Epithelial cell contact-induced alterations in *Salmonella enterica* serovar Typhi lipopolysaccharide are critical for bacterial internalization

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Summary

The invasion of *Pseudomonas aeruginosa* and *Salmonella enterica* serovar Typhi into epithelial cells depends on the cystic fibrosis transmembrane conductance regulator (CFTR) protein as an epithelial receptor. In the case of *P. aeruginosa*, the bacterial ligand for CFTR is the outer core oligosaccharide portion of the lipopolysaccharide (LPS). To determine whether serovar Typhi LPS is also a bacterial ligand mediating internalization, we used both *P. aeruginosa* and serovar Typhi LPS as a competitive inhibitor of serovar Typhi invasion into the epithelial cell line T84. *P. aeruginosa* LPS containing a complete core efficiently inhibited serovar Typhi invasion. However, neither killed wild-type Typhi cells nor purified LPS were effective inhibitors. LPS from mutant Typhi strains defective in O side-chain synthesis, but with an apparently normal core, was capable of inhibiting invasion, but LPS obtained from a deeper rough mutant strain with alterations in fast-migrating core oligosaccharide failed to inhibit invasion. Lastly, exposure of wild-type serovar Typhi to T84 cultures before heat killing resulted in a structural alteration in its LPS that allowed the heat-killed cells to inhibit invasion of wild-type serovar Typhi. These data indicate that the serovar Typhi LPS core, like the *P. aeruginosa* LPS core, is a ligand mediating internalization of bacteria by epithelial cells, and that exposure of this ligand on wild-type Typhi is induced by the bacteria's interaction with host cells.

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Introduction

Salmonella enterica serovar Typhi initiates infection by translocating from the gastrointestinal lumen to the submucosa. We have shown recently that the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride ion channel expressed on many secretory epithelia, is an intestinal epithelial cell receptor for serovar Typhi (Pier *et al.*, 1998). Cell lines lacking functional CFTR internalize fewer serovar Typhi bacteria than do cell lines expressing normal CFTR. Additionally, transgenic mice heterozygous for the murine ΔF508 *Cftr* allele (resulting in reduced quantities of cell surface CFTR) translocate 86% fewer serovar Typhi to the submucosa after inoculation into the gastrointestinal (GI) lumen compared with wild-type mice (Pier *et al.*, 1998), and mice homozygous for the ΔF508 *Cftr* allele essentially translocate no serovar Typhi cells to the GI submucosa (Pier *et al.*, 1998). The receptor site on CFTR for serovar Typhi has been determined to be the first predicted extracellular loop, encompassing amino acids 103–117. Monoclonal antibodies to CFTR peptide 103–117 and the corresponding synthetic CFTR peptide prevent the binding of serovar Typhi to this extracellular domain and inhibit bacterial uptake.

To characterize further the early interactions between serovar Typhi and intestinal epithelial cells, we sought to determine whether serovar Typhi lipopolysaccharide (LPS) is a bacterial ligand that is necessary for invasion into epithelial cells. LPS was of interest, as we have shown (Pier *et al.*, 1996a,b; Zaidi *et al.*, 1996) that LPS serves as the CFTR ligand in the case of *Pseudomonas aeruginosa*, which uses the same portion of CFTR as a host cell receptor (Pier *et al.*, 1997). Mutations in biosynthetic genes that result in a truncation of the *P. aeruginosa* LPS core polysaccharide prevent bacterial binding and internalization by epithelial cells. Mutations resulting in only the loss of synthesis of the *P. aeruginosa* LPS O side-chain, while retaining the complete outer core oligosaccharide structure, are without effect (Pier *et al.*, 1996a,b; 1997).

In this report, we show that LPS isolated from *in vitro*-grown wild-type serovar Typhi cells did not inhibit bacterial ingestion, whereas LPS from *in vitro*-grown *P. aeruginosa* did. Further investigation into this phenomenon revealed that a phenotypic change in the serovar Typhi LPS was

needed to expose the microbial ligand, and this change occurred secondary to bacterial contact with epithelial cells. Constitutive production of the serovar Typhi LPS ligand was found on a mutant strain with a *mudJ* insert in the *hisD* gene just upstream of the O side-chain biosynthetic locus. This strain synthesized an LPS with greatly diminished amounts of O side-chains, but with an apparently intact LPS outer core. In contrast, LPS from a mutant strain with a *mudJ* insert in the *cps*G gene that encodes a phosphomannomutase enzyme totally lacked O side-chains, expressed a core region with faster electrophoretic mobility and failed to inhibit serovar Typhi cell entry into epithelial cells. Collectively, these data suggest that a dynamic interaction occurs between the host epithelium and serovar Typhi, resulting in exposure of the bacterial ligand on the LPS that is needed for CFTR-mediated bacterial uptake.

Results

Effect of LPS extracted from P. aeruginosa *or serovar Typhi on internalization of live serovar Typhi by T84 cells*

Given that amino acids 103–117 in the CFTR protein function as the host cell's molecular receptor for both *P. aeruginosa* (Pier *et al.*, 1996a,b; 1997) and serovar Typhi (Pier *et al.*, 1998), and that the *P. aeruginosa* ligand for CFTR is the outer core oligosaccharide on the LPS (Pier *et al.*, 1996a,b), we anticipated that the serovar Typhi LPS would also function as the bacterial cell ligand for entry into T84 cells. To test this assumption, we compared the ability of 10 μg of LPS purified from *P. aeruginosa* strains producing complete or incomplete core oligosaccharide to inhibit ingestion of three strains of wild-type serovar Typhi cells by the human T84 colonic epithelial cell line (Table 1; Fig. 1). This amount of LPS competitor was chosen based on the estimate of Raetz (1990) as to the number of molecules of LPS per cell of Gram-negative organisms ($\approx 2 \times 10^6$ molecules per organism). Thus, there would be 4×10^{12} molecules of LPS on the surface of 2×10^6 cfu of live invading serovar Typhi. The total volume of 2×10^6 serovar Typhi cells, calculated by assuming ellipsoids 1 μm long and 0.5 μm in both axial directions, is $\approx 2.6 \times 10^5\ \mu m^3$ $\{2 \times 10^6 \times (4/3[\pi(0.25\ \mu m)(0.25\ \mu m)(0.5\ \mu m)])\}$. If there are 4×10^{12} LPS molecules in this volume, there would be an effective concentration of $\approx 1.5 \times 10^{19}$ LPS molecules ml^{-1} ($1\ ml = 10^{12}\ \mu m^3$). This corresponds to a molar concentration of 25 mM. Using the molecular weight of *P. aeruginosa* complete-core LPS (≈3500 Da) to determine the concentration of competitor when 10 μg of LPS is suspended in 0.1 ml ($\approx 1.7 \times 10^{16}$ LPS molecules ml^{-1} or 28 μM), we estimated that this concentration of inhibitor LPS was ≈ 0.1% of the effective concentration of the surface-bound LPS on serovar Typhi cells. As shown in Fig. 1, we observed that serovar Typhi entry into epithelial cells could be inhibited by *P. aeruginosa* LPS containing a complete core oligosaccharide (LPS from strain PAC 557) but not by the control *P. aeruginosa* LPS with an incomplete core (strain PAC1R*algC::tet*). The inhibition effect of PAC 557 LPS was diminished with lower amounts of inhibitory LPS; however, no inhibition was observed with PAC1R*algC::tet* LPS at any concentration tested (data not shown). This indicates that the *P. aeruginosa* LPS core oligosaccharide expresses a ligand that inhibits entry of serovar Typhi into epithelial cells.

Surprisingly, however, the LPS isolated from strain Ty2 itself was not able to inhibit invasion of live serovar Typhi (data not shown). To determine whether the lack of inhibition by serovar Typhi Ty2 LPS was attributable to the conditions used for its purification, we next attempted to inhibit the invasion of live serovar Typhi using whole bacterial cells grown in culture and then heat killed (65°C, 2 h) before the inhibition assay. As controls, we used as inhibitors heat-killed *P. aeruginosa* strains PAC 557 and PAC1R*algC::tet*, the respective cellular sources for the inhibitory and non-inhibitory LPS. For this experiment, we used a 50-fold

Table 1. Bacterial strains used in this study.

Strain	Phenotype	Source or reference
Salmonella enterica serovar Typhi		
Ty2	Wild type, smooth LPS	J. Robbins (Bethesda, MD, USA)
7251		ATCC
167		ATCC
Ty21a	Vaccine strain, chemical mutant, rough LPS	ATCC
MEI 120	*mudJ* insert in the *hisD* gene. Diminished amounts of O side-chains, but retains outer core	This study
MEI 126	*mudJ* insert in *cps*G gene (encodes phosphomannomutase) Lacks O side-chains and has a fast-migrating LPS component, which differs in mobility from the fast-migrating component of wild-type serovar Typhi	This study
Pseudomonas aeruginosa		
PAC557	LPS rough, complete outer core	Rowe and Meadow (1983)
PAC1R *algC*::tet	LPS rough, lacks outer core sugars	Coyne *et al.* (1994)

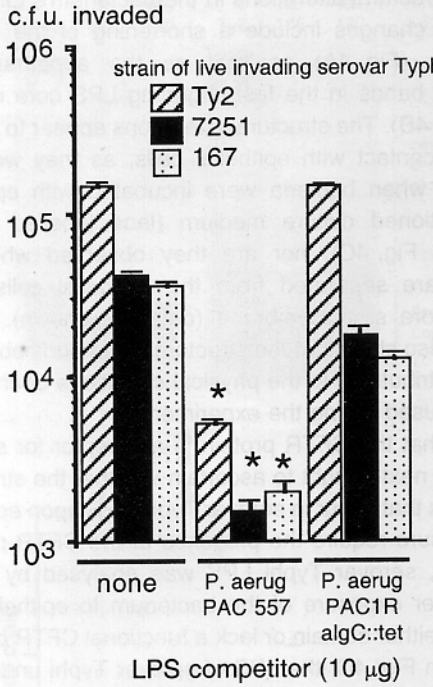

Fig. 1. Inhibition of invasion of three strains of serovar Typhi into epithelial cells by rough *P. aeruginosa* (P aerug) LPS. LPS used were from *P. aeruginosa* expressing a complete core oligosaccharide (strain PAC 557) and incomplete core oligosaccharide lacking the cell entry ligand (strain PAC1RalgC::tet). The competitor LPS were used to inhibit the invasion into epithelial cells by serovar Typhi strains Ty2, 7251 or 167. Bars represent means and error bars the SD. *Significantly different from no competitor ($P < 0.01$, ANOVA and Fisher PLSD).

excess of heat-killed cells over live serovar Typhi cells because of the loss of motility of the killed cells, which reduces their ability to compete with the live serovar Typhi cells for entry into T84 cells. Uptake of serovar Typhi Ty2 was inhibited by *P. aeruginosa* cells containing a complete LPS core polysaccharide, but not by *P. aeruginosa* expressing LPS with an incomplete core, nor by serovar Typhi Ty2 cells (Fig. 2). In addition, antibiotic-killed serovar Typhi cells, which would contain heat-labile surface antigens, also did not inhibit live Ty2 bacteria from being ingested by T84 cells (data not shown). Thus, the serovar Typhi ligand for entry into epithelial cells was not present on organisms grown in LB broth *in vitro*.

Effect of exposure of serovar Typhi Ty2 to T84 cells on expression of bacterial ligands for cell entry

It was hypothesized that heat-killed serovar Typhi Ty2 cells and purified serovar Typhi Ty2 LPS failed to inhibit epithelial cell uptake of live serovar Typhi because live bacteria enter epithelial cells using a ligand not expressed until after the bacterial cells interact with host epithelial cells. Therefore, serovar Typhi Ty2 bacteria were exposed to T84 epithelial cells for 4 h, extracellular and intracellular bacteria recovered, bacterial concentrations determined, and then the organisms were heat killed. Using these epithelial cell-exposed bacteria as inhibitors of ingestion of live serovar Typhi Ty2, we found that bacteria exposed to epithelial cells expressed a cellular ligand capable of inhibiting internalization of live serovar Typhi Ty2 (Fig. 3). This inhibition by T84-exposed serovar Typhi Ty2 cells is evident whether the competitor Ty2 was obtained from the outside of the T84 cells or had been internalized by the T84 cells before their recovery, although the extracellular bacterial cells were somewhat more potent inhibitors of ingestion than were the fully internalized serovar Typhi Ty2 cells. To exclude the possibility that induction of the cell entry ligand resulted from a change in pH when bacteria were transferred from the bacterial growth medium to the cell culture medium, as has been shown to underlie induction of type III secretion by *Salmonella enterica* serovar Typhimurium (Daefler, 1999), we grew serovar Typhi cells in LB, washed them, exposed them to tissue culture medium at pH 8, then heat killed them and used them as inhibitors of ingestion of wild-type serovar Typhi Ty2. The pH 8-exposed serovar Typhi cells did not inhibit ingestion of wild-type serovar Typhi, whereas the control inhibitor, serovar Typhi cells exposed to T84 cells, was highly inhibitory (data not shown).

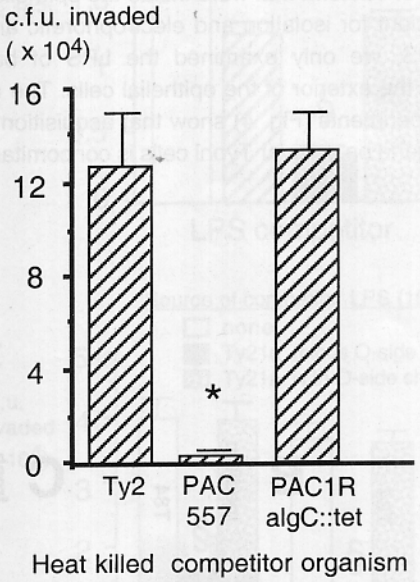

Fig. 2. Inhibition of invasion of serovar Typhi strain Ty2 into epithelial cells by 2×10^8 cfu of intact, heat-killed serovar Typhi strain Ty2 cells or by intact, heat-killed *P. aeruginosa* cells. *P. aeruginosa* strain PAC 557 expresses a complete core oligosaccharide, whereas strain PAC1RalgC::tet expresses an incomplete core LPS lacking the cell entry binding site. Bars represent means and error bars the SD. *Significantly different from Ty2 competitor ($P < 0.001$, ANOVA and Fisher PLSD).

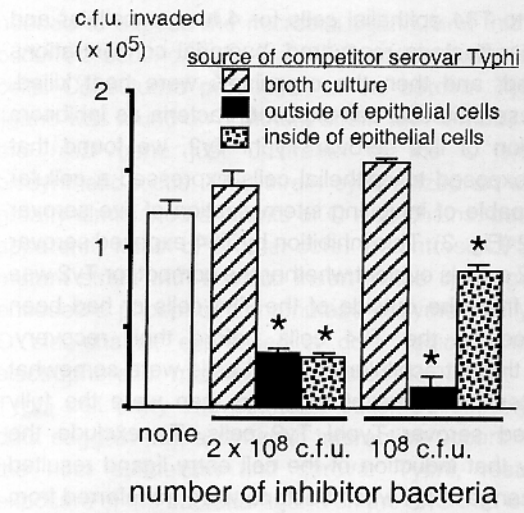

Fig. 3. Inhibition of invasion of serovar Typhi strain Ty2 into epithelial cells by intact, heat-killed Ty2 when the competitor is recovered from either broth culture or after a 4 h exposure to epithelial cells. Serovar Typhi was recovered from the exterior or the interior of infected T84 cells, washed with PBS, heat killed and used as a competitor. Bars represent means and error bars the SD. *Significantly different from no competitor ($P \leq 0.02$, ANOVA and Fisher PLSD).

To determine the molecular basis of the alteration in the cell entry ligand that occurs during interaction of serovar Typhi with epithelial cells, we analysed by SDS–PAGE the LPS structure expressed by serovar Typhi before and after exposure of the bacteria to epithelial cells. Because the number of bacteria that were inside the epithelial cells was insufficient for isolation and electrophoretic analysis of their LPS, we only examined the LPS of bacteria adherent to the exterior of the epithelial cells. The results of these experiments (Fig. 4) show that acquisition of the cell entry ligand on serovar Typhi cells is concomitant with several structural alterations in the bacterium's LPS. The observed changes include a shortening of the LPS O side-chain (Fig. 4A) as well as the appearance of additional bands in the fast-migrating LPS core component (Fig. 4B). The structural alterations appear to require bacterial contact with epithelial cells, as they were not observed when bacteria were incubated with epithelial cell-conditioned culture medium (lane labelled 'conditioned' in Fig. 4C), nor are they observed when the bacteria are separated from the epithelial cells by a 0.1 μm pore size membrane (data not shown). These controls also show that the structural alterations observed are not attributable to the physical conditions or chemical reagents used during the experiment.

Given that the CFTR protein is a receptor for serovar Typhi, we next sought to ascertain whether the structural alterations that occur in serovar Typhi LPS upon epithelial cell exposure require the presence of the CFTR protein. Therefore, serovar Typhi LPS was analysed by SDS–PAGE after exposure of the bacterium to epithelial cell lines that either contain or lack a functional CFTR protein. As seen in Fig. 4C, the LPS of serovar Typhi undergoes identical structural alterations whether the bacterium is exposed to CFT1-LCFSN (expressing wild-type CFTR) or CFT1-ΔF508 (containing only the ΔF508 *Cftr* allele) cells. These data show that the structural shifts in LPS that accompany acquisition of the cell entry ligand do not rely on bacterial interactions with CFTR, consistent with the view that LPS alterations must occur before and independently of the interaction with CFTR.

Serovar Typhi LPS mutants define an epithelial cell binding site on the LPS

One major difference between *Salmonella* LPS and *P.*

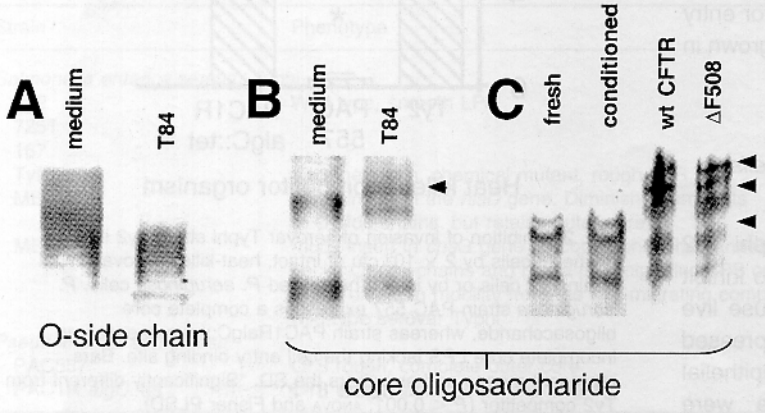

Fig. 4. Co-culture of serovar Typhi strain Ty2 with epithelial cells results in structural alterations in the serovar Typhi LPS.
A. LPS was recovered from serovar Typhi strain Ty2 that had been cultured with either fresh T84 growth medium or T84 cells and analysed by SDS–PAGE under conditions that allowed resolution of the O side-chain. Note that exposure to epithelial cells results in a shortening of the O side-chains.
B. LPS prepared as described in (A) was analysed by SDS–PAGE under conditions that allowed resolution of the core oligosaccharide. Note that exposure to epithelial cells results in the appearance of a novel band marked by an arrowhead.
C. A similar experiment was performed using the isogenic epithelial cell lines LCFSN (with a wild-type copy of the *Cftr* gene) and CFT1-ΔF508 (carrying only the ΔF508 *Cftr* allele). LPS was recovered from serovar Typhi strain Ty2 that had been cultured with either fresh growth medium ('fresh'), cell-conditioned medium ('conditioned'), LCFSN ('wt CFTR') cells or CFT1-ΔF508 cells ('ΔF508').

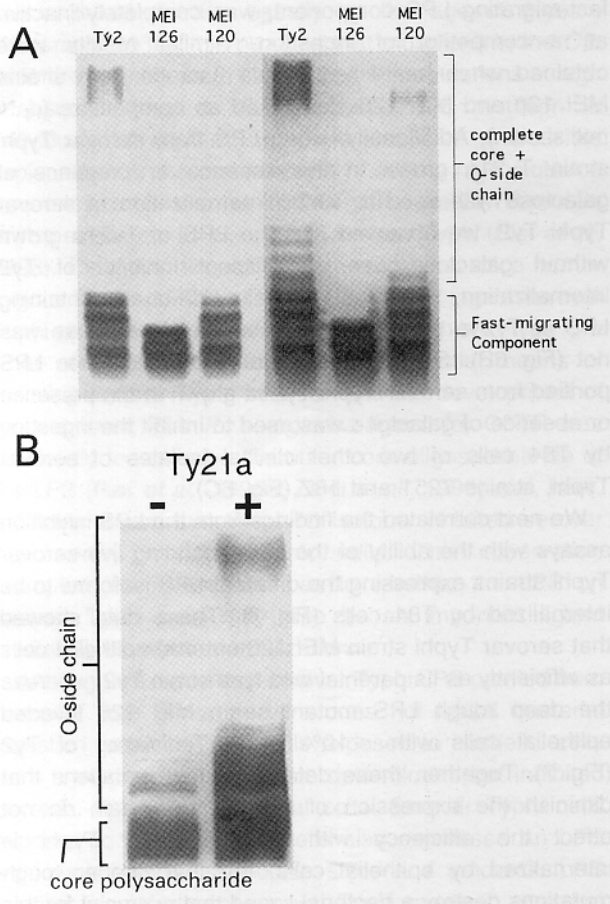

Fig. 5. Phenotypes of serovar Typhi LPS.
A. SDS–PAGE of LPS from serovar Typhi Ty2 and mutant strains MEI 126 and MEI 120. The two panels shown depict two gels loaded with different quantities of LPS and silver stained to different intensities. The panel on the left demonstrates the mobility differences in the fast-migrating components of the LPS from these three strains, whereas the panel on the right shows that strain MEI 120 does in fact produce O side-chain, albeit in lower amounts.
B. The LPS banding pattern of the mutant strain Ty21a grown in LB (lane labelled '–'), or in LB with exogenous galactose added to 0.5% (lane labelled '+').

aeruginosa LPS is that the LPS isolated from smooth enteric strains is heavily substituted with O side-chains (Peterson and McGroarty, 1985), whereas over 90% of LPS molecules on the surface of *P. aeruginosa* lack O side-chains (Rivera *et al.*, 1988). We hypothesized that the serovar Typhi LPS O side-chain present on broth-grown wild-type Typhi cells blocks the LPS core from binding to its epithelial cell receptor, and that exposure of serovar Typhi to epithelial cells results in loss of O side-chains or in the expression of newly synthesized LPS containing a smaller amount of O side-chain. To test this

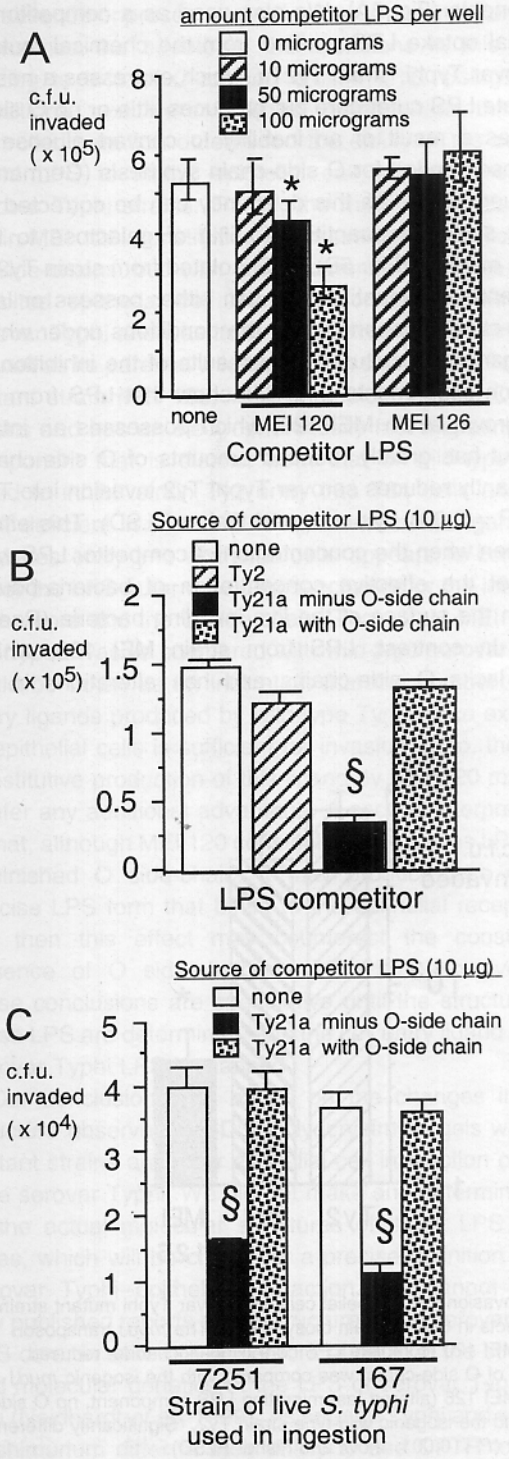

Fig. 6. Inhibition of invasion of serovar Typhi strain Ty2 into epithelial cells by mutant serovar Typhi LPS.
A. LPS from strain MEI 120 (deficient in O side-chain but containing a complete core oligosaccharide) was compared with LPS from the isogenic strain MEI 126 (lacking O side-chain and with a unique fast-migrating component).
B and C. LPS from mutant serovar Typhi strain Ty21a grown in LB (minus O side-chain) or in LB containing 0.5% galactose (with O side-chain) was used to inhibit the uptake of live serovar Typhi strain Ty2 (B) and that of live serovar Typhi strains 7251 and 167 (C). Bars represent means and error bars the SD. * and §Significantly different from no competitor (ANOVA and Fisher PLSD). $*P = 0.0001$; $§P < 0.01$.

hypothesis, we isolated LPS from different mutant strains of serovar Typhi with defects in O side-chain substitution and used these LPS to inhibit competitively epithelial cell invasion by wild-type serovar Typhi strain Ty2. MEI 120 and MEI 126 are two *mudJ* transposon mutant strains derived from serovar Typhi strain Ty2 (Table 1), which produce LPS deficient in O side-chains and with differing mobilities in SDS–PAGE of the faster migrating LPS components (Fig. 5A). We also used as a competitor of bacterial uptake LPS purified from the chemical mutant of serovar Typhi, strain Ty21a, which expresses a nearly complete LPS outer core but produces little or no O side-chain as a result of an inability to convert glucose to galactose needed for O side-chain synthesis (Germanier and Fuer, 1975). As this deficiency can be corrected by adding sublethal quantities (0.5%) of galactose to the culture medium (Fig. 5B), LPS isolated from strain Ty21a represents a competitor that can either possess or lack O side-chains, depending on the conditions under which the organism is cultured. The results of the inhibition of ingestion experiments (Fig. 6A) show that LPS from LB broth-grown strain MEI 120, which possesses an intact core but has greatly reduced amounts of O side-chain, significantly reduces serovar Typhi Ty2 invasion into T84 cells ($P = 0.001$ by ANOVA and Fisher PLSD). This effect was seen when the concentration of competitor LPS was $\approx 1\%$ of the effective concentration of bacteria-bound LPS on the surface of the live invading bacteria (Raetz, 1990). In contrast, LPS from strain MEI 126, which totally lacks O side-chains and has alterations in its fast-migrating LPS component, was completely inactive as a competitor of ingestion. Similar results were obtained when heat-killed, whole bacteria from strains MEI 120 and MEI 126 were used as competitors (data not shown). Additionally, when LPS from serovar Typhi strain Ty21a grown in the absence or presence of galactose was used to inhibit internalization of serovar Typhi Ty2, we observed that the LPS of Ty21a grown without galactose was an efficient inhibitor of Ty2 internalization, whereas the O side-chain-containing LPS of Ty21a grown in the presence of galactose was not (Fig. 6B). Similar results were obtained when LPS purified from serovar Typhi Ty21a grown in the presence or absence of galactose was used to inhibit the ingestion by T84 cells of two other clinical isolates of serovar Typhi, strains 7251 and 167 (Fig. 6C).

We next correlated the findings from the LPS inhibition assays with the ability of the corresponding live serovar Typhi strains expressing the different LPS isoforms to be internalized by T84 cells (Fig. 7). These data showed that serovar Typhi strain MEI 120 entered epithelial cells as efficiently as its parental wild-type strain Ty2, whereas the deep rough LPS mutant strain MEI 126 invaded epithelial cells with $< 10\%$ of the efficiency of Ty2 (Fig. 7). Together, these data show that mutations that diminish the expression of LPS O side-chain do not effect the efficiency with which serovar Typhi is internalized by epithelial cells, but that deeper rough mutations destroy a bacterial ligand that is crucial for this event.

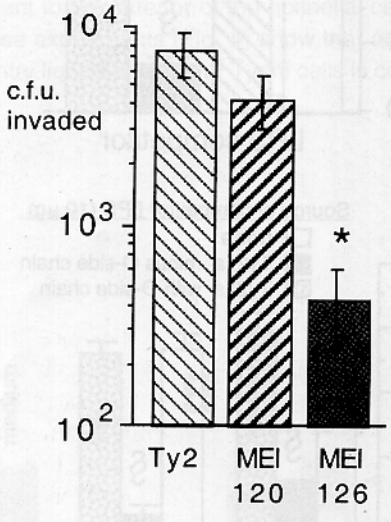

Fig. 7. Invasion into epithelial cells of serovar Typhi mutant strains with defects in O side-chain biosynthesis. The *mudJ* transposon mutant MEI 120 (complete LPS core oligosaccharide, reduced amounts of O side-chain) was compared with the isogenic *mudJ* mutant MEI 126 (altered fast-migrating LPS component, no O side-chain) and the isogenic wild-type strain Ty2. *Significantly different from Ty2 ($P < 0.001$, ANOVA and Fisher PLSD).

Discussion

The CFTR protein has been studied intensely on account of its role in the aetiology of the autosomal recessive disorder of cystic fibrosis (CF; Hundrieser *et al.*, 1990; Schwartz *et al.*, 1990; Tsui, 1990; Livshits *and* Kravchenko, 1996; Tummler *et al.*, 1996). The normal physiological function of this protein is to regulate the secretion of ions across epithelia of many tissues. The loss of CFTR protein function has dramatic negative effects on pancreatic function, nutrient absorption, the development of the male reproductive system and the composition and consistency of secretions of the skin and airways (Andersen, 1938; Andersen and Hodges, 1946; di Sant'Agnese *et al.*, 1953; McIntosh, 1954). We have shown previously that, in addition to this role, the CFTR protein also functions as a receptor for *P. aeruginosa* (Pier *et al.*, 1996a,b; 1997), the primary cause of morbidity and mortality among these patients. The role of the CFTR–*P. aeruginosa* interaction in bacterial uptake may explain why this one species of bacterium has emerged as such a prominent respiratory pathogen in CF patients.

Given that, before modern medical management, CF

was a lethal disease usually by the age of 2 years (Andersen, 1938), it is puzzling that mutations in the *Cftr* gene are maintained in certain human populations at high frequencies (e.g. 4–5% for the ΔF508 mutation in individuals of eastern European descent). A possible explanation for the maintenance of such a high mutation frequency came from the discovery that the CFTR protein is also a receptor for *S. enterica* serovar Typhi (Pier *et al.*, 1998). Cells and tissues from humans and mice homozygous for the ΔF508 allele of *Cftr* did not ingest serovar Typhi cells as well as cells and tissues with wild-type *Cftr* (Pier *et al.*, 1998), indicative of a potential means of resisting serovar Typhi infection and the development of typhoid fever. The *P. aeruginosa* ligand for CFTR was previously found to be the outer core oligosaccharide of the LPS (Pier *et al.*, 1996a; Zaidi *et al.*, 1996) and, here, we have shown that this LPS isoform inhibited serovar Typhi entry in T84 colonic cells. This observation prompted the investigation as to whether the LPS on serovar Typhi cells may also be an important ligand mediating bacterial internalization.

Although we report that serovar Typhi LPS purified from bacterial cells grown *in vitro* does not inhibit bacterial ingestion, serovar Typhi cells exposed to epithelial cells for 4 h acquired a heat-stable determinant that inhibited bacterial uptake by epithelial cells. Serovar Typhi cells exposed to T84 epithelial cells also expressed an LPS with diminished O side-chain sizes and amounts. LPS purified from strains of serovar Typhi grown *in vitro* that have greatly diminished amounts of high-molecular-weight O side-chains, but possess a complete core oligosaccharide, inhibited bacterial ingestion with an efficiency comparable with that of *P. aeruginosa* complete core LPS. This mutant strain did not require interaction with epithelial cells to express the CFTR ligand. Thus, it appears that the LPS on serovar Typhi cells grown in LB that is highly substituted with O-antigens does not express one of the major ligands for T84 epithelial cell ingestion, but that this ligand is expressed after interaction of serovar Typhi with epithelial cells. It must be noted, however, that we have not been able to perform inhibition-of-invasion assays using competitor LPS isolated from bacterial–epithelial co-cultures, because of inability to isolate sufficient quantities of LPS from such co-cultures. The data presented do, however, demonstrate a correlation between LPS structural shifts (diminished O side-chain and appearance of novel core oligosaccharide bands) and the acquisition of the cell entry ligand that can competitively inhibit invasion.

The literature does contain many examples of LPS variation and its role in virulence (Demarco de Hormaeche *et al.*, 1991a,b; Cowley *et al.*, 1996; Guard-Petter *et al.*, 1996). The results of these previous studies suggest several roles for LPS variation, including the development of serum resistance, the modulation of the host immune response (Cowley *et al.*, 1996) and the regulation of bacterial protein export (Guard-Petter *et al.*, 1996). The data in the current study potentially indicate yet another biological role for the alteration of LPS structure during infection.

Particularly noteworthy were the results with LPS purified from serovar Typhi strain Ty21a cells grown in the absence or presence of galactose. The latter condition overcomes the effects of the mutations in this strain leading to loss of O-antigen on the LPS. Using these conditions, we were able to demonstrate that this controlled variation determined the ability of the serovar Typhi LPS to inhibit epithelial cell uptake of several serovar Typhi strains. Finally, the mutant serovar Typhi strain MEI 120, constitutively expressing LPS deficient in O side-chains but with an apparently complete core, inhibited epithelial cell internalization of live wild-type serovar Typhi and was itself internalized by T84 cells just as well as its parental wild-type (Ty2) strain. LPS from the mutant strain MEI 126, which completely lacks O side-chains and has an electrophoretically fast-migrating LPS component that is distinct from that of wild-type LPS, failed to inhibit strain Ty2 entry into T84 cells and was itself deficient in invasion. Thus, the bacterial ligand that mediates entry into epithelial cells appears to comprise either the LPS outer core oligosaccharide or the interface of the core and the O side-chain. That strain MEI 120 is not hyperinvasive compared with wild-type serovar Typhi could be taken as an indication that the number of cell entry ligands produced by wild-type Typhi once exposed to epithelial cells is sufficient for invasion. If so, then the constitutive production of this ligand by MEI 120 may not confer any additional advantage. A second interpretation is that, although MEI 120 constitutively produces LPS with diminished O side-chain, it may produce less of the precise LPS form that binds to the epithelial receptor. If so, then this effect may counteract the constitutive absence of O side-chain on MEI 120. However, all these conclusions are speculative until the structures of these LPS are determined, and the cell entry ligand on the serovar Typhi LPS is mapped.

Our conclusions are based on the changes in LPS structure observed in SDS–polyacrylamide gels with the mutant strains and after epithelial cell interaction of wild-type serovar Typhi. We did not make any determinations of the actual molecular structures of these LPS molecules, which will be critical for a precise definition of the serovar Typhi–epithelial interaction. We cannot locate any published reports of the structure of the serovar Typhi LPS core, in spite of the plethora of data on the structure and molecular genetics of the LPS of serovar Typhimurium (Neidhardt *et al.*, 1999). As serovar Typhi and serovar Typhimurium differ in their ability to use CFTR to enter

epithelial cells (Pier *et al.*, 1998), and as serovar Typhimurium has an LPS core that differs electrophoretically from that of Ty2 (J. B. Lyczak, unpublished observations), it is likely that the LPS core of serovar Typhimurium is different from that of serovar Typhi. In addition, given that *P. aeruginosa* LPS core binds the same part of CFTR as does serovar Typhi LPS core, it is likely that parts of these two structures will be similar. We are currently producing large amounts of the serovar Typhi LPS from strains Ty2, MEI 120 and MEI 126, both to determine the chemical structure of the serovar Typhi LPS core and to elucidate the critical saccharide residues involved in the interaction of serovar Typhi LPS with its epithelial cell receptor.

Other ligand–receptor interactions are probably also important in serovar Typhi–host epithelial cell binding and may also be important for generating the cell entry ligand on the LPS after bacterial contact with epithelial cells. Pili (Horiuchi *et al.*, 1992), flagella and fimbriae (Dibb-Fuller *et al.*, 1999) have been shown to contribute to the initial interaction of this bacterium with host epithelial cells. Pili are thought to function in the adhesion of bacteria to epithelial cell surfaces. Pilin production during broth culture of two different *Salmonella* serovars was found to correlate with mannose-sensitive adhesion to epithelial cells. In this study, the addition of excess D-mannose to bacterial–epithelial co-cultures was found to reduce bacterial invasion to a level comparable with targeted disruption of the pilin gene (Horiuchi *et al.*, 1992). At least two types of fimbriae, SEF17 and SEF21, are important in bacterial–epithelial adhesion. Although both SEF17- and SEF21-dependent adhesion to epithelial cells could be blocked by fimbriae-specific antibodies, only SEF21-dependent adhesion could be blocked by D-mannose, suggesting that the two fimbriae bind to different epithelial cell receptors (Dibb-Fuller *et al.*, 1999). Early bacterial–epithelial interactions such as these probably lead to assembly of the type III secretion apparatus (Ginocchio *et al.*, 1994; Zierler and Galan, 1995), necessary for bacterial invasion into the epithelial cell. The data presented in this report are consistent with a model of early bacterial–epithelial interaction involving multiple ligands, as although: (i) competitor LPS is able to inhibit invasion by serovar Typhi, it is not capable of inhibiting invasion totally; and (ii) the LPS of mutant strain MEI 126 cannot inhibit bacterial internalization, this strain is itself capable of low-level invasion into epithelial cells (i.e. MEI 126 is not completely non-invasive). Furthermore, the fact that different wild-type strains of serovar Typhi are inhibited to different degrees by LPS could be taken as additional evidence that other ligand–receptor interactions are involved in invasion, and that the differential effect of LPS inhibition against different wild-type strains of serovar Typhi is a result of the variable importance of these alternative ligand–receptor interactions in the invasion process.

In summary, we have identified serovar Typhi LPS deficient in O side-chains as a major bacterial ligand for epithelial cell ingestion. Additionally, upon interaction with host epithelial cells, the LPS of serovar Typhi undergoes a phenotypic alteration that is essential for its interaction with its epithelial cell receptor. Experiments performed with LPS mutant strains of serovar Typhi suggest that this phenotypic alteration involves the unmasking of a binding site that may reside on or near the LPS outer core oligosaccharide. Studies are currently under way to determine the chemical structures of the serovar Typhi LPS core oligosaccharide involved in binding to epithelial cells.

Experimental procedures

Bacteria

Bacterial strains used in these experiments are listed in Table 1. Ty2 is a wild-type, invasive serovar Typhi strain that has a smooth LPS phenotype. Ty21a is a chemical mutant of Ty2 that has a defective *galE* gene, making it unable to convert glucose to galactose and, therefore, unable to produce LPS O side-chain (Germanier and Fuer, 1975). Ty21a invades epithelial cells as well as wild-type serovar Typhi. Strains 167 and 7251 are two clinical isolates of serovar Typhi. Although their genotype is unknown, these two strains invade epithelial cells as efficiently as the well-characterized laboratory strain Ty2.

MEI 126 and MEI 120 are two LPS rough mutants of strain Ty2 generated by transposon mutagenesis. These mutants were selected from a bank of *mudJ* transposon mutants by their susceptibility to phage Ffm, which binds to rough, but not smooth, LPS. Strain MEI 126 is hypoinvasive and has a deep rough LPS phenotype. Strain MEI 120 is invasive and expresses very small amounts of O side-chain on its LPS, but has a complete LPS core oligosaccharide. MEI 126 and MEI 120 have been shown to have *in vitro* growth rates that are virtually identical, both to each other and to their wild-type parental strain,Ty2 (data not shown), and have each been shown by Southern blot analysis to contain only a single *mudJ* insert in their genomes (data not shown). MEI 126 has a transposon insertion into the *cpsG* gene, which was accompanied by a deletion of virtually the entire *wba* gene cluster (responsible for O side-chain synthesis). Although polar effects cannot be entirely ruled out at this point, it has been shown in the case of MEI 126 that O side-chain production can be restored by complementation with a plasmid-borne wild-type copy of the *wba* cluster. In the case of mutant strain MEI 120, the transposon insertion lies immediately upstream of the *wba* gene cluster. The extreme reduction in O side-chain production in this mutant is therefore probably the result of a polar transcriptional effect on the *wba* cluster.

Growth of bacteria for cellular uptake assays

For all cellular uptake experiments, serovar Typhi was grown in Luria–Bertani (LB) broth on a rotator at 37°C for 6 h. A sample of 100 μl of the 6 h culture was then used to inoculate 10 ml of LB and a second culture grown overnight at 37°C without agitation. In some experiments, strain Ty21a was grown in LB containing 0.5% galactose to restore O side-chain synthesis.

Heat-killed bacteria were prepared using cells of serovar Typhi or *P. aeruginosa* grown in LB or trypticase soy broth respectively. Heating was at 65°C for 2 h. In addition, heat-killed serovar Typhi Ty2 cells were prepared from organisms that had first been exposed to T84 epithelial cells (see below) for 4 h. The extracellular serovar Typhi were recovered directly by washing the epithelial cell monolayers. The intracellular serovar Typhi were recovered after 1 h of treatment of cell monolayers with gentamicin (200 μg ml^{-1}; Gibco) and washing three times to remove antibiotic before epithelial cell lysis (0.1% Triton X-100) to release intracellular organisms. Heat-killed bacterial suspensions were plated on MacConkey agar (Allegiance Healthcare) to verify lack of viability.

LPS purification

LPS was purified from overnight agar plate cultures of serovar Typhi or *P. aeruginosa* as described previously (Zaidi *et al.*, 1996). Briefly, bacteria grown on tryptic soy agar plates (Allegiance Healthcare) were lysed in 1% sodium lauryl sarcosine. LPS was recovered from the supernatant by precipitation with 95% ethanol, resuspended in water and then incubated overnight at 37°C with DNase I (0.1 mg ml^{-1}; Sigma) and RNase A (0.1 mg ml^{-1}; Worthington Biochemical), followed by a 2 h, 56°C digestion with protease (0.1 mg ml^{-1}; Sigma) and proteinase K (0.1 mg ml^{-1}; Gibco). LPS was then pelleted in an ultracentrifuge at 100 000 *g* for 3 h, resuspended in water and centrifuged again under the same conditions. The pellet was resuspended in water and lyophilized. All preparations used showed < 1% contamination with nucleic acid or protein by ultraviolet light absorbance and showed only the expected banding pattern on silver-stained gels.

Cell lines

The T84 human colon carcinoma cell line (ATCC) was used for the epithelial cell uptake assays. T84 cells were grown at 37°C, 5% CO_2 in a 1:1 mixture of Ham's F-12 (Gibco) and DMEM (Mediatech Cellgro), containing 10% (non-heat-inactivated) fetal calf serum (FCS; Intergen).

One day before a bacterial ingestion experiment, T84 cells were recovered from 75 cm^2 tissue culture flasks by trypsin–EDTA treatment, washed and seeded into 96-well tissue culture plates at 1×10^5 cells per well in the aforementioned growth medium. Cultures were incubated overnight at 37°C in 5% CO_2.

CFT1-LCFSN and CFT1-ΔF508 human airway epithelial cell lines were used to determine the CFTR dependence of epithelium-induced LPS alterations. These cells were grown at 37°C, 5% CO_2 in Ham's F12 medium (Gibco) containing 10 mg ml^{-1} insulin, 1 mM hydrocortisone, 3.75 mg ml^{-1} endothelial cell growth supplement, 25 ng ml^{-1} epidermal growth factor, 30 nM triiodo-L-thyronine, 5 mg ml^{-1} transferrin, 10 ng ml^{-1} cholera toxin, 150 mg ml^{-1} neomycin B, 100 units ml^{-1} penicillin G, 250 ng ml^{-1} amphotericin B and 100 mg ml^{-1} streptomycin.

Epithelial cell ingestion assay

Cultures of serovar Typhi cells grown overnight to an approximate OD_{650} of 0.3 were centrifuged and resuspended in Ham's F-12 medium (Gibco) containing 10% heat-inactivated FCS. The OD_{650} of this suspension was adjusted to 0.3–0.35. The actual concentration of serovar Typhi in this suspension was determined at the time of the initiation of infection of T84 cells by serially diluting the suspension in F-12 + 10% FCS and plating each dilution on MacConkey agar for bacterial enumeration.

To inhibit serovar Typhi uptake by epithelial cells, the T84 monolayers were incubated with purified LPS (10–100 μg per well) or with heat-killed *P. aeruginosa* or serovar Typhi (2×10^6–2×10^8 cfu per well) for 1 h at 37°C, followed by inoculation with 2×10^5–5×10^6 cfu of live serovar Typhi. If the heat-killed bacteria had been recovered from epithelial co-cultures previously, they were washed extensively with PBS, pH 7.2, to remove detergent and/or contaminants from the previous co-culture, before being used as competitive inhibitors.

Plates were then incubated at 37°C, 5% CO_2 for 3 h. To ascertain the number of intracellular bacteria at the end of the incubation, the monolayer was washed twice and then incubated with F-12 medium containing carbenicillin (200 μg ml^{-1}; Gibco) for 1 h at 37°C. Strains MEI 126 and MEI 120 are resistant to gentamicin normally used in these assays, but control experiments (not shown) have demonstrated that these two antibiotics yield similar results in this assay system. The overall health of the epithelial cells was evaluated at the end of this incubation. The detachment of a large number of the epithelial cells from the culture vessel or the failure of the epithelial cells to exclude trypan blue at this stage of the experiment was considered cause for aborting the experiment, as this would make it impossible to distinguish reduced bacterial invasion from impaired exclusion of the antibiotic. After washing to remove the carbenicillin, the T84 cells were lysed in 0.5% Triton X-100 to release intracellular bacteria, and the lysates were diluted and plated on MacConkey agar for bacterial enumeration. The number of bacteria that were ingested under each condition was compared by ANOVA and pairwise differences determined by the Fisher predicted least significant difference (PLSD).

SDS–PAGE analysis of epithelial cell-induced alterations in serovar Typhi LPS

Serovar Typhi strain Ty2, grown in LB broth (as described above for cellular uptake assays), was incubated for 4 h at 37°C with epithelial cell monolayers, fresh epithelial cell growth medium or epithelial cell-conditioned medium. After this incubation, total bacteria (intra- and extracellular) were recovered from the cultures, lysed by boiling in SDS–PAGE

sample buffer and digested for 1 h at 60°C with proteinase K (20 mg ml^{-1} final concentration). The LPS samples were then separated on 20% polyacrylamide gels and stained with silver.

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