nadA and *nadB* of *Shigella flexneri* 5a are antivirulence loci responsible for the synthesis of quinolinate, a small molecule inhibitor of *Shigella* pathogenicity

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The evolution of bacterial pathogens from commensal organisms involves virulence gene acquisition followed by pathoadaptation to the new host, including inactivation of antivirulence loci (AVL). AVL are core ancestral genes whose expression is incompatible with the pathogenic lifestyle. Previous studies identified *cadA* (encoding lysine decarboxylase) as an AVL of *Shigella* spp. In this study, AVL of *Shigella* were identified by examining a phenotypic difference from its non-pathogenic ancestor, *Escherichia coli*. Unlike most *E. coli* strains, *Shigella* spp. are nicotinic acid auxotrophs, the pathway for the *de novo* synthesis of NAD being uniformly defective. In *Shigella flexneri*, this defect is due to alterations in the *nadA* and/or *nadB* genes encoding the enzyme complex that converts L-aspartate to quinolinate, a precursor to NAD synthesis. Quinolinate was found to inhibit invasion and cell-to-cell spread of *Sh. flexneri* 5a and its ability to induce polymorphonuclear neutrophil transepithelial migration. Virulence of other *Shigella* species was also inhibited by quinolinate. Introduction of functional *nadA* and *nadB* genes from *E. coli* K-12 into *Sh. flexneri* 5a restored its ability to synthesize quinolinate but also resulted in strong attenuation of virulence in this strain. The results define *nadA* and *nadB* as AVL in *Shigella* and validate the concept of pathoadaptive evolution of bacteria from commensal ancestors by inactivation of AVL. They also suggest that studies focusing on this form of bacterial evolution can identify novel inhibitors of virulence in other bacterial pathogens.

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INTRODUCTION

The evolution of bacterial pathogens from their commensal ancestors is believed to occur by the acquisition of genes that allow the organism to colonize a new ecological niche. A 'quantum leap' in the evolution of a bacterium occurs

when new genes are acquired *en bloc* via horizontal transfer of plasmids or bacteriophages (Groisman & Ochman, 1996). The presence of virulence genes in 'pathogenicity islands' reflects this form of gene acquisition (Lee, 1996). Thus, horizontal gene transfer can radically transform an organism from a benign resident of a eukaryotic host into a pathogen capable of colonizing a new niche within the host and, incidentally, causing harm to the host.

A complement to the process of bacterial pathogens' evolution by gene acquisition is the progressive adaptation of the bacteria to the new niche by mutation and selection for improved fitness. This process is called pathoadaptive evolution (Sokurenko *et al.*, 1999). An important element of this process is the selection of 'black holes' in pathogen genomes, that is, the inactivation or loss of genes that are

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Abbreviations: AVL, antivirulence loci; CR, Congo red; EIEC, enteroinvasive *E. coli*; EPEC, enteropathogenic *E. coli*; fMLP, *N*-formylmethionyl leucyl phenylalanine; PMN, polymorphonuclear neutrophil; QUIN, quinolinate; TSB, tryptic soy broth; T3SS, type III secretion system.

incompatible with, and even antagonistic to, the new pathogenic lifestyle (Maurelli, 2007). These incompatible genes, which we define as antivirulence loci (AVL), are present in the genome of the non-pathogenic ancestor but absent or inactive in the pathogen because expression of AVL in the pathogen is detrimental to expression of some virulence phenotype. Thus, deletion or mutational inactivation of AVL is selected for in the pathogen.

Bacteria of the genus *Shigella* are the causative agents of bacillary dysentery in humans and primates. In the early stage of the disease, *Shigella* produce several enterotoxins that are believed to facilitate their passage from the small intestine to the colon (Fasano *et al.*, 1997). Once in the colon, the bacteria invade colonic epithelial cells, replicate intracellularly, and spread to adjacent cells. Intracellular bacterial growth and tissue destruction lead to an intense inflammatory response with migration of polymorphonuclear neutrophils (PMNs) to the site of infection (Sansonetti *et al.*, 1999).

We previously demonstrated the contribution of 'black holes' to the evolution of *Shigella* from the non-pathogenic gut commensal *Escherichia coli* (Maurelli *et al.*, 1998). The *cadA* gene, encoding lysine decarboxylase in *E. coli* K-12, was shown to be part of a large region uniformly altered in all *Shigella* spp., and its expression is incompatible with several phenotypes of *Shigella* virulence (Day *et al.*, 2001; McCormick *et al.*, 1999). These observations support the concept of bacterial pathogen evolution by selection for increased fitness through gene inactivation or deletion.

The genetic similarities between *Shigella* and *E. coli* are sufficient to justify grouping them in the same genus. *Shigella* spp. may even be considered clones, or subgroups, of *E. coli* (Pupo *et al.*, 2000). However, there are several well-known metabolic differences that distinguish the organisms and aid in their clinical differentiation. Among these are auxotrophic requirements of *Shigella* that are not found among isolates of *E. coli*. Most clinical isolates of *Shigella* fail to grow on minimal medium. Of these auxotrophic isolates, 98% grow on minimal medium supplemented with methionine, tryptophan and nicotinic acid (Ahmed *et al.*, 1988). Many strains of *Shigella* require only nicotinic acid supplementation for growth on minimal medium. This requirement is due to mutations in *nadA* and/or *nadB* in *Sh. flexneri* (Gemski *et al.*, 1971; Mantis & Sansonetti, 1996).

The L-aspartate-dihydroxyacetone phosphate pathway is used by *E. coli* K-12 for *de novo* synthesis of NAD (Fig. 1). L-Aspartate oxidase, encoded by *nadB*, forms a multi-enzyme complex with quinolinate synthetase A, the product of *nadA*. This enzyme complex catalyses the oxidation of L-aspartate to iminoaspartate, which is then condensed with dihydroxyacetone phosphate (DHAP) to form quinolinate (QUIN). Quinolinate phosphoribosyltransferase, the product of *nadC*, converts QUIN to nicotinic acid mononucleotide, which enters the pathway for NAD synthesis. In the absence of functional *nadA* and/or *nadB*, QUIN is not made and no nicotinic acid mononucleotide is available to synthesize NAD. However, exogenous nicotinic acid can be converted to nicotinic acid mononucleotide by the action of nicotinate phosphoribosyltransferase, the product of *pncB*.

Fig. 1. L-Aspartate-dihydroxyacetone phosphate pathway for *de novo* NAD synthesis in *E. coli* K-12 [adapted from Flachmann *et al.* (1988)].

(Fig. 1). This pathway bypasses the need for *nadA* and *nadB* and synthesis of QUIN and is the basis of the nicotinic acid requirement for *Shigella* growth on minimal medium (Gemski *et al.*, 1971).

The following study determined whether the auxotrophic requirement for nicotinic acid in *Sh. flexneri* represents another example of pathoadaptation through selection for loss of a gene function that is incompatible with virulence. We hypothesized that selection for pathoadaptation has led to inactivation of *nadA* and/or *nadB* in *Shigella* and enteroinvasive *E. coli* (EIEC). Our results demonstrate that *nadA* and *nadB* are AVL and that QUIN, an intermediate in the biosynthesis of NAD, is a potent small molecule inhibitor of several virulence phenotypes of *Shigella*.

METHODS

Bacterial strains and media. Strain M90T is the wild-type strain of *Sh. flexneri* 5a (Sansone *et al.*, 1982) used throughout this study. Other strains of *Shigella* used were: 2457T (*Sh. flexneri* 2a) (Formal *et al.*, 1958) and its avirulent, plasmid-cured derivative strain BS103 (Maurelli *et al.*, 1984); BS504 (*Sh. dysenteriae* 1), BS512 (*Sh. boydii*), and BS514 (*Sh. sonnei*). BS176 is a spontaneous virulence-plasmid-cured derivative of M90T (Sansone *et al.*, 1986). Overnight cultures for virulence assays were inoculated from single pigmented colonies growing on tryptic soy broth (TSB, Bacto) plates with 1.5 % agar and 0.025 % Congo red (CR; Sigma) at 37 °C (Maurelli *et al.*, 1984) and grown in Luria–Bertani (LB) medium at 37 °C. The wild-type strain of *Salmonella typhimurium* employed was χ 3306 (Gulig & Curtiss, 1987). ATM266 is an EIEC strain (Harris *et al.*, 1982). E2348/69 is a strain of enteropathogenic *E. coli* (EPEC) obtained from Gail Hecht (University of Illinois at Chicago, USA). *E. coli* K-12 MC4100 was the source of DNA for PCR reactions to amplify the *nadA* and *nadB* loci. Ye37 is a *Yersinia enterocolitica* isolate WA obtained from Peter Feng (FDA, College Park, MD, USA). BS813 is the *nadA*⁺ *nadB*⁺ transformant of M90T.

For plating efficiency determination of M90T, M9 glucose minimal medium supplemented with methionine (10 μ g ml⁻¹) and tryptophan (20 μ g ml⁻¹) was used. When required, 1 mM QUIN (Fluka), or 10 μ g ml⁻¹ nicotinic acid (Sigma) was added.

Molecular techniques. The primers used for amplification of the *nadA* and *nadB* loci were as follows: *nadAPF* 5'-CAAGCAACTCTAT-GTCGGTGG-3' and *nadAM* 5'-TGGCAAGGCCAATACACAGC-3' allowed amplification of the entire *nadA* locus and its promoter, while *nadBPF* 5'-CAAAGGGTTAGAGTGCTCTCG-3' and *nadBM* 5'-CG-TGGGCCAGACCAGAACTATTCC-3' allowed amplification of the entire *nadB* locus and its promoter, using Taq DNA polymerase (Qiagen).

For cloning, we used the commercially available plasmids pGEM-T (Promega) and pBluescript II-SK (Stratagene). Transformants were obtained by electroporation of competent cells from cold water treatment and screened for ampicillin resistance. Ampicillin-resistant clones were then confirmed by PCR and their phenotype was monitored by determining plating efficiency on minimal medium containing the appropriate supplements.

Invasion assays. Invasion assays were performed as described previously (Sandlin *et al.*, 1996) with some alterations. Bacteria were grown overnight at 37 °C then diluted 1:50 into 5.0 ml LB and grown with agitation at 37 °C to OD₆₀₀ ~0.6. Standardized culture volumes were removed, diluted, titered, and used to infect semi-

confluent monolayers of mammalian cells at 37 °C in Dulbecco's modified essential medium (DMEM) with or without the indicated concentrations of QUIN, nicotinic acid or nicotinic acid mononucleotide (Sigma). The input inoculum was $\sim 1 \times 10^8$ c.f.u. in a volume of 1 ml. After 30 min, infected monolayers were washed extensively with 1 $\times$ PBS to remove both the QUIN and extracellular bacteria and incubated for an additional 90 min in DMEM with 50 μ g gentamicin ml⁻¹. Monolayers were subsequently washed with PBS and lysed in the presence of 0.5 % Triton X-100. Released intracellular bacteria were plated on TSB-CR agar and colonies were counted to determine the percentage invasion compared to the input titre. The same protocol was used to test the effect of QUIN on intracellular growth of bacteria except that the lysis step was performed after 0.5, 1.5, 2.5, 3.5, 4.5 and 5.5 h.

Alternatively, instead of lysing the infected cells, monolayers were fixed with methanol and stained with Giemsa 45, 90, 135 or 180 min after final gentamicin treatment, and examined by light microscopy for evidence of bacterial invasion. At least 200 infected cells were counted. The frequency of protrusion formation, indicating intracellular spreading capacity of the bacteria, was determined as the percentage of cells with internalized bacteria that displayed cytoplasmic protrusions.

Plaque assay. Plaque assays were performed essentially as described previously (Oaks *et al.*, 1985) with some alterations. Bacterial cultures were grown overnight, diluted 1:50 into 5.0 ml fresh LB, and grown with agitation at 37 °C to OD₆₀₀ ~0.6. Standardized culture volumes were removed, diluted, titered, and used to infect confluent monolayers of HeLa cells for 2 h at 37 °C in a 6.0 % CO₂ atmosphere. The input inoculum was $\sim 1 \times 10^4$ c.f.u. in a volume of 100 μ l. Agarose overlays with or without the indicated concentrations of QUIN were then applied to infected monolayers and allowed to incubate at 37 °C in a 6 % CO₂ atmosphere for an additional 72 h. Neutral red staining allowed for enumeration of plaques to determine the plaque-forming efficiency compared to the input titre. Plaque diameter was calculated as the average of 10 plaques.

PMN transepithelial migration assay. PMN migration was assessed as previously described (McCormick *et al.*, 1998). For assessment of *Sh. flexneri*-induced PMN migration in the presence of QUIN, 25 μ l washed bacterial suspension (6×10^8 bacteria) was added directly to the basolateral surface (upper compartment) of inverted T84 cell monolayers for 90 min at 37 °C. Next, the T84 cell monolayers were washed to remove non-adherent bacteria and QUIN (5 or 100 μ M) was added to both the apical and basolateral reservoirs for 30 min. PMNs isolated from volunteers were subsequently added to the basolateral surface (1×10^6 per monolayer) and assessed for PMN transmigration in the continued presence of QUIN. Transmigration was quantified by assaying for myeloperoxidase, the PMN azurophilic marker, and is expressed as cell equivalents as described previously (McCormick *et al.*, 1998; Parkos *et al.*, 1991). A 1 μ M solution of *N*-formylmethionyl leucyl phenylalanine (fMLP; Sigma), a potent PMN chemoattractant, was used as a control.

Protein extraction and preparation. To study the amount of expressed proteins, ~20 ml of exponential cultures of M90T grown with or without 0.4 mM QUIN were standardized using OD₆₀₀ measurement and harvested by centrifugation (13 000 g, 5 min). Supernatants were filtered through a 0.45 μ m pore size filter (Millipore) and secreted proteins were precipitated by the addition of trichloroacetic acid (10 % final concentration), followed by acetone washing. The final pellet of precipitated proteins was resuspended in 200 μ l sample buffer. Whole-cell lysates were generated by washing the pellets once with 20 ml ice-cold, sterile 1 $\times$ PBS, followed by centrifugation of 1 ml aliquots and suspension in 500 μ l Laemmli sample buffer (Bio-Rad).

To study the effect of QUIN on CR-induced secretion via the type III secretion system (T3SS), whole-cell fractions and supernatants of CR-treated *Sh. flexneri* were prepared as described previously (Baharani *et al.*, 1997). Briefly, 40 ml samples from exponential cultures of M90T were harvested by centrifugation (13000 g, 5 min). Each pellet was resuspended in 2 ml sterile PBS and split into two 1 ml aliquots incubated with 30 µg CR ml⁻¹ for 1 h at 37 °C, with or without 0.4 mM QUIN. After the incubation period, the samples were centrifuged again (13 000 g, 5 min). Whole-cell lysates and precipitated proteins from the supernatants were prepared as described above.

To study the effect of QUIN on the low-calcium-induced T3SS of *Y. enterocolitica* we used the same protocol with the exception that CR was replaced by 5 mM EGTA to chelate cations from the medium.

SDS-PAGE and immunoblotting. All samples were boiled and 10 µl of each were loaded on 10% acrylamide SDS-PAGE gels as described by Laemmli (1970). After electrophoresis, proteins were transferred to a nitrocellulose membrane. Immunoblotting procedures were carried out with mouse monoclonal antibodies 2F1 and 2G2, which recognize IpaB and IpaC, respectively (Mills *et al.*, 1988). Horseradish peroxidase-labelled sheep anti-mouse IgG (Amersham Bioscience) was used as secondary antibody and visualized by enhanced chemiluminescence with the visualizer Western blot detection kit (Upstate) in a LAS-3000 system using Image reader LAS 3000 v2.1 software (Fuji). The images obtained were analysed using Image Gauge v4.22 software (Fuji) allowing quantification of the signals observed. For the study of the effect of QUIN on the calcium-deprivation-induced T3SS of *Y. enterocolitica*, SDS-PAGE gels were stained with Imperial protein stain (Pierce).

RESULTS

QUIN inhibits *Shigella* invasion of HeLa cells

If an intermediate in the *de novo* pathway of NAD synthesis can attenuate *Shigella* pathogenesis, the selective pressure for pathoadaptation to inactivate one or more of the genes encoding enzymes in the pathway could explain the presence of mutations in *nadA* and/or *nadB* in *Shigella* spp. and EIEC. To test the hypothesis that *nadA* and/or *nadB* are AVL whose expression leads to the synthesis of an inhibitor of *Shigella* virulence, the ability of M90T to invade HeLa cells was assayed in the presence and absence of several intermediates in the NAD biosynthetic pathway.

Table 1. Effect of NAD biosynthetic pathway intermediates on the invasive phenotype of *Sh. flexneri* 5a

Treatment*	Invasion (%)†
None	0.11 ± 0.023
1 mM nicotinic acid	0.125 ± 0.015
1 mM nicotinic acid mononucleotide	0.1 ± 0.019
0.5 mM QUIN	0.0024 ± 0.0004

*The compounds were present in the invasion assay only during the 30 min invasion period.

†Percentage of bacteria recovered from infected wells after gentamicin treatment relative to the number of bacteria used for invasion. Values shown are mean ± SD from at least two independent experiments.

As shown in Table 1, 0.5 mM QUIN almost completely inhibited invasion of HeLa cells by M90T, whereas 1 mM nicotinic acid or nicotinic acid mononucleotide had no adverse effect on invasion. Addition of 1 mM QUIN caused no visible changes in HeLa cell morphology and up to 2.5 mM QUIN caused no change in pH of the tissue culture medium.

We next measured the inhibitory effect of QUIN over a range of concentrations on *Shigella* invasion into HeLa cells. These experiments showed that QUIN inhibited *Shigella* invasion in a dose-dependent fashion and that the presence of as little as 400 µM QUIN almost completely inhibited invasion (Fig. 2).

QUIN inhibits intercellular spread of *Shigella*

Another hallmark of *Shigella* pathogenesis is its ability to spread from cell to cell within a confluent monolayer after the initial invasion step. A prerequisite for intercellular spread is the intracellular motility mediated by the ability of internalized *Shigella* to polymerize actin monomers in the cytoplasm (Bernardini *et al.*, 1989). The formation of cytoplasmic protrusions extending from infected cells and containing one or more bacteria at the end is a reliable measure of intracellular motility. To examine the effect of QUIN on protrusion formation in *Shigella*-infected cells, bacteria were first allowed to invade semi-confluent HeLa monolayers and then 400 µM QUIN was added and the monolayers incubated at 37 °C. At various time points, the monolayers were fixed, stained and examined for protrusions emanating from infected cells. Fig. 3(a) shows that QUIN-treated monolayers displayed the same frequency of protrusion formation from *Shigella*-infected cells as untreated monolayers. Thus, QUIN does not inhibit the actin-based intracellular motility of *Shigella*.

The ability to spread from cell to cell within a confluent monolayer is a virulence phenotype measured by the plaque assay (Oaks *et al.*, 1985). Since QUIN blocked

Fig. 2. Effect of QUIN on invasion of HeLa cells by M90T measured by the gentamicin protection assay. Percentage invasion was calculated as described in Methods. The results represent the mean ± SD of data obtained from two to four independent experiments for each concentration of QUIN, relative to the results obtained for M90T without QUIN (i.e. 1.0).

Fig. 3. Effect of QUIN on intra- and intercellular spreading by M90T in HeLa cell monolayers. See Methods for details. (a) Effect of QUIN on protrusion formation. The results represent the mean $\pm$ SD of data obtained from two independent experiments. (b) Effect of QUIN on plaque-forming efficiency. The results represent the mean $\pm$ SD of data obtained from three independent experiments for each concentration of QUIN (*, $P < 0.05$). (c, d) Effect of QUIN on plaque size: (c) shows the mean $\pm$ SD of plaque size measurements from the three experiments recorded in (b); (d) shows representative pictures of the plaques obtained for the control without QUIN as well as for three different QUIN concentrations (indicated in the upper right corner of each picture).

Shigella invasion it was added to infected monolayers only after an initial period in order to allow the bacteria to invade. Fig. 3(b) shows that QUIN impaired plaque formation in a dose-dependent manner and at much lower concentrations than were required to block invasion. The mean plaque diameter formed by M90T in the cell monolayer in the absence of QUIN was 1.53 mm. Fig. 3(c) shows that the mean plaque size decreased with increasing concentrations of QUIN. This effect of QUIN on plaque size was accompanied by a marked effect on plaque shape, with QUIN causing the formation of faint and irregularly shaped plaques compared to the large, well-defined plaques of the control (Fig. 3d). Therefore, the major post-invasion effect of QUIN is a strong inhibition of the ability of the bacteria to spread from cell to cell, although it had no effect on the intracellular motility of *Shigella*.

QUIN inhibits *Shigella*-induced transepithelial migration of PMNs

A model system for the ability of *Shigella* to induce the severe inflammatory response typical of dysentery is the induction of PMN transepithelial migration across a monolayer of polarized T84 cells (McCormick *et al.*, 1998). We tested QUIN for its effect on the ability of *Shigella* to induce transepithelial migration of PMNs after invasion of a monolayer of polarized epithelial cells. Since QUIN inhibited invasion of M90T into polarized T84 cells (Table 2), the assay was modified to allow the bacteria to invade the polarized T84 cells for 90 min before different concentrations of QUIN were added to the monolayers. Fig. 4 demonstrates that QUIN has a potent inhibitory effect on the ability of *Shigella* to induce transepithelial migration of PMNs.

Table 2. Effect of QUIN on virulence phenotypes of *Sh. flexneri*, *Sal. typhimurium* and EPEC

Strain	Treatment	Invasion (%)	PMN migration*
<i>Sh. flexneri</i> M90T	Buffer alone	0.044	8.15×10^3 †
<i>Sh. flexneri</i> M90T	100 µM QUIN	0.012	0.28×10^3 †
<i>Sal. typhimurium</i> χ 3306	Buffer alone	0.123	39.24×10^4
<i>Sal. typhimurium</i> χ 3306	100 µM QUIN	0.118	45.53×10^4
EPEC E2348/69	Buffer alone	ND‡	4.2×10^4
EPEC E2348/69	100 µM QUIN	ND‡	4.8×10^4

*PMN migration was quantified as described in Methods.

†Results from Fig. 4.

‡ND, not done. EPEC strains are non invasive but still induce PMN migration.

Since QUIN was present in the medium throughout the experiment even after the PMNs were added, we tested the possibility that inhibition was due to a general effect of QUIN on PMN mobility. Control experiments showed that up to 100 µM QUIN had no significant effect on PMN transepithelial migration in response to fMLP ($61.9 \pm 6.1 \times 10^4$ cell equivalents in the absence of QUIN vs $57.3 \pm 11.4 \times 10^4$ and $51 \pm 4.6 \times 10^4$ cell equivalents with 5 and 100 µM QUIN, respectively).

QUIN is a specific inhibitor of *Shigella* virulence

We tested another *Sh. flexneri* serotype as well as representative strains of *Sh. dysenteriae*, *Sh. boydii*, *Sh. sonnei* and EIEC to determine whether our observations on the inhibition of virulence by QUIN could be extended to

Fig. 4. Effect of QUIN on the ability of M90T to induce transepithelial migration of PMNs across T84 monolayers. See Methods for details. The three middle columns show PMN transmigration responses induced by wild-type *Sh. flexneri* 5a M90T. BS103, a virulence-plasmid-cured *Sh. flexneri* strain, served as a negative control. The second negative control (-) represents PMN transmigration to buffer in the absence of bacteria or chemotactic stimulus. (*) Both control PMN values were below the detection limit of the assay (700 PMNs). Data are expressed as mean $\pm$ SD for three monolayers in a single experiment and are representative of three separate experiments ($P < 0.05$). CE, cell equivalents.

the four species of *Shigella* as well as to the closely related EIEC. As shown in Fig. 5, 400 µM QUIN largely inhibited invasion of mammalian cells by all these strains.

We next examined whether the inhibitory properties of QUIN on the virulence phenotypes of *Shigella* spp. could be extended to related invasive pathogens. *Sal. typhimur-*

Fig. 5. Effect of QUIN on invasion of mammalian cells by representative strains of *Shigella* spp. and EIEC. Invasion of L2 cell monolayers (for *Shigella*) or Hep2 cell monolayers (for EIEC) was measured by the gentamicin protection assay. Hep2 cells were used for EIEC because EIEC are cytotoxic to L2 cells. Percentage invasion was calculated as described in Methods. The results represent the mean $\pm$ SD of data obtained from two to four independent experiments; (+) and (-) indicate the presence or absence of 400 µM QUIN during the infection. The data are shown in separate panels with different scales on the y-axes due to the different levels of invasion efficiency observed among the species of *Shigella* and EIEC.

ium was chosen since, like *Shigella*, it invades mammalian cells in tissue culture and induces a strong PMN transepithelial migration response upon invasion of T84 cells. Whereas *Sh. flexneri* 5a invasion of T84 cells was reduced by 73 % with as little as 100 μ M QUIN (Table 2), the same concentration of QUIN had no effect on invasion of T84 cells by *Sal. typhimurium* strain χ 3306 (Table 2).

While *Shigella* induction of PMN transepithelial migration was significantly inhibited by as little as 5 μ M QUIN (Fig. 4), *Sal. typhimurium* still induced PMN migration in the presence of 100 μ M QUIN (Table 2). We also tested E2348/69, an EPEC strain that induces PMN transepithelial migration in this model system. It was still able to induce PMN transepithelial migration in the presence of 100 μ M QUIN (Table 2). These data strongly suggest that the antivirulence properties of QUIN observed in this assay are limited to *Shigella* spp.

Effect of QUIN on intracellular growth and virulence factor expression in *Shigella*

One possible explanation for the observations that QUIN inhibited cell-to-cell spread and plaque formation, yet did not inhibit intracellular motility of the bacteria is that QUIN inhibits intracellular growth or survival of *Shigella*. However, when we measured the effect of QUIN on growth of *Shigella* after invasion of HeLa cells, we found that intracellular growth of the bacteria was not inhibited by 400 μ M QUIN (data not shown).

Since *Shigella* can utilize QUIN for growth on minimal medium (data not shown) by converting QUIN to nicotinic acid mononucleotide by the action of the *nadC* gene product (Fig. 1), we can assume that QUIN can enter the bacterial cell. Therefore, we wished to determine whether the antivirulence effects result from direct repression of *Shigella* virulence gene expression by QUIN. We used Western blot analysis of the IpaB and IpaC virulence proteins expressed during exponential culture of *Sh. flexneri* M90T with or without QUIN to address this question. The amount of protein detected in the pellet (non-secreted proteins) plus the amount of protein TCA-precipitated from the supernatant (secreted proteins) were compared for the two conditions. We found less than a twofold reduction in the amount of IpaB and IpaC expressed in the presence of QUIN compared to the amount expressed during growth without QUIN (1.68 ± 0.12 - and 1.55 ± 1.03 -fold reduction for IpaB and IpaC, respectively). By comparison with the well-studied growth temperature regulation of *Shigella* virulence, low growth temperature (30 °C) causes 60-fold reduced expression of *ipaB* as compared to expression at 37 °C (Andrews *et al.*, 1991; Hromockyj & Maurelli, 1989; McCormick *et al.*, 1998). Therefore, it is not likely that the inhibitory effect of QUIN on *Shigella* virulence is due to QUIN interaction at the level of virulence gene transcription/translation.

Effect of QUIN on secretion of invasion effectors via the *Shigella* type III secretion system

Invasion, intracellular and intercellular spread, and the ability to induce PMN transepithelial migration all require the secretion of Ipa proteins from *Shigella*. Mutations in the T3SS, which mediates secretion of these invasion effectors, abolish these virulence phenotypes (McCormick *et al.*, 1998; Menard *et al.*, 1993; Schuch *et al.*, 1999). We therefore used Western blot analysis of M90T culture supernatants to determine whether QUIN could alter the secretion of Ipa proteins via the T3SS. When wild-type *Sh. flexneri* are incubated with CR, secretion of Ipa proteins through the T3SS is induced (Parsot *et al.*, 1995). Fig. 6 shows that incubation of M90T with 400 μ M QUIN strongly impaired the secretion of IpaB and C that is normally observed when bacteria are incubated with CR. Quantitative analysis showed that the secretion of IpaB and C in the presence of QUIN was reduced 81.9 % and 82.8 %, respectively.

We next wanted to examine the specificity of these inhibitory properties of QUIN on the induced secretion of virulence factors. *Y. enterocolitica* was chosen since it is also an enteric pathogen with well-studied T3SS induction by calcium deprivation (Straley *et al.*, 1993). Coomassie staining of SDS-PAGE-analysed protein extracts obtained from *Yersinia* cultures showed that addition of QUIN to the growth medium did not affect the amount of virulence effectors detected in the supernatant of a calcium-deprived culture (data not shown), which suggests that the effects of QUIN on T3SS-induction are specific to *Shigella* spp.

Fig. 6. Effect of QUIN on CR-induced secretion of IpaB and IpaC. Protein extracts were obtained as described in Methods. Identical volumes of pellet and supernatant extracts were subjected to SDS-PAGE and immunoblotted with antibodies directed against IpaB and IpaC. (+) and (-) indicate the presence or absence of 400 μ M QUIN during induction.

***nadA*⁺ *nadB*⁺ transformants of *Sh. flexneri* 5a are attenuated in virulence**

We wanted to study the effect of reconstructing the NAD pathway in *Shigella*. The wild-type (functional) *nadA* and *nadB* genes with their native promoters from *E. coli* K-12 were cloned together in pBluescript and transformed into *Sh. flexneri* 5a strain M90T in order to determine whether expression of these putative AVL in the wild-type *Shigella* would attenuate virulence. The *nadA*⁺ *nadB*⁺ transformant of M90T, BS813, was able to grow on minimal medium in the absence of nicotinic acid, confirming the functional activity of the transformed genes. Invasion assays with BS813 in HeLa cells showed a 100-fold reduction of invasion capacity compared to M90T ($0.0023 \pm 0.0003\%$ vs $0.21 \pm 0.03\%$, respectively). In fact, BS813 gave results similar to BS176, the non-invasive plasmid-cured derivative of M90T (i.e. $0.0019 \pm 0.0001\%$), and to M90T in the presence of 0.5 mM QUIN (Table 1). BS813 was also unable to form plaques in the plaque assay on HeLa cells, even at very high input concentrations (plaque-forming efficiency $<10^{-8}$). PMN transmigration experiments could not be performed with BS813 due to the assay's dependence on efficient invasion of *Shigella* into T84 cells. However, invasion and plaque assay results are sufficient to confirm the antivirulence nature of *nadA* and *nadB* when functional alleles of these genes are expressed in *Shigella*.

DISCUSSION

The striking difference between *Shigella* and *E. coli* in their ability to synthesize NAD was our rationale for examining the genes in this pathway in *Shigella* for AVL properties. *Shigella* is a nicotinic acid auxotroph in that the bacterium is unable to synthesize NAD via the *de novo* pathway but can still synthesize NAD from exogenous nicotinic acid (Fig. 1). Mutations in *nadA* and *nadB* are responsible for this auxotrophy in *Sh. flexneri* 2a (Gemski *et al.*, 1971) and our analysis of these loci in other strains of *Shigella* and EIEC strongly suggested them as good AVL candidates for *Shigella* (A.-L. Prunier and others, unpublished).

Several intermediates of the NAD synthesis pathway were tested for their capacity to interfere with the invasive phenotype of *Shigella* and only QUIN was shown to have an effect. QUIN inhibited three essential phenotypes of *Shigella* virulence: bacterial invasion, cell-to-cell spread and induction of PMN transepithelial migration. Moreover, we found that QUIN strongly inhibited CR-induced T3SS secretion of the *Shigella* virulence factors IpaB and IpaC, which are key to the entry of the bacteria into eukaryotic cells and provide a pore for the delivery of other effectors (Enninga *et al.*, 2005; Mounier *et al.*, 1997).

Interference with *Shigella*-induced actin polymerization could explain how QUIN blocks the entry step. However, any interference of this type must be limited to the early invasion stage, as we observed no gross alteration of the

bacteria's ability to polymerize actin in the cytoplasm of the host cell after invasion. When bacteria were allowed to invade cells before addition of QUIN, they produced normal numbers and lengths of cytoplasmic protrusions that contained bacteria. This observation demonstrated the ability of intracellular bacteria to polymerize actin normally in the presence of QUIN.

The plaque assay showed that QUIN also blocked a late stage in *Shigella* infection, cell-to-cell spread. Since intracellular bacteria were motile in the presence of QUIN and appeared capable of producing cytoplasmic extensions that could 'invade' adjacent cells, the inhibitory effect of QUIN on cell-to-cell spread is not on actin-based motility per se but on some subsequent step in the spreading process. QUIN could be blocking the actual 'invasion' of the bacteria-containing protrusion into the adjacent cell. Alternatively, QUIN could be blocking the ability of the bacteria to lyse the protrusion and escape into the cytoplasm of the adjacent cell.

The failure to spread to adjacent cells is consistent with the inhibition of another virulence phenotype by QUIN, the induction of PMN transepithelial migration. Previous observations in our laboratory have shown that an *icsA* mutant of *Sh. flexneri* is severely impaired in its ability to induce transepithelial migration of PMNs (Fernandez *et al.*, 2001). This mutant is unable to catalyse polymerization of actin in the host cytoplasm and, as such, is unable to spread intercellularly. Therefore, induction of PMN transepithelial migration is dependent not only on invasive ability and expression of a functional T3SS (McCormick *et al.*, 1999; Zurawski *et al.*, 2006), but also on the ability to spread to adjacent cells.

While QUIN displays a remarkable ability to inhibit invasion of L2 cells by all four species of *Shigella* (Fig. 5), its inhibitory properties appear to be restricted to *Shigella* spp. *Sal. typhimurium* is also an invasive pathogen of the intestinal epithelium and is closely related to *E. coli* and *Shigella*. Both *Shigella* and *Sal. typhimurium* invade a variety of cell types in tissue culture, while their respective *in vivo* targets are different, and they both require the activities of T3SS secretory pathways. Yet QUIN dramatically inhibits *Shigella* invasion of tissue culture cells whereas it has no effect on invasion by *Sal. typhimurium*.

Analogous results were observed in the PMN transepithelial migration assay where QUIN blocked PMN signalling by *Shigella* but had no effect on signalling by *Sal. typhimurium*. Similarly, a strain of EPEC was insensitive to QUIN and induced PMN transepithelial migration in the presence of 100 μ M QUIN. It should be noted that, unlike *Shigella*, *Sal. typhimurium* has a completely functional pathway for *de novo* NAD synthesis and synthesizes QUIN (Penfound & Foster, 1996). The EPEC strain used in this study is a nicotinic acid prototroph and, presumably, also has a functional pathway for *de novo* NAD synthesis. The model of pathoadaptive evolution by inactivation of AVL predicts that since these pathogens can synthesize

QUIN, virulence of *Sal. typhimurium* and EPEC should be insensitive to inhibition by QUIN. Our results confirm this prediction.

The precise mechanism by which QUIN acts to inhibit *Shigella* virulence is still unknown. It is possible that QUIN acts directly on the mammalian cell to inhibit *Shigella* invasion. However, pretreatment of HeLa cell monolayers with 0.4 mM QUIN for up to 18 h prior to infection with M90T had no effect on bacterial invasion of the monolayer cells (data not shown). In addition, pretreatment of the bacteria with QUIN before addition to the cell monolayer was ineffective at blocking bacterial invasion. In fact, inhibition of invasion was only observed when QUIN was present during incubation of the bacteria with the cell monolayer. QUIN did not affect growth of *Sh. flexneri* in HeLa cells, and it did not significantly impair the expression of IpaB and IpaC virulence factors. It is possible that a bacterial or host target for the action of QUIN only becomes exposed once the bacterium comes in contact with the eukaryotic cell and is subsequently inhibited by QUIN.

QUIN shows a strong inhibitory effect on the induction of T3SS by CR, as shown by reduced levels of secreted IpaB and IpaC when the compound is added during the secretion assay (Fig. 6). The fact that all the *Shigella* virulence properties monitored in this study (invasion, cell-to-cell spread, and induction of PMN migration) are inhibited by QUIN is consistent with this last observation, since all of these phenotypes require a functional T3SS. As the mediator of T3SS induction by CR has yet to be identified in *Shigella*, we cannot deduce from this observation the precise mechanism by which QUIN exercises its effect. However, this result is consistent with the hypothesis that a bacterial target for the action of QUIN (probably a T3SS component) only becomes exposed once the virulence properties of the bacterium are induced, either by growth at 37 °C and addition of CR, or by contact with the eukaryotic cell. Furthermore, the QUIN target is specific to *Shigella*, since we saw no evidence in this work for the impairment of T3SS function in EPEC, *Sal. typhimurium* or *Y. enterocolitica*.

We have proposed two main criteria for the identification of AVL in pathogens. First, the AVL must be present and expressed in closely related or ancestral species occupying the non-virulent ancestral niche but absent from (or mutated in) pathogenic clones living in host tissues. Second, expression of the AVL by the pathogen in host tissues must attenuate virulence (Maurelli, 2007). Therefore, in order to prove that *nadA/B* fulfil the definition of AVL, we restored the functionality of *nadA/B* in *Shigella*. We observed a consistent inability of the resulting strain, BS813, to invade tissue culture cells. These results, combined with the results of virulence assays that exhibit a strong inhibitory effect of QUIN on *Shigella* virulence, demonstrate powerful pathoadaptive selection against the expression of the *nadA* and *nadB* genes in *Shigella* spp. and show that they are AVL for *Shigella*.

In summary, we have identified a new set of AVL for *Shigella* spp., and our results support the concept of bacterial pathogen evolution via inactivation of AVL. The results also demonstrate the potential of genomic and phenotypic screening for differences between bacterial pathogens and related commensals as a powerful tool for discovery of AVL. Nordfelth *et al.* (2005) recently screened a 9400 compound library to identify small molecules that specifically inhibited the T3SS of *Y. pseudotuberculosis*. In our study, we used a totally different approach and also identified a small molecule that specifically blocks T3SS of *Shigella*. Thus, identification of AVL can provide a powerful tool to discover new compounds that specifically inhibit bacterial virulence and therefore could be used in the treatment of infectious diseases. Recognition of pathoadaptive mutation as a process of bacterial pathogen evolution and application of the sort of converse comparative genomics we describe in this report (i.e. identification of genes and/or phenotypes present in a non-pathogenic ancestor and absent in the bacterial pathogen) should enable investigators to identify AVL in other pathogens. This information will provide new avenues for investigation of areas ranging from pathogenic mechanisms to the identification of novel antimicrobial agents.

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