

The interaction between a non-pathogenic and a pathogenic strain synergistically enhances extra-intestinal virulence in *Escherichia coli*

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Finding two or more genotypes of a single species within an infected sample is a not infrequent event. In this work, three *Escherichia coli* strains of decreasing extra-intestinal virulence (pathogenic B2S and B1S strains, and the avirulent K-12 MG1655 strain) were tested in septicaemia and urinary tract infection (UTI) mouse models, either separately or in pairs. Survival was monitored and bacteria were counted in various organs. Serum interleukin (IL)-6, tumour necrosis factor alpha (TNF α) and IL-10 were measured. We show that a mix of high amounts of B1S or of MG1655 with low amounts of B2S killed more rapidly (B1S), or killed more mice (MG1655), than either high amounts of B1S, high amounts of MG1655 or low amounts of B2S separately in the mouse septicaemia model. This bacterial synergy persisted when high amounts of dead or abnormal-LPS K-12 cells were injected together with a low amount of B2S. In both septicaemia and UTI models, significantly more bacteria were recovered from the organs of mice injected with the MG1655/B2S mix than from those of mice injected with the inocula separately. Consistently, in the septicaemia model, more IL-6 was secreted before death by the mice that were injected with the mix of bacteria than by the mice that were injected with the inocula separately. The synergistically enhanced mortality in the case of co-infection in the septicaemia model persisted in *RFc γ ^{-/-}*, *Myd88^{-/-}* and *IL-6^{-/-}* knockout mice. This synergistically increased virulence resulting from the interaction between an avirulent and a pathogenic strain of the same bacterial species raises questions about the role of avirulent bacteria in the development of some extra-intestinal infections.

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INTRODUCTION

Social interactions have been described in all living entities. Countless examples can be given of interactions involving the simplest organisms, such as small parasites, protozoa and even prokaryotes (Brogden *et al.*, 2005). In some cases these interactions are involved in the development of a particular pathological process. For example, interactions have been described between a bacterium and a parasite [*Streptococcus bovis* and *Strongyloides stercoralis* (Link & Orenstein, 1999)], a fungus [*Staphylococcus aureus* and *Candida albicans* (Roux *et al.*, 2009)], a virus [*Streptococcus pneumoniae* and influenza (Sun & Metzger, 2008)] and another bacterium [*Treponema vincenti* and *Fusobacterium*

nucleatum in Vincent's angina (Brook, 2005; Stammers, 1944)]. Another interesting example of bacterial association is the case of polymicrobial bacteraemia, which frequently associates several *Enterobacteriaceae* and results in higher mortality (Cooper *et al.*, 1990; Pittet *et al.*, 1993).

Interactions between clonal individuals of a single bacterial species are also well documented. Socio-microbiology has known a major development over the last two decades (Parsek & Greenberg, 2005). One of the earlier and best-described examples is that of quorum sensing in *Vibrio fischeri* (Fuqua *et al.*, 1994). The quorum sensing in *Pseudomonas aeruginosa* has been shown to regulate the expression of some virulence factors (Passador *et al.*, 1993). It also appears to control biofilm formation (Davies *et al.*, 1998), the role of which is well documented in human pathology. In some cases, the benefits of the interaction are evenly distributed between the two parties. In others, one of the two parties involved in the interaction obtains a

Abbreviations: ExPEC, extra-intestinal pathogenic *E. coli*; FcR, Fc receptor; IL, interleukin; MyD88, myeloid differentiation factor 88; TLR, Toll-like receptor; TNF α , tumour necrosis factor alpha; UTI, urinary tract infection.

greater advantage than the other (West *et al.*, 2006). This has been perfectly described in *P. aeruginosa*, in which species co-operators and cheaters have been found (Buckling *et al.*, 2007). Therefore, micro-organism interactions appear to be frequently linked to virulence.

Very little is known about monospecies polyclonal interactions, i.e. interactions involving divergent strains of the same species. Even so, finding two or more genotypes of a single species within an infected sample is a not infrequent event. Wendt *et al.* (1998) analysed 29 cases of monobacterial Gram-negative bacteraemia in which at least two morphologically different isolates could be identified. Nine of these were polyclonal as shown by pulsed gel electrophoresis. Johnson *et al.* (2003) subjected several *E. coli* clones isolated from four samples of extra-intestinal infections to molecular phylotyping. One out of the four samples contained two divergent strains, one from the A phylogenetic group and one from the D phylogenetic group. More recently, such polymicrobial infections were extensively searched for in 19 patients with visceral *Escherichia coli* infections and were found in four patients (Levert *et al.*, 2010).

E. coli is the most important anaerobic facultative commensal of the lower intestinal tract of all vertebrates (Tenaillon *et al.*, 2010). However, some strains, due to specific virulence genes, are able to produce a very wide range of infections within the host (Kaper *et al.*, 2004). The infections can be intestinal as well as extra-intestinal, and include septicaemia, urinary tract infections (UTIs), meningitis and pneumonia (Russo & Johnson, 2003). *E. coli* is responsible for more than two million human deaths worldwide every year (Kosek *et al.*, 2003; Russo & Johnson, 2003). Commensal and potentially extra-intestinal pathogenic *E. coli* (ExPEC) strains frequently coexist in the intestinal tract of the same host (Escobar-Páramo *et al.*, 2004; Moreno *et al.*, 2009), and their relative proportions vary greatly in humans.

In this work, we assessed the potential role in *E. coli* extra-intestinal virulence of within-species interactions between non-pathogenic and pathogenic strains, using mouse septicaemia and UTI models.

METHODS

Bacterial strains. *E. coli* strain B2S was isolated from the pus of a human liver abscess during a surgical procedure in 1984. It belongs to the B2 phylogenetic group. B2S is serotype O6, is resistant to streptomycin and contains the following extra-intestinal virulence factors [out of a panel of 20 tested genes (Johnson *et al.*, 2006)]: *chuA*, *sfa/foc*, *iroN*, *papC*, *papG* (III), *hlyC*, *cnf1*, *hra*, *usp*, *ompT*, *malX*, *fyuA* and *irp2*. *E. coli* strain B1S was isolated from the same pus of a human liver abscess as B2S. It belongs to the B1 phylogenetic group and is serotype O8. It possesses the *aer*, *fyuA* and *irp2* extra-intestinal virulence genes, and is resistant to aminopenicillins. Strain K-12 MG1655 is a laboratory-derived commensal *E. coli* strain. It belongs to the A phylogenetic group, is serotype O16 and has no antibiotic resistance or virulence factors. Strain CFT073 is a uropathogenic *E. coli* strain. It belongs to the B2 phylogenetic group and is serotype O6.

It possesses the following virulence factors: *neuC*, *kpsE*, *sfa/foc*, *iroN*, *aer*, *iha*, *papC*, *papG* (II), *hlyC*, *sat*, *ireA*, *usp*, *ompT*, *malX*, *fyuA* and *irp2*. It has no antibiotic resistance. In addition, a K-12 BW25113 strain deleted for *msbB* was obtained from the Keio collection (Baba *et al.*, 2006).

Preparation of dead *E. coli* cells. Exponential-phase bacteria grown in Luria–Bertani (LB) broth were washed once in 0.9% NaCl. The bacteria were then resuspended in absolute ethanol. They were left at room temperature for 3 h, vortexing from time to time. They were washed once in 0.9% NaCl and then used to prepare bacterial mixes. Bacterial death was checked by plating 10^8 cells on an LB agar plate, and no bacteria were found to survive. Dead bacteria were also checked by Gram staining. No significant lysis was observed in the washed dead bacteria suspension.

Preparation of *E. coli* membranes. Exponential-phase bacteria grown in LB broth were washed once in sterile double-distilled water. They were sonicated three times for 1 min (and left for 1 min in an ice/water slurry between each sonication). The preparation was then centrifuged for 7 min at 6000 r.p.m. (3800 g). The supernatant was transferred to a fresh tube and centrifuged for 2 h at 14 000 r.p.m. (20 800 g). The supernatant was removed and the membranes were resuspended in a volume of 0.9% NaCl equal to that of the initial exponential-phase culture. The number of bacteria surviving in the membrane preparation was checked by plating on LB agar plates. Fewer than 100 c.f.u. ml⁻¹ were recovered out of 10^8 bacteria used to make the preparation. The membrane preparation was then used to make membrane/bacteria mixes.

Mice. Six-week-old OF1 outbred females were obtained from Charles River. Seven-week-old CBA/j females, six-week-old BALB/c females and five-week-old C57BL/6 females were obtained from Janvier. Six- to eight-week-old male or female BALB/c, *fcRγ*^{-/-} mice were obtained from the authors' own laboratory (Pinheiro da Silva *et al.*, 2007). Six- to eight-week-old male or female C57BL/6, *myd88*^{-/-} mice were obtained from the authors' own laboratory (Kawai *et al.*, 1999). Six- to eight-week-old male or female C57BL/6 IL-6^{-/-} mice were obtained from Gérard Eberl (Institut Pasteur, Paris, France) (Doisne *et al.*, 2009). All animal experiments were performed in compliance with the recommendations of the French Ministry of Agriculture and were approved by the French Veterinary Services (accreditation no. A 75-18-05).

Septicaemia model. Exponential-phase bacteria grown in LB broth were washed twice in 0.9% NaCl. Bacterial mixes were prepared prior to injection in 0.9% NaCl. OF1 (wild-type), C57BL (wild-type or knockout for *myd88* or the IL-6 gene) or BALB/c (wild-type or knockout for *fcRγ*) mice were challenged subcutaneously with 0.2 ml of bacterial mix (containing between 10^5 and 10^8 bacteria, depending on the experiment). Mice had free access to food and water (Picard *et al.*, 1999). Time to death was recorded until day 7. Mice that survived more than 7 days were sacrificed by cervical dislocation. All dead mice were frozen (see below). All mouse experiments were repeated at least twice. No 'day of the experiment' effect was observed and the results of all the repeats of the same bacterial mix were pooled. Frozen mice were left at room temperature until sufficiently defrosted to allow dissection (less than 1 h for 14–16 g mice). Spleens, hearts, livers and kidneys were recovered and weighed. One millilitre of 0.9% NaCl buffer was added to each organ, which was then homogenized with a tissue blender (Ultra-Turrax T25, Fisher Scientific) for 1 min. Bacterial counts were determined on LB agar plates with or without antibiotics in order to distinguish between the various strains. The results are expressed as log₁₀[c.f.u. (g organ)⁻¹]. For survival and organ bacterial count experiments, a minimum of 10 mice was used in each group of infected mice (maximum 60 mice).

Because freezing and thawing of mice could differentially affect the survival of bacteria of different strains, six groups of mice were injected with the six bacterial challenges used in this study (Fig. 4). In each group, half of the mice were dissected immediately after death to recover the spleen and the other half were frozen after death, thawed and then dissected. The number of bacteria isolated from the spleens of mice that had been frozen was not statistically different from that found in the spleens of mice that had been dissected immediately after death (data not shown). This absence of a difference was found for all strains (MG1655, B1S and B2S) and on all media (LB, LB+streptomycin and LB+amoxicillin) used in this study. The freezing of mice was therefore subsequently used for logistic simplification.

Mouse anaesthetic mix. The anaesthetic mix contained 0.5 ml 2% xylazine (Bayer), 1.5 ml 10% ketamine chlorhydrate (Merial) and 18 ml sterile water. A dose of 0.2 ml per 10 g of mouse was injected into the peritoneum.

Ascending UTI model. The ascending, unobstructed UTI mouse model has been described previously (Labat *et al.*, 2005). Briefly, 10^7 or 10^9 bacteria suspended in 50 μ l 0.9% NaCl were injected through a urethral catheter into the bladder of seven-week-old anaesthetized female CBA mice. The catheter was removed immediately after inoculation. Animals were sacrificed 48 h after inoculation and kidneys and bladders were aseptically removed. Organs were homogenized with a tissue blender for 1 min. Bacterial counts were determined on LB agar plates with or without antibiotics in order to distinguish between the various strains. The results are expressed as \log_{10} [c.f.u. (g organ) $^{-1}$]. Five mice were injected with each bacterial mix and UTI experiments were repeated twice. The results of the repeats were pooled.

Serum collection for cytokine determination. Infected BALB/c mice were anaesthetized 14 h after the bacterial injection (immediately before the death of the first mice). Blood was collected by heart aspiration (after thoracotomy if necessary) in a syringe containing approximately 10 μ l 25 000 U heparin ml $^{-1}$ (Choay). Blood samples were transferred into Eppendorf tubes and left on the bench for coagulation for at least 2 h. Samples were then centrifuged for 10 min at 14 000 r.p.m. (20 800 g), at 4 °C. Supernatants were transferred to fresh Eppendorf tubes and centrifuged again (20 min at 14 000 r.p.m., 4 °C). Sera were kept at -20 °C until use. ELISA kits were used to determine cytokines (IL-6, IL-10 and TNF α) according to the manufacturer's instructions (R&D Systems, Inc.). For cytokine determination experiments, a minimum of five mice was used in each group.

Statistical analysis. Kaplan–Meier estimates of mouse survival were performed using Stata 8.0 software (Stata Corp.). The equality of the survivor function across groups was estimated by the log rank test (Stata 8.0). Bacterial counts and cytokine level differences were assessed using non-parametric *t* tests.

RESULTS

In this paper, 'high amounts' of a bacterial strain means 10^8 (septicaemia model) or 10^9 (UTI model) cells and is abbreviated as '10 8 -' or '10 9 -', respectively, and 'low amounts' of a bacterial strain means 10^6 (septicaemia model) or 10^7 (UTI model) cells, and is abbreviated as '10 6 -' or '10 7 -', respectively. Since no deaths occurred after day 3, all survival curves presented in Figs 1–3 show the first 72 h of the experiments only.

The interaction between a commensal and a pathogenic strain of *E. coli* synergistically enhances mortality in a mouse septicaemia model

Four groups of OF1 mice were injected with four different bacterial mixes: high amounts (10^8 cells) of B1S (10 8 -B1S), high amounts (10^8 cells) of B2S (10 8 -B2S), low amounts (10^6 cells) of B2S (10 6 -B2S) and 'mixed infection' (10^8 cells of B1S mixed together with 10^6 cells of B2S prior to subcutaneous injection: 10 8 -B1S + 10 6 -B2S). Survival was then recorded (Fig. 1a). Both 10 8 -B2S and 10 8 -B1S rapidly killed all the mice, indicating that both strains were virulent. However, 10 8 -B1S killed the mice significantly less rapidly than 10 8 -B2S: at more than 20 h and less than 18 h ($P < 10^{-4}$), respectively, which corresponds to a previously described important cut-off in the mouse septicaemia model (Picard *et al.*, 1999). Therefore, B1S was slightly less virulent than B2S. There was also a dose effect, since 10 6 -B2S killed fewer than 25% of the mice ($P < 10^{-5}$ when compared with 10 8 -B2S). When high amounts of the less virulent strain (10 8 -B1S) were mixed together with low amounts of the more virulent strain (10 6 -B2S) and then injected into the mice (10 8 -B1S + 10 6 -B2S), the mortality curve was slightly, but significantly, shifted to the left ($P = 0.02$ for comparison between 10 8 -B1S + 10 6 -B2S and 10 8 -B1S). This seemed to indicate that there was an additive effect of B1S and B2S virulence. The same experiment was repeated with the non-pathogenic K-12 MG1655 (MG) strain as a replacement for B1S. Fig. 1(b) confirms that MG1655 is avirulent (Johnson *et al.*, 2006): even when high amounts were injected into the mice, they all survived. When mice were injected with the 10 8 -MG + 10 6 -B2S mix, the 7-day survival rate dropped to $9.5 \pm 3.4\%$ ($P < 10^{-5}$). Since neither 10 8 -MG nor 10 6 -B2S alone was able to kill more than 25% of the mice, this high mortality rate evidenced a synergy in extra-intestinal virulence between MG1655 and B2S.

This bacterial synergy was dose-dependent. When the amount of B2S was progressively decreased from 10^6 to 10^4 cells, the survival increased (Fig. 1c, $P \leq 0.01$ for any comparison of paired inocula). Similarly, when the amount of MG was progressively decreased from 10^8 to 10^6 cells, survival also increased (Fig. 1d, $P \leq 0.02$ for any comparison of paired inocula).

In order to test whether this bacterial interaction relied on a specific function of MG1655, the same experiment was repeated with dead MG1655 (\dagger MG). All the mice injected with the 10 8 - \dagger MG + 10 6 -B2S bacterial mix were very rapidly killed ($P < 10^{-5}$ when compared with 10 6 -B2S, Fig. 2a). No difference was observed between the survival of the mice that were injected with 10 8 -B2S and that of the mice that received the mixed infection.

E. coli membrane lipopolysaccharide (LPS) is a well-known virulence factor (Leon *et al.*, 2008; Parrillo *et al.*, 1990). When injected into mice in sufficient quantity it is lethal and mimics septic shock. In order to test whether the above

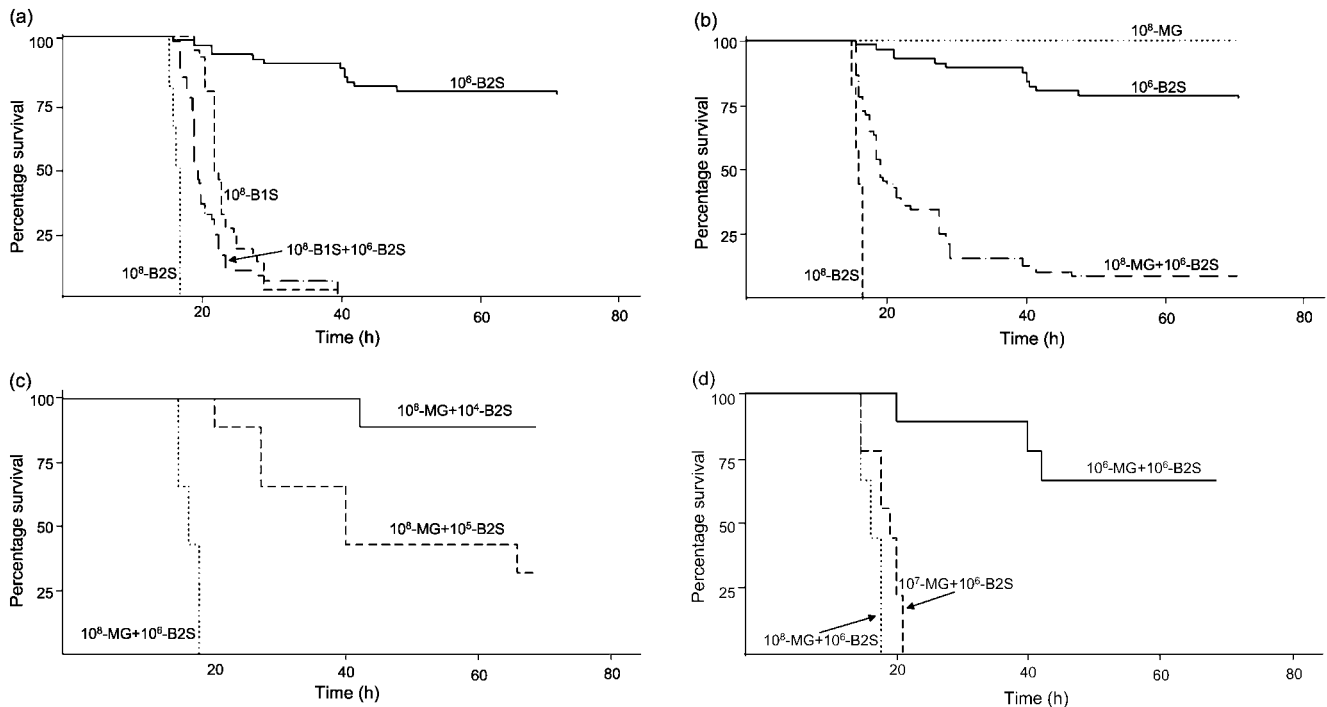


Fig. 1. OF1 mice survival after subcutaneous injection with different mixtures of wild-type *E. coli* isolates. (a) Solid line, mice injected with 10^6 cells of strain B2S; short-dashed line, mice injected with 10^8 cells of strain B1S; long-dashed line, mice simultaneously injected with 10^8 cells of the B1S strain and 10^6 cells of the B2S strain; dotted line, mice injected with 10^8 cells of strain B2S. (b) Dotted line, mice injected with 10^8 MG1655 cells; solid line, mice injected with 10^6 cells of strain B2S; long-dashed line, mice simultaneously injected with 10^8 MG1655 cells and 10^6 cells of strain B2S; short-dashed line, mice injected with 10^8 cells of strain B2S. (c) Mice were simultaneously injected with 10^8 MG1655 cells and 10^4 (solid line), 10^5 (dashed line) or 10^6 (dotted line) cells of strain B2S. (d) Mice were simultaneously injected with 10^6 cells of the B2S strain and 10^6 (solid line), 10^7 (dashed line) or 10^8 (dotted line) MG1655 cells.

bacterial synergy was due to LPS, we used an *msbB*-deficient mutant of *E. coli* strain K-12. LPS from the *msbB*⁻ strain lacks the myristoyl fatty acid moiety of the lipid A (Somerville *et al.*, 1996). High amounts of *msbB*-deficient *E. coli* were not able to kill any mice (Fig. 2b). Conversely, 10^8 -*msbB*⁻ K-12 + 10^6 -B2S mixed infection killed 80% of the mice in less than 18 h and the remainder within the next day ($P < 10^{-5}$ when compared with 10^6 -B2S). The mixed infection was therefore highly virulent. No statistical difference was found between the survival of the mice that received the mixed infection and that of the mice that received 10^8 -B2S. This indicates that wild-type LPS is not necessary to reproduce the bacterial synergy between the non-pathogenic and B2 strains.

To assess the localization of the factor responsible for the synergy, we injected a mix of MG1655 membranes (prepared from 10^8 cells) and 10^6 B2S living cells (Fig. 2c). Membranes alone were not lethal to mice. The injection of a mix of MG1655 membranes and of B2S was partially able to reproduce the synergy, since the survival of the mice was inferior to that of mice injected with 10^6 -B2S ($P = 0.006$). The survival of mice injected with 10^8 -MG + 10^6 -B2S tended to be inferior to that of mice

injected with MG membranes + 10^6 -B2S (non-significant trend: $P = 0.06$).

In order to test whether the bacterial synergy could be extended to other *E. coli* strains, the lethality test was also repeated with a B2 urosepsis strain, CFT073 (CFT), and MG1655 (Fig. 3). Again, the survival of mice injected with the 10^8 -MG + 10^6 -CFT mix was significantly lower than that of both groups of mice injected with either 10^8 -MG or 10^6 -CFT ($P < 0.02$ in both cases). This indicates that the bacterial synergy is not specific to two strains isolated from a polyclonal human sample.

Organ bacterial counts are higher in the case of mixed infection

In order to exclude the theoretical possibility of intestinal microbiota contamination of the spleens during sepsis or after death, the bacterial species and phylogenetic group were checked using a rapid PCR determination method for 60 randomly picked clones recovered from the spleens (Clermont *et al.*, 2000). At least 10 clones from each group of bacterial challenges were tested. Only *E. coli* was found on the plates (data not shown). The clones from LB

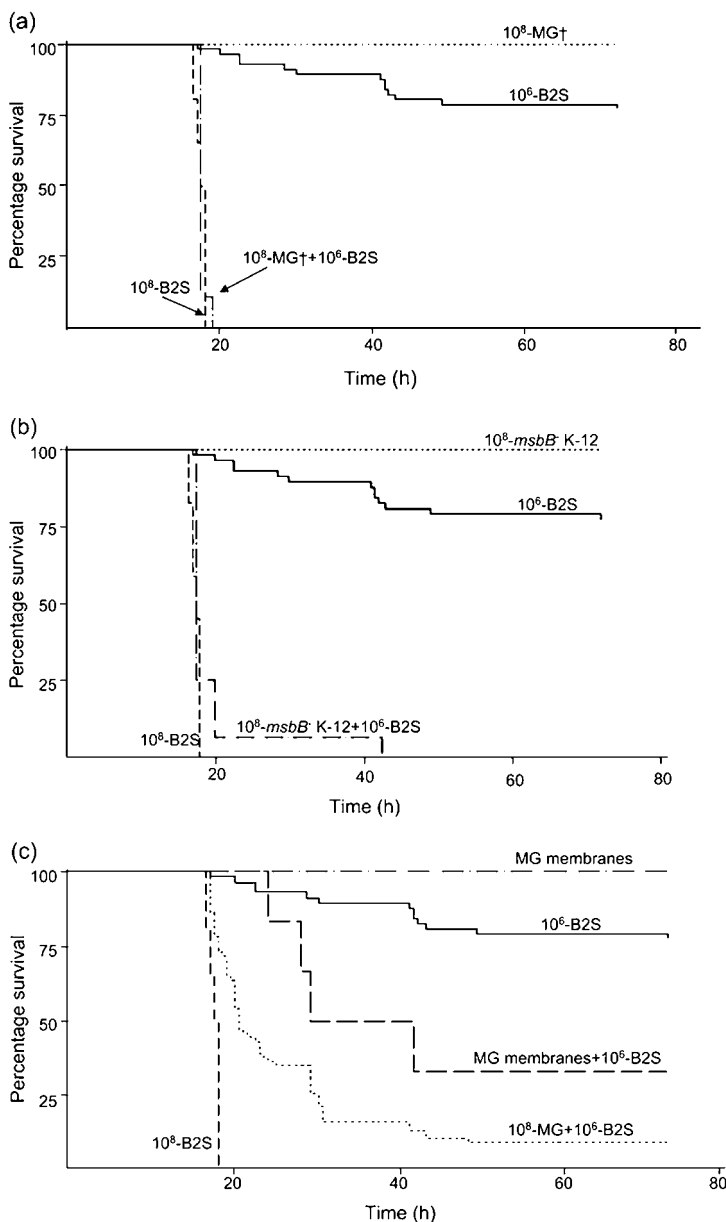


Fig. 2. OF1 mice survival after subcutaneous injection with different mixtures of modified *E. coli* strains. (a) Dotted line, mice injected with 10^8 dead MG1655 cells; solid line, mice injected with 10^6 cells of strain B2S; dashed and dotted line, mice simultaneously injected with 10^8 dead MG1655 cells and 10^6 cells of strain B2S; short-dashed line, mice injected with 10^8 cells of strain B2S. (b) Dotted line, mice injected with 10^8 *msbB*-deficient K-12 cells; solid line, mice injected with 10^6 cells of strain B2S; dashed and dotted line, mice simultaneously injected with 10^8 *msbB*-deficient K-12 cells and 10^6 cells of strain B2S; short-dashed line, mice injected with 10^8 cells of strain B2S. (c) Dashed and dotted line, mice injected with a membrane preparation from 10^8 MG1655 cells; solid line, mice injected with 10^6 cells of strain B2S; long-dashed line, mice simultaneously injected with a membrane preparation from 10^8 MG1655 cells together with 10^6 cells of strain B2S; dotted line, mice simultaneously injected with 10^8 MG1655 cells and 10^6 cells of strain B2S; dashed line, mice injected with 10^6 cells of strain B2S.

agar + streptomycin plates all belonged to the B2 phylogenetic group (presumably B2S); the clones from LB agar + amoxicillin plates all belonged to the B1 phylogenetic group (presumably B1S). Clones from the A phylogenetic group were the exception. They were found only on LB agar plates from MG1655/B2S mixed infections (together with a vastly greater number of B2 cells) and were presumed to be strain MG1655.

Fig. 4(a) shows the bacterial counts obtained from the spleens of OF1 mice that were injected with the different bacterial mixes. When mice were injected with 10^8 -B2S or 10^8 -B1S, 5.9 ± 1.4 and $5.2 \pm 1.1 \log_{10}[\text{c.f.u. (g organ)}^{-1}]$ were recovered from the spleens, respectively ($P=0.01$). When the mice were injected with 10^6 -B2S or with 10^8 -B1S + 10^6 -B2S, significantly fewer bacteria were found in

the spleens: 2.5 ± 2.7 and $3.9 \pm 1.3 \log_{10}[\text{c.f.u. (g organ)}^{-1}]$, respectively ($P < 10^{-7}$ in both cases compared with 10^8 -B2S). The spleens of mice injected with 10^8 -B1S + 10^6 -B2S contained more bacteria than those of mice that were injected with 10^6 -B2S alone ($P < 0.001$), but fewer bacteria than the spleens of mice injected with 10^8 -B1S ($P < 0.00001$). In the organs of B1S/B2S co-infected mice, bacteria were also counted on LB + agar + streptomycin ($30 \mu\text{g ml}^{-1}$) or amoxicillin ($50 \mu\text{g ml}^{-1}$) media in order to distinguish B2S (streptomycin-resistant) and B1S (amoxicillin-resistant) strains. Strain B2S in the spleens of B1S/B2S co-infected mice was more abundant than in the spleens of mice injected with 10^6 -B2S [3.6 ± 1.2 and $2.5 \pm 2.7 \log_{10}[\text{c.f.u. (g organ)}^{-1}]$, respectively; $P=0.02$]. Conversely, B1S in the spleens of B1S/B2S co-infected mice [$2.8 \pm 1.6 \log_{10}[\text{c.f.u. (g organ)}^{-1}]$] was less abundant than

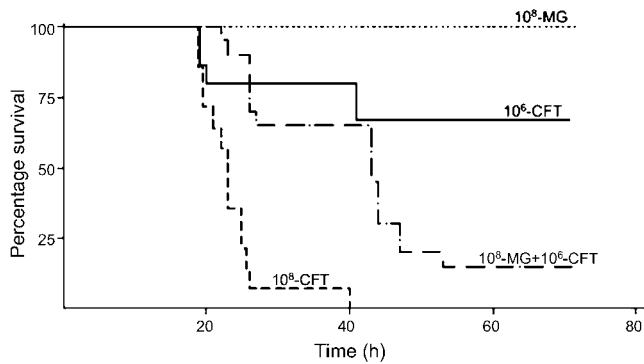


Fig. 3. OF1 mice survival after subcutaneous injection with CFT073 and/or MG1655 *E. coli* strains. Dotted line, mice injected with 10^8 MG1655 cells; solid line, mice injected with 10^6 CFT073 cells; dashed and dotted line, mice simultaneously injected with 10^8 MG1655 cells and 10^6 CFT073 cells; dashed line, mice injected with 10^8 CFT073 cells.

in the spleens of mice injected with a 10^8 -B1S single infection ($P < 0.00001$). Finally, more B2S than B1S was found in the spleens of co-infected mice [3.6 ± 1.2 and $2.8 \pm 1.6 \log_{10}[\text{c.f.u. (g organ)}^{-1}]$, respectively; $P = 0.03$]. Taken together, these results indicate that in the case of B1S/B2S co-infection, the two strains compete with each other (fewer total bacteria were found), although B2S is favoured (there were more B2S in the mixed infection than in the low amount of B2S group) to the detriment of B1S (there were fewer B1S in the mixed infection than in the high amount of B1S group).

When the mice were injected with 10^8 -MG, almost no bacteria were found in the spleens: $0.4 \pm 0.8 \log_{10}[\text{c.f.u. (g organ)}^{-1}]$. When the mice were injected with 10^8 -MG + 10^6 -B2S, $4.9 \pm 1.5 \log_{10}[\text{c.f.u. (g organ)}^{-1}]$ was recovered from the spleens. This bacterial count was significantly lower than that obtained with 10^8 -B2S ($P = 0.0004$), but was significantly higher than that obtained with 10^6 -B2S ($P < 0.0005$) or 10^8 -MG ($P < 0.00001$) alone, which indicates that MG1655 is able to favour the development of a spleen infection. In the MG/B2S co-infection group of mice, the bacteria were also counted on LB agar + streptomycin ($30 \mu\text{g ml}^{-1}$) plates in order to count the number of B2S cells (streptomycin-resistant). Spleen B2S counts in the mixed infection group of mice were $4.6 \pm 1.6 \log_{10}[\text{c.f.u. (g organ)}^{-1}]$. This count was higher than that obtained with 10^6 -B2S alone ($P < 10^{-5}$), but statistically was no lower than the count obtained on LB agar alone (total bacterial count) in the mixed infection group of mice. Forty-four colonies were randomly picked on the LB agar plates from six mice that received the mixed infection. Their phylogenetic groups were determined by a rapid PCR method (Clermont *et al.*, 2000). Forty-two (95%) colonies were from the B2 phylogenetic group (data not shown). This indicates that most of bacteria collected from the spleens of the MG/B2S mixed infection group of mice were B2S. Therefore, MG1655 favours the development

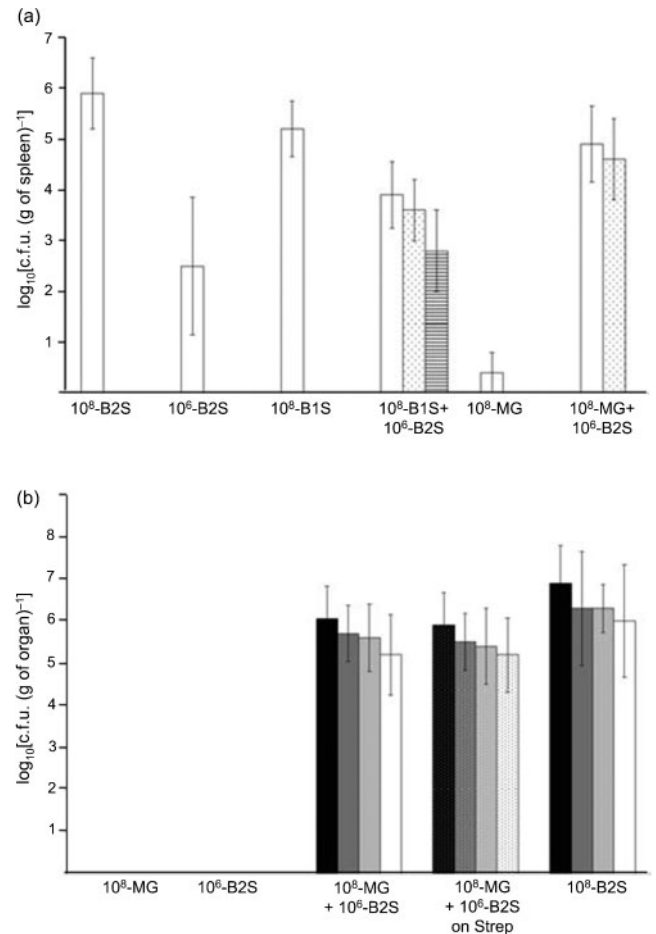


Fig. 4. Bacterial counts in organs of infected OF1 mice. (a) Bacterial counts in the spleens of infected mice. The *E. coli* mix with which the mice were injected is indicated at the bottom of each column. 10^8 -B1S, 10^8 -B2S and 10^8 -MG, mice were injected with 10^8 cells of strain B1S, B2S or MG1655, respectively; 10^6 -B2S, mice were injected with 10^6 cells of strain B2S; 10^8 -B1S + 10^6 -B2S and 10^8 -MG + 10^6 -B2S, mice were injected with a mix of 10^6 cells of strain B2S together with 10^8 cells of strain B1S or MG1655, respectively. The mean $\log_{10}[\text{c.f.u. (g spleen)}^{-1}]$ is shown; error bars, SD. White bars, *E. coli* were counted on LB agar plates (total bacterial counts); stippled bars, *E. coli* cells were counted on LB agar + streptomycin ($30 \mu\text{g ml}^{-1}$) [only B2S c.f.u. were counted (counts performed for the B1S/B2S and MG1655/B2S mixed infection only)]; hatched bars, *E. coli* cells were counted on LB agar + amoxicillin ($50 \mu\text{g ml}^{-1}$) [only B1S c.f.u. were counted (counts performed for the B1S/B2S mixed infection only)]. (b) Bacterial counts in various organs of infected mice. The *E. coli* mix with which the mice were injected is indicated at the bottom of each column. 10^8 -B2S and 10^8 -MG, mice injected with 10^8 cells of strain B2S or MG1655, respectively; 10^6 -B2S, mice injected with 10^6 cells of strain B2S; 10^8 -MG + 10^6 -B2S, mice injected with a mix of 10^6 cells of strain B2S together with 10^8 MG1655 cells. The mean $\log_{10}[\text{c.f.u. (g organ)}^{-1}]$ is shown; error bars, SD. Black bars, bacterial counts in the heart; dark-grey bars, bacterial counts in the kidneys; light-grey bars, bacterial counts in the liver; white bars, bacterial counts in the spleen. Stippled bar, *E. coli* cells were counted on LB agar + streptomycin ($30 \mu\text{g ml}^{-1}$) [only B2S c.f.u. were counted (counts performed for MG1655/B2S mixed infections only)].

of B2S infection, although it is not able to take advantage of the B2S co-infection to grow during the infectious process.

Interestingly, more bacteria were found in the 10^8 -MG + 10^6 -B2S group of mice than in the 10^8 -B1S + 10^6 -B2S group of mice [4.9 ± 1.5 and 3.9 ± 1.3 \log_{10} [c.f.u. (g organ) $^{-1}$], respectively; $P < 0.005$]. The interaction between the two pathogenic strains from this study is therefore less virulent than the interaction between a pathogenic and a non-pathogenic strain. This is probably due to the B1S/B2S competition detailed above.

In a subgroup of 16 OF1 mice only, bacteria were counted in the spleen, heart, kidneys and liver (Fig. 4b). Bacterial counts were perfectly homogeneous in all organs. Bacterial counts obtained in all the organs were comparable, within one order of magnitude, with those recovered from the spleens. For all the organs, co-infection with 10^8 -MG + 10^6 -B2S led to recoveries of more than 5 \log_{10} [c.f.u. (g organ) $^{-1}$]. This confirms that the spleen bacterial count is a good surrogate for the intensity of septicaemia. In the group of mice that were injected with 10^8 -MG + 10^6 -B2S, bacteria were also plated on LB agar + streptomycin in order to enumerate B2S cells. Counts on LB agar and on LB agar + streptomycin were not statistically different. In this particular group of 16 mice, due to chance, no bacteria were recovered from any organ in the groups of mice injected with 10^8 -MG or with 10^6 -B2S, and this finding was also very common in the much higher number of spleen dissections performed for Fig. 4(a).

The interaction between a commensal and a pathogenic strain of *E. coli* synergistically exacerbates UTI in a mouse model

In order to test whether the synergy was restricted to one specific mouse model, four different bacterial mixes were injected into the bladders of CBA/j mice. After 48 h, bacteria were counted in the bladder and kidneys (Fig. 5). The number of bacteria in \log_{10} [c.f.u. (g organ) $^{-1}$] found in the bladder and kidneys of the mice that were injected with 10^9 -B2S (5.0 ± 1.0 and 5.7 ± 1.2 , respectively) was higher than the bacterial count in the bladder and kidneys of the mice injected with 10^7 -B2S (2.4 ± 2.7 , $P = 0.002$, and 1.9 ± 1.9 , $P = 0.0002$, respectively) or 10^9 -MG (3.4 ± 1.4 , $P = 0.0004$, and 3.1 ± 0.9 , $P < 0.0001$, respectively).

The number of bacteria in \log_{10} [c.f.u. (g organ) $^{-1}$] found in the bladder and kidneys of mice injected with the 10^9 -MG + 10^7 -B2S mix (6.8 ± 2.7 and 6.4 ± 2.8 , respectively) was higher than the bacterial counts in the bladder and kidneys of the mice injected with either 10^7 -B2S ($P = 0.0007$ and $P < 0.0002$, respectively) or 10^9 -MG ($P = 0.001$ and $P = 0.002$, respectively). This implies that the MG1655/B2S co-infection in the bladder synergistically results in a more severe infection of the urinary tract in mice. The bacterial synergy observed in the septicaemia model is therefore also reproduced in the UTI model.

Finally, in the 10^9 -MG + 10^7 -B2S mixed infection group of mice, bacteria were also plated on LB agar + streptomycin

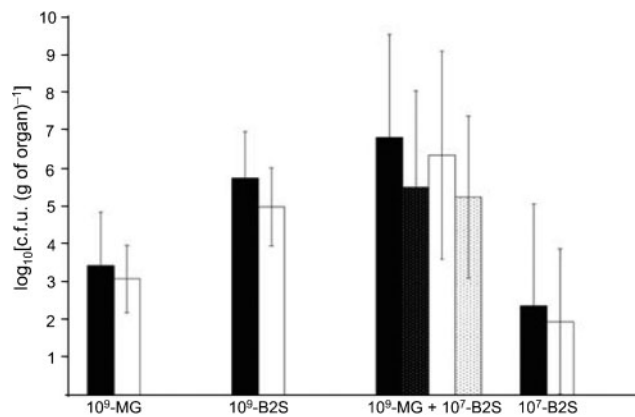


Fig. 5. Kidney and bladder bacterial counts after inoculation of different mixtures of *E. coli* strains into the bladder of CBA mice. The *E. coli* mix injected into the bladder is indicated at the bottom of each column. 10^9 -B2S and 10^9 -MG, mice were injected with 10^9 cells of strain B2S or MG1655, respectively; 10^7 -B2S, mice injected with 10^7 cells of strain B2S; 10^9 -MG + 10^7 -B2S, mice injected with a mix of 10^7 cells of strain B2S together with 10^9 MG1655 cells. The mean \log_{10} [c.f.u. (g organ) $^{-1}$] is shown; error bars, SD. Black bars, bacterial counts in the bladder; white bars, bacterial counts in the kidneys; stippled bars, *E. coli* cells were counted on LB agar + streptomycin ($30 \mu\text{g ml}^{-1}$) [only B2S c.f.u. were counted (counts performed for the MG1655/B2S mixed infection only)].

($30 \mu\text{g ml}^{-1}$) in order to determine the number of B2S cells. More B2S was found in the bladder and kidneys of the mixed infection group of mice (5.5 ± 2.5 and 5.2 ± 2.1 , respectively) than in the bladder and the kidneys of the mice that were injected with 10^7 -B2S alone ($P = 0.005$ and $P = 0.0006$). This shows that MG1655 favours the development of B2S in both organs in the case of co-infection. The number of B2S cells in the bladder and kidneys of the mice that received the mixed infection was also higher than the number of MG1655 found in the bladder and kidneys of mice that were injected with 10^9 -MG alone ($P = 0.02$ and $P = 0.005$, respectively). This indicates not only that MG1655 favours B2S UTI but also that MG1655 is outcompeted by B2S in the kidney. In the mixed infection group of mice, no statistical difference was found between the counts on LB (total bacteria) and the counts on LB + streptomycin (B2S only).

Bacterial synergy elicits higher IL-6 secretion

In our septicaemia experiments, there was a very short delay between bacterial challenge and mouse death. This clearly indicates that the host response probably mostly involves innate immunity. In order to characterize the inflammatory state of infected mice, two pro-inflammatory cytokines (TNF α and IL-6) and one anti-inflammatory cytokine (IL-10) were quantified in the serum 14 h after the subcutaneous bacterial challenge (Fig. 6).

No significant difference was found in the secretion of IL-10 between the groups of mice (Fig. 6a).

TNF α expression was significantly higher in the group of mice injected with 10^8 -B2S (700 ± 603 pg ml $^{-1}$) than in the groups that were injected with saline buffer, 10^6 -B2S or 10^8 -MG (0 pg ml $^{-1}$, $P < 0.04$ in all three cases). TNF α expression in the group of mice that received 10^8 -MG + 10^6 -B2S (574 ± 571 pg ml $^{-1}$) was not high enough to reach statistical significance with respect to all the other groups of mice (Fig. 6b).

Wide differences were obtained in the expression of IL-6 (Fig. 6c). The groups of mice that were injected with saline buffer, 10^6 -B2S or 10^8 -MG expressed little IL-6 (0 , 383 ± 410 and 325 ± 215 pg ml $^{-1}$, respectively). The 10^8 -MG + 10^6 -B2S and 10^8 -B2S groups expressed significantly increased amounts of IL-6: 2193 ± 2066 pg ml $^{-1}$ ($P = 0.02$ when compared with 10^6 -B2S or 10^8 -MG) and 7749 ± 1700 pg ml $^{-1}$ ($P < 0.0001$ when compared with 10^8 -MG + 10^6 -B2S), respectively. This indicates that the 10^8 -MG + 10^6 -B2S mixed infection elicits an IL-6 secretion that neither 10^8 -MG nor 10^6 -B2S alone is able to induce. This result defines a synergistic inflammatory response between MG1655 and B2S. In conclusion, IL-6, the major cytokine of the acute phase of the inflammatory response, is only increased when a pathogenic non-pathogenic interaction occurs or when high amounts of the most virulent strain are used.

The high secretion of IL-6 in the case of mixed infection is a consequence and not a cause of the synergistic effect

Wild-type C57BL mice and C57BL mice deficient for IL-6 secretion were injected with the four following bacterial mixes: 10^8 -B2S, 10^5 -B2S, 10^8 -MG and 10^8 -MG + 10^5 -B2S [because IL-6 $^{-/-}$ mice were more sensitive to *E. coli* infection (100% mortality with 10^6 B2S, data not shown), the inoculum used for the 'low dose' of B2S had to be decreased to 10^5 instead of 10^6 bacteria]. Fig. 7(a) represents the Kaplan–Meier survival estimates obtained with the IL-6 $^{-/-}$ knockout mice. We observed no mortality in the 10^8 -MG group, low mortality in the 10^5 -B2S group, and very high mortality in the 10^8 -B2S and 10^8 -MG + 10^5 -B2S groups. Therefore, the bacterial synergy does not rely on high IL-6 secretion. High IL-6 secretion in wild-type OF1 mice is a consequence of the co-infection. It is not the cause of the enhanced mortality in the mixed infection group of mice.

The bacterial synergy is independent of Toll-like receptors (TLRs) that involve myeloid differentiation factor 88 (MyD88) as well as of Fc receptors (FcRs)

TLRs and FcRs are fundamental effectors of innate immunity, which is why we tested their possible involvement in the bacterial synergy that we have described. The same bacterial mixes (10^8 -B2S, 10^6 -B2S, 10^8 -MG and 10^8 -MG + 10^6 -B2S) were tested on two knockout mouse

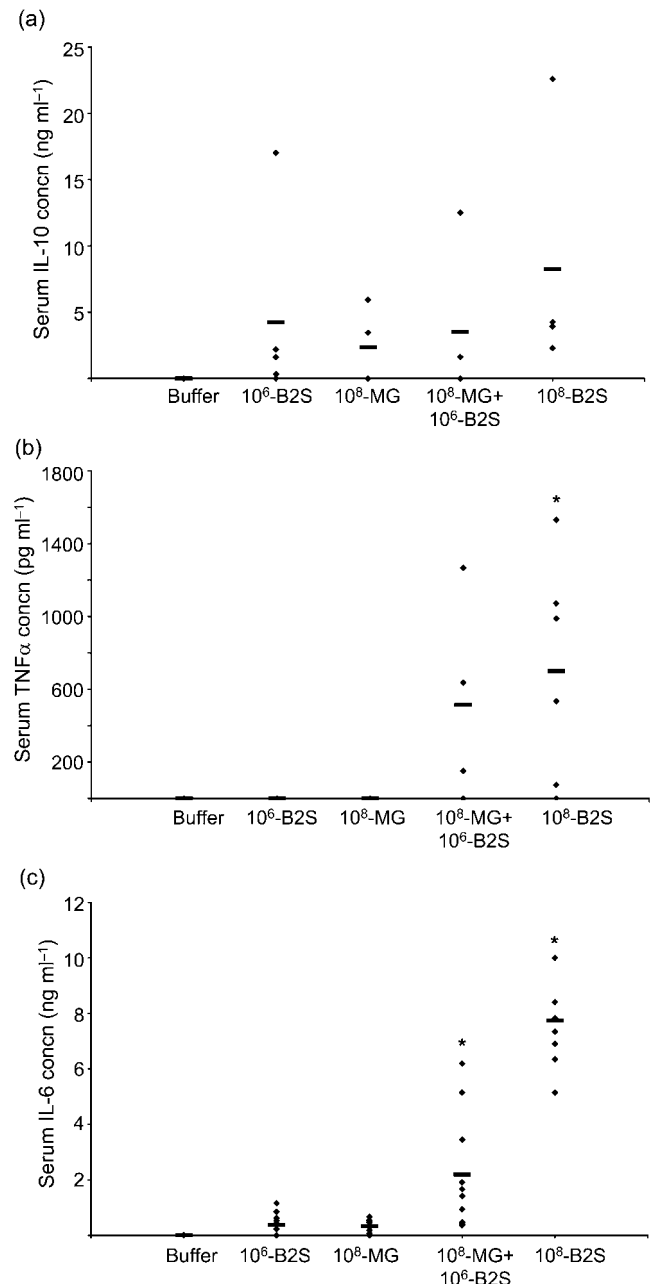


Fig. 6. OF1 mouse serum cytokine levels 14 h after bacterial injection. The *E. coli* mix with which the mice were injected subcutaneously is indicated at the bottom of each column. Buffer, mice injected with 0.9% NaCl buffer only; 10^8 -B2S and 10^8 -MG, mice injected with 10^8 cells of strain B2S or MG1655, respectively; 10^6 -B2S, mice injected with 10^6 cells of strain B2S; 10^8 -MG + 10^6 -B2S, mice injected with a mix of 10^8 cells of strain MG1655 together with 10^6 cells of strain B2S. Diamonds, single measurements; horizontal bars, mean values; asterisk, significant difference ($P < 0.05$). (a) IL-10 serum quantification (in ng ml $^{-1}$); (b) TNF α serum quantification (in pg ml $^{-1}$); (c) IL-6 serum quantification (in ng ml $^{-1}$).

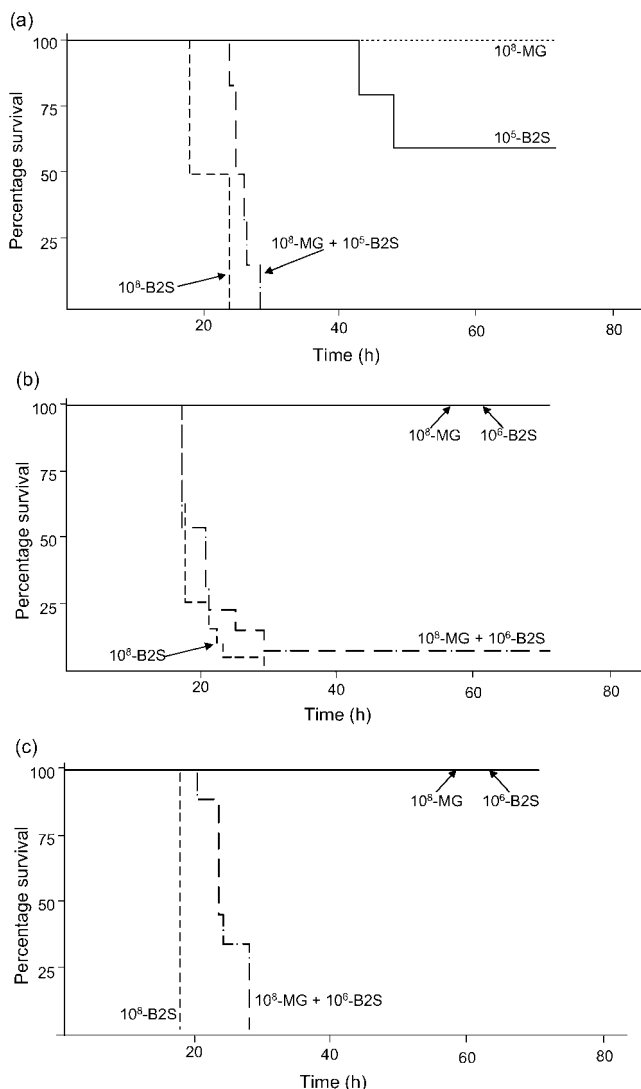


Fig. 7. Survival of various knockout mice after subcutaneous injection with mixtures of MG1655 and/or B2S. (a) Survival of C57BL/6 IL-6^{-/-} mice after subcutaneous challenge. Dotted line, mice injected with 10⁸ MG1655 cells; solid line, mice injected with 10⁶ cells of strain B2S; dashed and dotted line, mice simultaneously injected with 10⁸ MG1655 cells and 10⁵ cells of strain B2S; dashed line, mice injected with 10⁸ cells of strain B2S strain. (b) Survival of C57BL/6 myd88^{-/-} mice after subcutaneous challenge. Solid line, mice injected with 10⁸ MG1655 cells or 10⁶ cells of strain B2S (the two lines are superimposed); dashed and dotted line, mice simultaneously injected with 10⁸ MG1655 cells and 10⁶ cells of strain B2S; dashed line, mice injected with 10⁸ cells of strain B2S. (c) Survival of BALB/c fcRγ^{-/-} mice after subcutaneous challenge. Solid lines, mice injected with 10⁸ MG1655 cells or 10⁶ cells of strain B2S (the two lines are superimposed); dashed and dotted line, mice simultaneously injected with 10⁸ MG1655 cells and 10⁶ cells of strain B2S; dashed line, mice injected with 10⁸ cells of strain B2S.

strains and their wild-type counterparts: wild-type C57BL (data not shown) and C57BL-myd88^{-/-} (Fig. 7b), and

wild-type BALB/c (data not shown) and BALB/c-fcRγ^{-/-} (Fig. 7c). Even in myd88^{-/-} and fcRγ^{-/-} mice, the bacterial synergy in the case of co-infection was reproduced. This indicates that the bacterial synergy is not restricted to a particular strain of mouse but can be reproduced in BALB/c, C57BL and OF1 mice. More importantly, it also indicates that neither the Rfγ nor the TLR pathway is necessary to the bacterial synergy observed.

DISCUSSION

While interactions involving the simplest organisms such as small parasites, protozoa and even prokaryotes have been described, it is not clear whether interactions occurring between avirulent and pathogenic micro-organisms would favour a particular pathological process. We have used septicaemia and UTI mouse models to identify a synergistic increase of extra-intestinal virulence in *E. coli* species. This effect occurred between two strains, one being avirulent (MG1655) but a hundred times more abundant in the mix, the other highly virulent (B2S) but a minority in the mix. This synergy had several consequences. First, it had clinical consequences, since the mixed infection killed more mice (or more rapidly) than its components separately. It also had bacteriological consequences: more bacteria were found in the organs of the mice that were injected (subcutaneously or in the bladder) with a mix of MG and B2S than in the organs of the mice that received these inocula separately. In the septicaemia model, the MG1655 avirulent strain was able to favour the development of B2S infection but remained avirulent, since it could not participate in the infectious invasion of the spleen. In fact, B2S represented 95% of 44 colonies randomly picked from the spleens of six mice that received the mixed infection. When two pathogenic strains (10⁸-B1S + 10⁶-B2S) were subcutaneously injected together, an additive effect was observed (the mice died more rapidly), but the two strains competed with each other, because fewer total bacteria were found in the spleens compared with the mice that were injected with 10⁸-B1S alone. However, although a 100-fold fewer B2S than B1S cells were injected into the mice in the B1S/B2S mixed infection, at the end of the infectious process, more B2S than B1S cells were found. This means that in the B1S/B2S co-infection, B2S is favoured by B1S to the detriment of B1S. Finally, the synergy had immunological consequences, since more IL-6 was secreted in the 10⁸-MG+10⁶-B2 group of mice than in the 10⁸-MG or 10⁶-B2S groups alone.

Because death occurred very rapidly (less than 24 h after the bacterial challenge in most cases), we believe that this bacterial interaction relies on the innate immunological response. Innate immunity is the consequence of the interaction between pathogen-associated molecular patterns (PAMPs) and host pathogen recognition receptors (PRRs) (Sansonetti, 2006). This has been well described in

the case of LPS, a component of the Gram-negative bacterial cell wall. LPS (the PAMP) mainly elicits an inflammatory response through a member of the TLR family, TLR4 (the PRR) (Beutler, 2000). Activation of TLR4 initiates a succession of events in inflammatory cells, resulting in the production of cytokines, among which are IL-6 and TNF α (Takeda *et al.*, 2003), which constitute a pro-inflammatory response. MyD88, a signal adaptor molecule, has been shown to be essential for signalling via all TLRs. Besides TLR signalling pathways, innate responses may also be regulated through direct interaction between *E. coli* and Fc γ RIII (also called CD16). This interaction downregulates other bacterial receptors, such as TLRs and MARCO, and thus favours sepsis (Pinheiro da Silva *et al.*, 2007). For these reasons we tested whether the TLR or FcR pathways were responsible for the bacterial synergy that we had observed. Since this synergy did not rely on wild-type LPS, it was important to test all TLRs and not only TLR4. We used mice deficient for MyD88 or Fc γ R γ (a subunit of all FcRs which includes Fc γ RIII). Neither TLRs nor Fc γ RIII appeared to be involved in the bacterial interaction that we describe.

The importance of innate immunity is further supported by the IL-6 secretion pattern. IL-6 has already been described as a cytokine that increases mortality in mouse sepsis models (Cohen, 2002), so the observed bacterial synergy could have been due to a cytokine storm (Sriskandan & Altmann, 2008; Wang & Ma, 2008). Signalling a danger to the host, such as the presence of an invading micro-organism, is of major importance because it is the only way the host can efficiently respond to and clear the danger. However, in some cases the response to the danger can be disproportionate and become a danger itself (Sansonetti, 2006). In our case, the bacterial synergy was reproduced in the absence of IL-6 secretion. We believe that this is strong evidence against the cytokine storm hypothesis, unless it relies on cytokines other than IL-6.

Another way to explain this bacterial interaction could be what we call the 'lure hypothesis'. In this model, the high amounts of the avirulent strain would overwhelm the host clearing system (mainly natural killer cells and macrophages). Host innate defences being saturated, the low amount of the pathogenic strain would be free to trigger the infectious process. In this case, the high amount of circulating IL-6 in the mixed infection group of mice would be the consequence and not the cause of the severe sepsis and lethality. This hypothesis is further supported by the fact that the bacterial synergy is reproduced in an amoeba model (Adiba *et al.*, 2010). In this model, neither 10⁸-MG nor 10⁶-B2S cells can kill the amoebae, and numerous lysis plaques are observed on plates due to amoeboid grazing. In contrast, 10⁸-B2S or a mix of 10⁸-MG + 10⁶-B2S cells are able to kill amoebae (no lysis plaque observed; S. Adiba, personal communication). Since amoebae are considered to have many functions in common with macrophages, this may be indirect evidence

in support of the lure hypothesis. The fact that ethanol-killed or *msbB*⁻ mutant MG1655 cells seem to produce a more efficient synergy could also be explained by the lure hypothesis. In fact, ethanol-fixed or modified membranes would be harder for macrophages to process. This could result in a less efficient clearance and therefore a facilitation of B2S virulence.

Interestingly, an exacerbation of virulence has been described in a mouse *Salmonella enterica* infection model (Foster *et al.*, 2008). Those authors showed that the virulence of strain C5 is increased by the co-administration of the attenuated *aroA* *S. enterica* serovar Typhimurium SL3261 vaccine strain. They also showed that this bacterial synergy relies on macrophages. The co-infection resulted in higher IL-10 secretion by macrophages and higher spleen and liver bacterial counts. The bacterial synergy was greatly reduced in *myd88*^{-/-} and in Toll/IL-1 receptor domain-containing adaptor-inducing IFN β (TRIF)^{-/-} mice. However, it is interesting to note that even though the loss of the Myd88 signal transduction pathway greatly reduced the effect of the synergy, it still persisted to some extent. Therefore, in their work as in ours, synergy could be reproduced even in *myd88*^{-/-} mice.

To our knowledge, this is the first description of a virulence-enhancing bacterial interaction between an avirulent and a naturally occurring pathogenic strain of the same species. We believe that this observation gives a new insight into *E. coli* extra-intestinal virulence. The possibility that a commensal avirulent strain favours the development of an extra-intestinal pathogenic strain is of great concern, considering the numbers of commensal bacteria contained in the intestinal tract of any mammal (Berg, 1996). It is now well established that *E. coli* is able to translocate from the intestinal tract to mesenteric lymph nodes, both in the mouse model (Berg & Garlington, 1979) and in humans (Reddy *et al.*, 2007). This bacterial translocation involves *E. coli* strains that do not exhibit any virulence factor (Clark *et al.*, 2005; Nazli *et al.*, 2004; Reddy *et al.*, 2007). Translocation seems to play a role in human pathology (MacFie *et al.*, 1999). Even though our septicaemia model is not suitable to address this question, our results emphasize the importance of studying the links between ExPEC and the endogenous microbiota. Is it possible that under particular conditions (immunosuppression, intestinal or systemic inflammation) the endogenous commensal bacterial reservoir of the host plays a role in the development of extra-intestinal infections? We believe that this fundamental question should be addressed. The avirulent strain might not be found in the infected sample, which would result in great difficulties in bringing this hypothetical phenomenon to light and in the underestimation of its frequency. The bacterial synergy was also observed in a mouse UTI model. Urinary pathogenic *E. coli* strains come from the digestive tract, and our results raise the question of a possible microbiota implication in the pathogenesis of UTIs. It is interesting to note that the pathological strains studied

(B2S and B1S) were isolated from a human liver abscess and therefore are probably ExPEC of intestinal origin. They are an example of a polyclonal monobacterial infection, indicating that our observations are probably reflected in human pathology.

Conclusion

This work shows that a bacterial infection cannot be reduced to a single organism (or to a population of genetically identical organisms) infecting a host. Different organisms from the same species can interact within the host. Many aspects of bacterial virulence still need to be examined. These encompass bacteria–host and also bacteria–bacteria interactions, and our study shows good examples of both. In an era of increasing antibiotic resistance, disrupting these fundamental interactions could lead to new and interesting ways to fight infections.

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