fractions of the respective proteins, whereas the overwhelming majority of the corresponding gene products was not differentially modified. In conclusion, our data argue against a specific adaptation of coccoid *Helicobacter* for particular tasks.

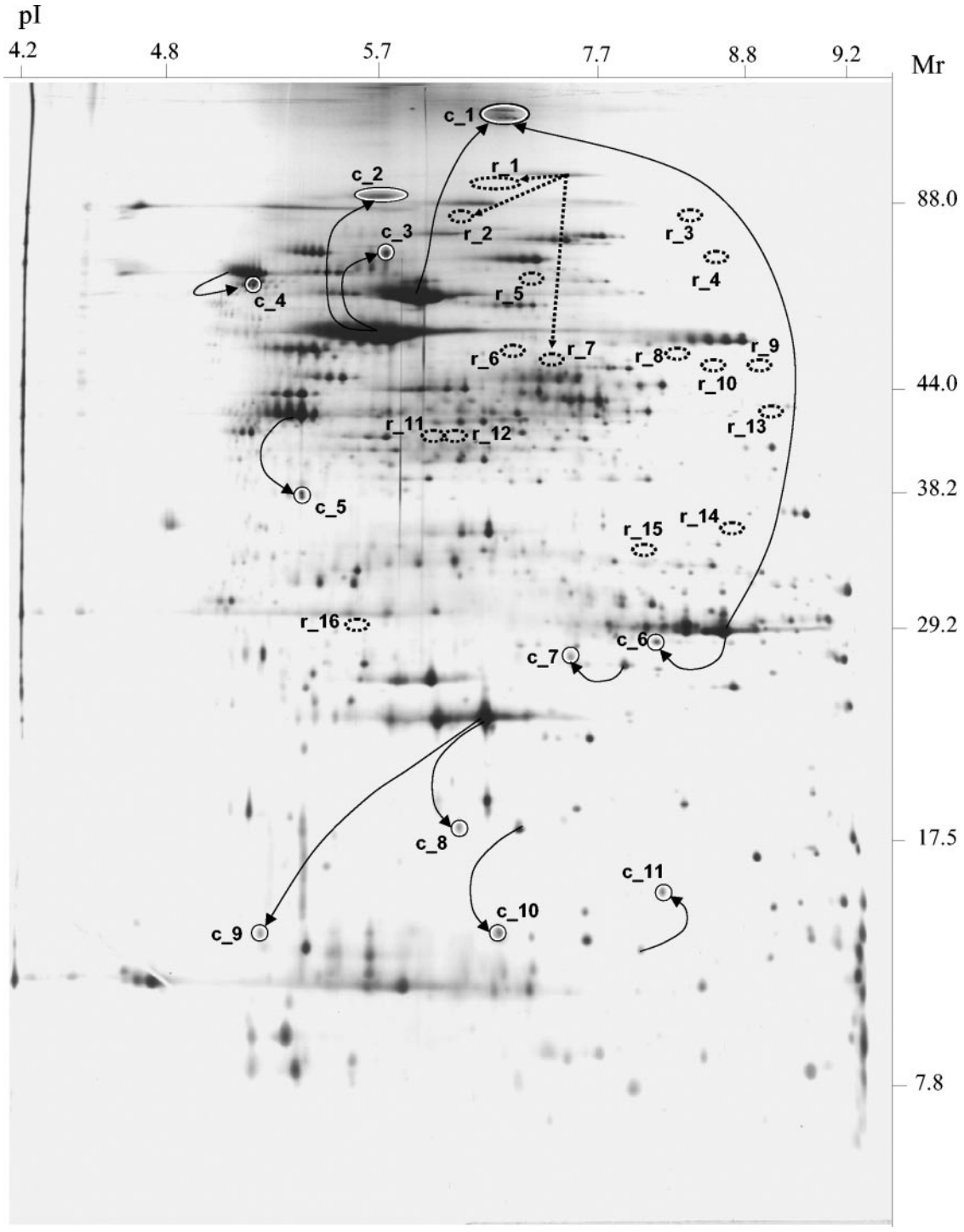

FIG. 2. Protein composition of coccoid *Helicobacter* cells as determined by two-dimensional gel electrophoresis of bacteria harvested after 7 days of liquid culture. Proteins were detected by silver staining. The spot numbers correspond to those shown in Table 1. Spots r_1 to r_16 (rod shape specific) are absent in coccoid *Helicobacter*, while spots c_1 to c_11 are coccoid specific. The straight arrows indicate relationships between major and minor variants of individual proteins. The dotted arrows connect the major CagA variant with three minor variants that are only detected in rod-shaped *Helicobacter*.

TABLE 1. Identified *H. pylori* proteins with differential presence in rod-shaped and coccoid cells

Spot no.[a]	Locus	Gene	Protein	Sequence coverage (%)	Theoretical mol wt	Theoretical pI
r_1	HP0547	*cagA*	CagA	30	132,306	8.8
r_2	HP0547	*cagA*	CagA	29	132,306	8.8
r_3	HP0887	*vacA*	Vacuolating cytotoxin	18	139,227	9.0
r_4	HP0407	*bisC*	Biotin sulfoxide reductase	42	90,014	9.1
r_5	HP1527		Hypothetical protein	25	54,750	6.6
r_6	HP0075	*ureC*	Urease protein C	39	49,055	6.4
r_7	HP0547	*cagA*	CagA	26	132,306	8.8
r_8	HP1019	*htrA*	Serine protease	47	47,954	9.0
r_9	HP0269		Conserved hypothetical ATP-binding protein	30	49,392	8.8
r_10	HP0605		Hypothetical protein	33	54,553	9.0
r_11	HP1179	*deoB*	Phosphopentomutase	38	46,151	5.9
r_12	HP0774	*tyrS*	Tyrosyl-tRNA synthetase	32	45,710	5.9
r_13	HP1126	*tolB*	Colicin tolerance-like protein	79	47,768	9.2
r_14	HP0854	*guaC*	GMP reductase	39	36,016	8.6
r_15	HP0353	*ansB*	L-Asparaginase II	46	35,516	8.6
r_16	HP0724	*fliH*	Flagellar export protein	15	29,303	5.4
c_1	HP0072/HP0073	*ureA*/*ureB*	Urease alpha subunit/urease beta subunit	52/37	26,523/61,645	8.5/5.6
c_2	HP0010	*groEL*	Chaperone and heat shock protein 60	29	58,228	5.6
c_3	HP0010	*groEL*	Chaperone and heat shock protein 60	28	58,228	5.6
c_4	HP0109	*dnaK*	Chaperone and heat shock protein 70	36	67,011	5.0
c_5	HP1205	*tufB*	Translation elongation factor EF-Tu	43	43,620	5.2
c_6	HP0072	*ureA*	Urease alpha subunit	53	26,523	8.5
c_7	HP0561	*fabG*	3-Ketoacyl-acyl carrier protein reductase	43	26,652	7.8
c_8	HP1563	*tsaA*	Alkyl hydroperoxide reductase	46	22,221	5.9
c_9	HP1563	*tsaA*	Alkyl hydroperoxide reductase	43	22,221	5.9
c_10	HP0390	*tagD*	Adhesin-thiol peroxidase	56	18,281	7.7
c_11	HP1246	*rps6*	Ribosomal protein S6	65	16,961	6.9

[a] r_1 to r_16 designate rod-shape-specific spots; c_1 to c_11 designate coccoid-shape-specific spots.

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