Outsider to insider
Resetting the natural host niche of commensal *E. coli* K-12

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ADDENDUM
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The status of *E. coli* K-12 as an exclusively non-invasive, non-pathogenic bacterium has almost been incontrovertible. Our recent finding that a mutation in one of its main architectural protein, HU, converts *E. coli* K-12 to an actively invasive form suggests that gaining host cell entry might be an expedient survival tactic for traditional commensals during certain altered host conditions. The mutant *E. coli* (SK3842) exhibits properties usually associated with pathogenic bacteria: host cell invasion, phagosomal disruption and intracellular replication. However, unlike the situation with some pathogens, internalized SK3842 imparts anti-apoptotic and cytoprotective effects rather than lethality on the host cell, both in vitro and in vivo. Here, we show that SK3842 also provides colonization resistance against other invasive pathogens—a trait not shared by the parental commensal strain. Thus, the altered lifestyle of SK3842 encompasses characteristics both from traditional pathogens as well as beneficial probiotic strains.

**E. coli** K-12—An Archetypal Extra-Cellular Commensal

Long-standing concepts about strict niche adherence by human commensal bacteria have been challenged by increasing incidences of commensals being recovered from sites that are remote from their original site of colonization. Commensal *E. coli,* the most abundant facultative anaerobe present in human intestine, is frequently isolated from distal intestinal and extra-intestinal sites in cases of a number of extra-intestinal diseases, ranging from colitis and IBD to sepsis and multiorgan dysfunction. It has been a widely-accepted theory that host cell entry by resident *E. coli* is a consequence of compromised immunity or epithelial barrier function or a property exclusive to certain specialized, disease-causing *E. coli* clones termed as extra-intestinal pathogenic *E. coli* (ExPEC). Laboratory or enteric *E. coli* K-12 strains are considered as enfeebled organisms, lacking strong colonizing or virulent characteristics, as a result of being maintained as a laboratory strain for many decades. Invasiveness and intracellular survival are not traits normally attributed to these strains. Transgression across host boundaries to a new or remote host location could be an expedient strategy of resident microbes as a counter-response to altered host conditions for functional and survival benefits. However, due to lack of major invasive modules and crypticity of existing invasive factors (e.g., curli fibers), the possibility of established commensals switching to an invasive lifestyle, without any significant genomic flux, has not been explored to a great extent. However, certain recent reports about active translocation of conventional extracellular *E. coli* strains across the intestinal barrier and non-ExPEC commensal *E. coli* strains being responsible for extra-intestinal infections raise the possibility of well-established commensal *E. coli* intruding across and into unconventional host niches by an active, microbe-driven process.

An Invasive Variant of *E. coli* K-12

We have recently shown that a mutational change in one of its major, histone-like architectural protein, HU, causes a reversal
of the normal mode of interaction of E. coli K-12 with mammalian intestinal epithelial cells. Previously, we had demonstrated that a gain-of-function double amino acid mutation in HU (E38K, V42L) in E. coli K-12 rendered wide-ranging global changes both in the physical architecture as well as the transcriptional output of the nucleus. This was reflected in the plethora of morphological and physiological changes in the mutant strain (SK3842), many of which were consistent with properties exhibited by E. coli under in vivo or within-host conditions. Upon further study, we confirmed that the overall cellular changes of the mutant SK3842 bacteria corresponds with a conversion of traditional extracellular E. coli K-12 strain into an actively invasive form (SK3842). Unlike the parental K-12 strain, and the majority of commensal and pathogenic E. coli groups, SK3842 can rapidly and efficiently invade cultured intestinal epithelial cells. SK3842 maintains its invasive characteristic even under in vivo (ileal loop assay) and ex vivo (intestinal explants assay) conditions, indicating that invasiveness is an intrinsic trait and a functional consequence of the entire gamut of cellular changes. Gain of invasive property by conventional non-invasive bacteria is generally attributed to the acquisition of extra-chromosomal genetic elements carrying the requisite host cell entry functions. Our studies have shown that, even without any gross genetic changes, non-pathogenic, extra-cellular E. coli possesses the remarkable metabolic and physiological flexibility to co-opt widely-divergent approaches for its host-microbe interaction program.

SK3842 invade non-phagocytic host cells by inducing their own endocytosis through cytoskeletal rearrangement which results in membrane ruffling and projection of microvilli that encapsulate the bacteria and internalize them. Activation of the quiescent agr locus in SK3842, which codes for cellu fibers, is one of the principal events involved in the adhesion/invasion of host cells. The internalized SK3842 cells show AgrD-dependent disruption of their surrounding endocytic vacuoles to prevent phago-lysosomal fusion and escape into the host cell cytoplasm, where they are able to survive and multiply.  

Invasive but Non-Lethal Intra-Cellular Lifestyle of SK3842  

The sequence of intestinal cell-SK3842 interaction events, starting from the initial bacterial attachment to intracellular bacterial behavior, is strongly reminiscent of traditional host cell-pathogenic bacteria interplay: (1) colonization of a privileged host site, (2) evasion of host defenses and (3) multiplication. The final and definitive act of the infection cycle by traditional pathogens is host cell damage. However, SK3842 exhibited no change in LD50 in mice experiments, indicating that there is no change in its virulence potential despite following many of the trademark actions of pathogenic bacteria. At the cellular level, SK3842 invasion engenders well-defined anti-apoptotic signaling pathways in the form of degradation of pro-apoptotic Bim and Puma proteins and upregulation of pro-survival Mcl-1 protein. SK3842 infection of Int-407 cells does not cause either host cell death or bacterial elimination even after an extended incubation of 72 h (Fig. 1). This indicates that SK3842 have evolved specific adaptations to survive within host cells for prolonged periods by manipulating the host cell machinery for its own benefit.

Induction of apoptosis in non-professional phagocytic cells is a host defense strategy to thwart invasive bacteria from establishing a protected intra-cellular niche; it serves to delete the infected and damaged host cells as well as remove the infecting agent and promote efficient pathogen clearance. Conventional pathogens have evolved intricate counteractive mechanisms to prevent host cell apoptosis to maintain its survival and replicative niche or use destruction of the host cell as a route to disseminate further into deeper mucosal layers. Despite being a derivative of an E. coli K-12 strain with no recognized intracellular lifestyle, SK3842 not only avoids being killed intracellularly but also circumvents and subverts the host cell machinery to inhibit epithelial cell death.

Exclusion of Invasive Pathogens by Internalized SK3842  

Maintenance of intestinal epithelial barrier integrity is a well-established role of luminal commensal microbes. Proposed mechanisms of commensal-mediated maintenance of intestinal epithelial barrier function include enhanced cyto-protection, decreased apoptosis and bacterial interference. SK3842 infection exerts strong anti-apoptotic effect as well as significant cytoprotection against external apoptosis-inducing agent like staurosporine. The finding that SK3842, despite converting from an exclusive extracellular existence to an almost obligate invasive form, retained its cellular protective functions was both unexpected and intriguing. But, depending on the subsequent course of events, protection of host cells from premature death could be an effect of either beneficial gut commensals or harmful pathogens. To further corroborate the lack of virulence capacity in

![Figure 1. Long-term survival of SK3842 inside Int-407 cells. Int-407 cells were incubated in presence of DAPI-stained SK3842 (1 h, MOI 100). Non-internalized bacteria were killed by gentamycin treatment. Infected Int-407 cells were labeled with Alexa 488-phalloidin and viewed under fluorescence microscope after 24 h (A) and 72 h (B) to visualize intra-cellular bacteria.](image-url)
SK3842 and probe its enhanced potential for maintenance of epithelial barrier integrity, we wanted to test whether SK3842 also offers colonization resistance against other invasive pathogens. Gut microbiota provides colonization resistance to incoming pathogens by competitive exclusion, such as occupation of attachment sites, competition for available nutrient and production of antimicrobial peptides. Internalization of a gut commensal inside the host epithelial cells is expected to eliminate most, if not all, of these competitive advantages. We investigated the inhibitory effect of SK3842 infection on *Shigella flexneri* invasion of intestinal epithelial cells. Pre-incubation of Int-407 cells with SK3842 (MOI 100) resulted in a sharp reduction in *Shigella flexneri* 2457T (N64) invasion efficiency (Fig. 2A) while MG1655 (parental wild-type *E. coli* K-12) had negligible effect on *Shigella* invasion. This demonstrates that internalization of SK3842 provides significant protection against other invasive pathogens; a property mostly exhibited by probiotic *E. coli* strains like Nissle 1917. Colonization resistance against pathogens,
especially gram-negative bacteria, is provided mainly by extra-cellular, obligate anaerobes in the gut.22 SK3842, which is an invasive form of a facultative anaerobic bacterium, imparts similar colonization resistance against invasive pathogens. Pre-incubation with SK3842 do not impact the internalization efficiency of SK3842 in a second round of infection (data not shown), revealing that the colonization resistance is specific to other bacterial species. We then checked whether the cross-resistance to infection of a different bacterial species by SK3842 was also valid in case of other intra-cellular bacteria. Using uninfected and N64-infected Int-407 cells we checked for the invasion efficiency of SK3842. As shown in Figure 2B, the presence of intracellular Shigella had negligible effect on the internalization of SK3842. This indicates that the competitive exclusion of other invasive bacteria by SK3842 is not a non-specific restriction arising out of spatial constraints of host cells. Intracellular presence of pathogenic bacteria like Shigella, which have no known colonization resistance attributes, has no significant effect on the entry of SK3842 into the host cells. Internalization of SK3842 inside the host cell was necessary for it to exert its cross-protective influence against Shigella infection. SK3842 culture was grown in the presence of the synthetic β-breaker peptide, NH₂-QPGGGNPP-COOH, which has been shown to inhibit SK3842 invasion and used to infect Int-407 cells. After removing the extracellular SK3842 cells, invasion of N64 into Int-407 cells was evaluated. At a SK3842 MOI of 10, N64 showed no significant reduction of invasion efficiency compared with control cells pre-incubated with the β-breaker peptide alone (Fig. 2C). At a SK3842 MOI of 100, N64 showed a reduction in invasion efficiency, which can be explained by the greater number of SK3842 cells which escaped the inhibitory effect of the β-breaker peptide at a higher MOI and invaded the host cells. These results suggest that SK3842 represent a unique variant of normal E. coli K-12 which induces its own uptake by host epithelial cells (a trait usually associated with gut pathogens) while providing enhanced protection to the invaded host cells against other pathogens and cell death signals (a trait normally associated with beneficial probiotic strains).

A New Paradigm of Host-Commensal Interaction

The concept of utilizing an architectural nucleoid-associated protein, like HU, as a conformation-based master regulator to switch between different lifestyles within the mammalian host is more rational from an evolutionary perspective. The hypothesis of “antagonistic pleiotropy” postulates that niche expansion leads to loss of fitness at the original niche. In case of SK3842, activation of invasion-related functions is accompanied by the suppression of a large number of carbon utilization and amino acid biosynthetic genes. If this fitness trade-off for entry into a new host site is implemented through permanent genetic changes, like in the case of many obligate pathogens, this transition will represent a permanent change in niche address and an “evolutionary dead-end.” For a traditional commensal species, for whom opportunities to make niche transitions are presented on extremely rare occasions, it is imperative to retain the metabolic and physiological flexibility to switch back to its original host colonization site.

Migration from a primary niche to a new host niche by a traditional commensal can give rise to three different outcomes: (1) Commensalism: the phenotypic switch can be viewed as an act of self-preservation which benefit the bacteria during periods of acute host-inflicted stress. (2) Mutualism: invasion and persistence inside host cells can help both the bacteria by providing a safe haven from other microbial predators in the intestinal lumen as well as the host by thwarting other pathogens from gaining entry inside the intestinal cells. (3) Pathogenesis: given that host cell invasion by pathogenic bacteria is almost invariably linked to disease pathogenesis, SK3842 can be viewed as a commensal-pathogen switch-over. The facts that SK3842 lacks a refined and forceful virulence arsenal like that of an obligate pathogen and there are no selective advantages of mounting a virulent attack against the host by a traditional commensal (host cell death and poor transmission to new hosts) are probably consistent with the finding that SK3842, despite invading intestinal mucosal cells, does not exhibit enhanced lethality in mouse model. However, SK3842 survives intra-cellularly for an extended period while safeguarding host epithelial cells against invasive pathogens and other potential cellular insults (mutualism). This suggests an active and methodical stratagem by these invasive commensal variants to manipulate host cell machinery for extended survival benefits both for itself and the host, rather than a fortuitous and arbitrary change of its own colonization site. Identification of more bacterial effectors, their host cell targets and the long-term outcome of the interplay, both from the host and bacterial perspectives, is needed to uncover this novel and uncharacterized host-commensal inter-action dynamics.

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