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Identification of *Salmonella typhimurium* genes responsible for interference with peptide presentation on MHC class I molecules: Δ*yej Salmonella* mutants induce superior CD8$^+$ T-cell responses

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Summary

***Salmonella*-derived epitopes are presented on MHC molecules by antigen-presenting cells, and both CD4$^+$ and CD8$^+$ T cells participate in protective immunity to *Salmonella*. Therefore, mechanisms that allow *Salmonella* to escape specific immune recognition are likely to have evolved in this bacterial pathogen. To identify *Salmonella* genes, which potentially interfere with the MHC class I (MHC-I) presentation pathway, Tn*10d* transposon mutagenesis was performed. More than 3000 mutants, statistically covering half of the *Salmonella* genome, were individually screened for altered peptide presentation by infected macrophages. Two mutants undergoing enhanced antigen presentation by macrophages were identified, carrying a Tn*10d* insertion in the *yej* operon. This phenotype was validated by specific inactivation and complementation experiments. In accordance with their enhanced MHC-I presentation phenotype, we showed that (i) specific CD8$^+$ T cells were elicited at a higher level in mice, in response to immunization with *yej* mutants compared to their parental strain in two different experimental settings; and (ii) *yej* mutants were superior vaccine carriers for heterologous antigens compared to the parental strain in a tumour model.**

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Introduction

Salmonella enterica serovar *typhimurium* (*S. typhimurium*) is a Gram-negative, facultative intracellular pathogen and its capacity to cause systemic disease in mice depends upon its ability to survive and replicate within macrophages (Fields *et al*., 1986).

T cells are critical for performing helper and effector functions in immunity against bacteria. Both CD4$^+$ and CD8$^+$ T-cell responses are elicited by Gram-positive and Gram-negative bacteria (Kaufmann, 1988; 1993; Harding *et al*., 1995). In many instances, such responses are critical for effective protection against bacterial pathogens (Starnbach *et al*., 1994; Shen *et al*., 1998; Lo *et al*., 1999; Mittrucker and Kaufmann, 2000; Raupach and Kaufmann, 2001). Peptides generated by proteolytic processing of bacterial proteins in antigen-presenting cells (APCs) are bound and displayed by polymorphic class I and II MHC molecules, for recognition by CD8$^+$ or CD4$^+$ T cells respectively (Starnbach and Bevan, 1994; Harding *et al*., 1995; Ojcius *et al*., 1998; Schaible *et al*., 1999; Hess *et al*., 2000). It has been demonstrated that macrophages can process and present exogenous bacterial antigens on MHC-I. The demonstration that CD8$^+$ T cells play a critical role in host defence to certain bacteria, particularly not only to those residing in the cytoplasm but also to those living in phagosomes, highlights the relevance of MHC-I antigen presentation pathway to antibacterial immunity (Rock *et al*., 1990; Pfeifer *et al*., 1993; Kovacsovics-Bankowski and Rock, 1995; Schaible *et al*., 1999; Maksymowych and Kane, 2000). The observations on CD8$^+$ T-cell induction by *Salmonella* motivated studies of *Salmonella* as a vaccine vector for loading foreign epitopes on MHC-I (Russmann *et al*., 1998; 2001; Shams *et al*., 2001; Igwe *et al*., 2002).

As *Salmonella* reside in macrophages and macrophages are involved in the induction of specific immune responses, mechanisms that allow *Salmonella* to escape specific immune recognition are likely to evolve in this intracellular bacterium. Reports on bacterial genes affecting MHC presentation are scarce and only few have been investigated. Wick *et al*. (1995), Svensson *et al*. (1997) and Wick and Ljunggren (1999) have shown that MHC-II

presentation to T cells occurred with greater efficiency in macrophages infected with PhoP$^-$ mutant strains compared to those infected with wild-type *Salmonella*. PhoP–PhoQ is a two-component system that governs virulence, mediates the adaptation to Mg^{2+}-limiting environments and regulates numerous cellular activities in various Gram-negative species. It consists of the inner membrane sensor PhoQ and the cytoplasmic regulator PhoP (Groisman, 2001). Yet, there are no reports on a specific gene that is regulated by this *phoPQ* regulon and interferes with presentation on MHC. A rare example of *Salmonella*'s interaction with MHC-I was found by Huang *et al*. (1997). They described a novel mechanism by which infection with invasive *Salmonella* and *Yersinia* species, but not with non-invasive mutants, led to alternative splicing of the pre-mRNA of two HLA-B27 (human MHC-I allele) transfected cell lines.

In this report, we present the first systematic search for *S. typhimurium* genes that interfere with the MHC-I presentation pathway. Of more than 3000 mutants screened, we identified two insertions in the *yejA* and *yejE* genes that interfere with antigen presentation on MHC-I. The *yej* operon mutants exhibited superior efficacy as vaccine delivery vehicles, compared to their parental strains, preventing growth of a tumour challenge.

Results

Screening method for genes influencing MHC-I antigen presentation

In order to select a *Salmonella* strain for insertional mutagenesis and screening, we tested the ability of macrophages to process and present CS093 (wild type), CS022 (PhoP^c), CS015 (PhoP$^-$) and SL7207 (AroA$^-$) *Salmonella* strains (Table 1); all strains were transformed with a pQE-OVA8 plasmid, encoding the OVA8 peptide [H-2K^b-restricted epitope, ovalbumin (OVA) 257–264 aa] under a prokaryotic promoter (Table 1). Results indicated that OVA8 peptide presentation by macrophages infected with recombinant CS015 strain is roughly four- and twofold higher compared to CS022 and CS093 strains respectively (Fig. 1, black bars). Presentation of *Salmonella*-expressed OVA8 peptide was significantly reduced by treatment of infected macrophages with cytochalasin D (CCD) that blocks phagocytic uptake. No adverse effect on presentation was observed when OVA8 was exoge-

Table 1. Compilation of strains and plasmids used in this study

	Description[a]	Source or reference
Strain		
CS093	Wild type	Miller *et al*., 1989
CS022	*pho-24* (PhoP-constitutive)	Miller *et al*., 1989
CS015	CSO22- *phoP102*::Tn*10d*-cam (PhoP$^-$)	Miller *et al*., 1989
SL7207	*ΔaroA*	Stocker *et al*., 1983
UQ55	CS093-*yejE*2319517::Tn*10d*-Tet	This study
UQ56	CS022-*yejE*2319517::Tn*10d*-Tet	This study
UQ57	CS015-*yejE*2319517::Tn*10d*-Tet	This study
UQ58	SL7207-*yejE*2319517::Tn*10d*-Tet	This study
UQ59	CS022-Δ*yejE*2319128::kan	This study
UQ60	CS022-Δ*yejF*2320155::kan	This study
UQ61	CS022-Δ*yejEF*2319128::kan	This study
NM73	CS093-*yejA*2317112::Tn*10d*-Tet	This study
NM74	CS022-*yejA*2317112::Tn*10d*-Tet	This study
Plasmid		
pSA11	Encodes FACS optimized GFP protein (amp^r)	Schlosser-Silverman *et al*., 2000
pQE-OVA8	Encodes $OVA_{257-265}$ SIINFEKL minigene (amp^r)	This study
pACQE60	pACYC177-derived vector with T5 promoter from pQE60	This study
pACQE-*yejE*	*S. typhimurium yejE* complementation vector (kan^r)	This study
pACQE-*yejF*	*S. typhimurium yejF* complementation vector (kan^r)	This study
pACQE-*yejEF*	*S. typhimurium yejEF* complementation vector (kan^r)	This study
pQE-GFP	Encodes FACS-optimized GFP protein under the T5 promoter	This study
pP_{yej}GFP	Derivative of pSA8; encodes FACS-optimized GFP protein under the *yej* operon promoter	This study
pP_{tac}GFP_OVA	Encodes a fusion gene of the GFP and an MHC-II-restricted 25-amino-acid fragment of OVA	Bumann, 2001
pP_{pagC}GFP_OVA	Encodes a fusion gene of the GFP and an MHC-II-restricted 25-amino-acid fragment of OVA	Bumann, 2001
pJLP-2H	Encodes the fusion protein CrI-OVA, which contains residues 257–277 of OVA (including OVA8)	Pfeifer *et al*., 1993
pNK972	P_{tac}-tnpA (amp^r)	Maloy *et al*., 1996
pKD46	araC-P_{araB}, γβ exo (amp^r)	Datsenko and Wanner, 2000

a. Numbers in the description of mutant strains, generated in this study, are the location according to the complete genome of *S. typhimurium* LT2 (NCBI accession number NC_003197) in which the insertional mutagenesis occurred.

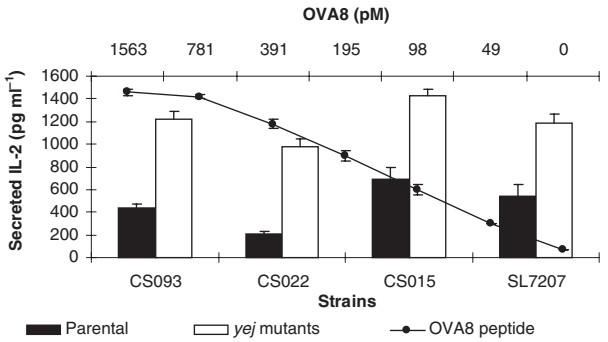

Fig. 1. Presentation efficiencies of parental strains and *yej* mutants. Indicated bacterial strains, all transformed with pQE-OVA8, were coincubated with BMA3.1A7 macrophages and tested for MHC-I-mediated presentation efficiency (H-2K^b-OVA8) as described in 'Experimental procedures'. Exogenous OVA8 peptide was added to non-infected macrophages in the same experiments at the indicated concentrations. Secretion of IL-2 by B3Z cells was assessed by ELISA with standard curve using recombinant mouse IL-2 (Peprotech, NJ). Background of pQE60-transformed strains in these assays was less than 10% and was subtracted accordingly. Results are from one representative experiment of five and are the average of two different colonies assayed in the same experiment in duplicates. Bars, ±SD. Differences between each parental and mutant strain pair and between CS093 and CS022, CS093 and CS015, and CS022 and CS015 are statistically significant with $P < 0.005$ (single-factor ANOVA test).

nously added to the treated cells [interleukin 2 (IL-2) measurements (ELISA) showed values of 0.66 ± 0.1 OD for untreated macrophages vs. 0.21 ± 0.05 OD for CCD-treated macrophages, whereas IL-2 secretion induced by 10 nM exogenous OVA8 peptide presentation was 0.89 ± 0.04 OD and 0.84 ± 0.03 OD respectively]. This indicates that the bacterial OVA8 is phagocytosed before presentation by the macrophages.

The low presentation efficiency of the CS022 strain enabled us to detect, with greater sensitivity, any presentation shift arising from mutation. Thus, in order to identify specific genes, which influence peptide presentation on MHC-I, we generated random insertion mutants in the CS022 strain. Freshly transduced mutant colonies, containing an OVA8 peptide-encoding plasmid, were separately seeded into Luria–Bertani (LB) medium in 96-well plates and grown overnight. After growth to saturation, bacteria were diluted and grown to mid-log phase. The bacteria were used to infect the BMA3.1A7 macrophage cell line (H-2^b) for 30 min. Thereafter, B3Z, a T-cell hybridoma recognizing OVA8 on MHC-I H-2K^b, was used to detect OVA8 peptide presentation via IL-2 secretion.

We screened more than 3000 separate colonies from different mutagenesis procedures of CS022/pQE-OVA8. Screening was performed twice and top 10 mutants of desired phenotype from each 96-well screening plate were further analysed. About 50 colonies, which showed consistently enhanced MHC-I-mediated presentation in 96-well screening plates, were further assayed in upscaled tests, with accurate measurements and titrations of multiplicity of infection (moi). Seven mutants that consistently showed enhanced presentation were chosen for further validation. P22 phage-mediated transduction was performed to transfer the Tn*10d*-Tet insertions of these mutants back to CS022 strain. Of the seven mutants picked, four lost their enhanced presentation phenotype. Therefore, their primary phenotype probably resulted from spontaneous mutations not associated with the induced insertion mutation.

Determination and validation of the phenotype of the mutants

To identify the genes disrupted by the transposon in the remaining three mutants, we developed a simple and rapid method based on direct sequencing of pure genomic DNA from the mutants (Qimron *et al*., 2003). One mutant that exhibited enhanced presentation efficiency had insertion in the *phoP* gene that resulted in a PhoP$^-$ phenotype, shown to enhance presentation (Fig. 1). Although this mutant did not lead to identification of a specific gene responsible for interference with MHC-I-mediated presentation, it confirmed the validity of the screening assay.

The other two colonies were mutated in the *yejE* and *yejA* genes of the *yej* operon. The CS022 (PhoP^c) strain is rather complicated with significant pleiotropic effects including lack of expression of the SPI-1-encoded type III secretion system and altered efficiency of phagocytic processing by activated macrophages. Therefore, to determine the validity of the phenotype of the *yej* mutation, we transferred the *yej*::Tn*10d*-Tet insertions to different *Salmonella* strains using P22 phage-mediated transduction. We first studied the *yejE*-mutated colony, identified earlier in the screen. On PhoP^c background (UQ56), as well as on wild-type (UQ55), PhoP$^-$ (UQ57) and AroA$^-$ (UQ58) backgrounds, this mutation significantly enhanced presentation of MHC-I-restricted peptides by macrophages (Fig. 1 and Table 2). Exogenously added OVA8 peptide was titrated in the same experiments in order to give an indication of the dynamic range available for measuring efficiency of presentation *in vitro*. For example, IL-2 secretion from B3Z cells incubated with UQ55/pQE-OVA8-infected macrophages was similar to IL-2 secretion following incubation with macrophages exogenously pulsed with 400 pM OVA8 peptide. On the other hand, IL-2 secretion following incubation with wild-type CS093/pQE-OVA8-infected macrophages resembled secretion induced by 50 pM OVA8-pulsed macrophages (Fig. 1). The obtained results are in the dose–response curve as mois of 1000 and of 10 were also tested and demonstrated higher and lower presentation capacity by macrophages respectively (data not shown). To verify that the *yej* effect was not restricted to OVA8 peptide expression

Table 2. Enhancement of MHC-mediated presentation following yej operon disruption[a]

	Plasmid		
	pQE-OVA8 (MHC-I epitope)	pJLP-2H (MHC-I epitope)	pP_{tac}GFP_OVA (MHC-II epitope)
Strain			
UQ55/CS093	2.4 ± 0.3*	ND	1.1 ± 0.1***
UQ56/CS022	3.9 ± 0.5**	1.9 ± 0.1**	0.95 ± 0.1***
UQ57/CS015	2.6 ± 0.2*	3.6 ± 1.1*	ND
UQ58/SL7207	2.3 ± 0.4**	ND	ND

a. Parental and *yej*-disrupted mutants were transformed with either pQE-OVA8 (expressing the OVA8 minigene), pJLP-2H (expressing the OVA fragment containing the OVA8 minigene and fused to CrI) or pPpagCGFP_OVA (expressing the OVA fragment containing the $OVA_{323–339}$ MHC-II-restricted peptide and fused to GFP). *Salmonella* harbouring mock plasmids were tested in the same assays to exclude non-specific presentation. Presentation assays were performed as described in 'Experimental procedures'. Presentation by infected macrophages was compared between each strain and its *yej*-disrupted mutant. The ratios of OD values of each mutant strain divided by its isogenic strain harbouring the indicated plasmids were calculated. Each result is the average fold enhancement ±SD. Data are based upon more than 10 independent experiments with different samples and from different colonies.
*$P < 0.05$; **$P < 0.001$; ***insignificant differences; single-factor ANOVA test.

from the pQE-OVA8, we tested the well-studied pJLP-2H plasmid encoding the fusion protein CrI-OVA, which contains residues 257–277 of OVA including the OVA8 epitope (Pfeifer *et al*., 1993). Again, *Salmonella*/pJLP-2H-infected macrophages presented OVA8 peptide better when the *Salmonella* contained the *yej* mutation (Table 2). Next, we studied C57BL/6 thioglycolate-induced peritoneal exudate cells as APCs, and OT-1 transgenic T cells as OVA8-K^b-specific T cells. The induction of better MHC-I-mediated presentation, caused by *yej* mutation in the *Salmonella*, was also observed in this experimental system (data not shown). We further tested the effect of *yej* mutation on MHC-II-mediated presentation. An OVA MHC-II-restricted epitope ($OVA_{323–339}$), expressed by the parental and the *yej* mutant *Salmonella*, in a parallel MHC-II presentation assay was presented similarly indicating that the *yej* influence was specific to MHC-I-mediated presentation (Table 2).

The *yejE* is part of the *yej* operon, which encodes other subunits of an ABC transporter (Fig. 2). Hence, we suspected that the insertion in *yejE* disrupted the function of the downstream *yejF* gene and that both gene products are necessary. Therefore, we determined the specific function of *yejE and yejF* genes using two complementary approaches. The first was to directly complement *yejE*, *yejF* and *yejEF* genes. The second approach was to separately delete *yejE*, *yejF* and *yejEF* (strains UQ59, UQ60 and UQ61 respectively) using a modified one-step gene inactivation method (Datsenko and Wanner, 2000). Figure 3A shows that both *yejE* and *yejF* are required for functional complementation because pACQE-*yejEF* was the only plasmid to restore function. This result is also supported by the specific gene inactivation of *yejE* and *yejF* as these directly inactivated mutants also exhibited enhanced presentation (Fig. 3B). Similar results were also obtained on CS093 background (data not shown). Thus, we deduce that both *yejE* and *yejF* are associated with interference of MHC-I-mediated presentation, and through the same mechanism.

The *yejA* mutant, identified later in the screen, was also studied on $PhoP^c$ and wild-type backgrounds (NM74 and NM73 respectively) and enhancement in MHC-I-mediated presentation was shown in both; complementation of the *yejA* mutant with *yejEF* genes did not reduce presentation (data not shown). Therefore, *yejA*, *yejB* or both are also required for repression of presentation. The

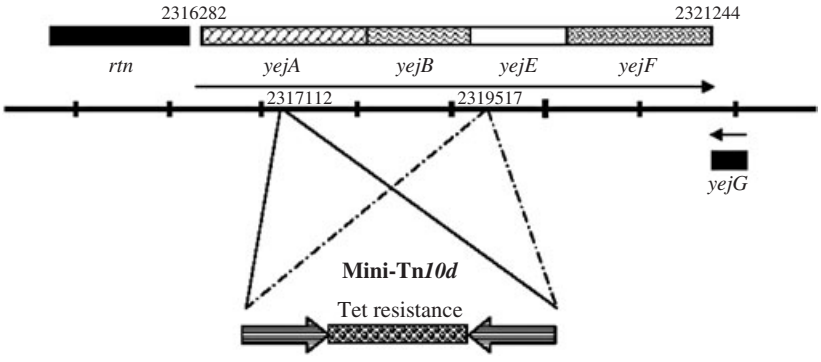

Fig. 2. Schematic description and location of the *yej* operon. Numbers indicate initiation and termination of the operon, as well as Tn*10d* insertions characterized in this study according to the complete genome of *S. typhimurium* LT2 (NCBI accession number NC_003197).

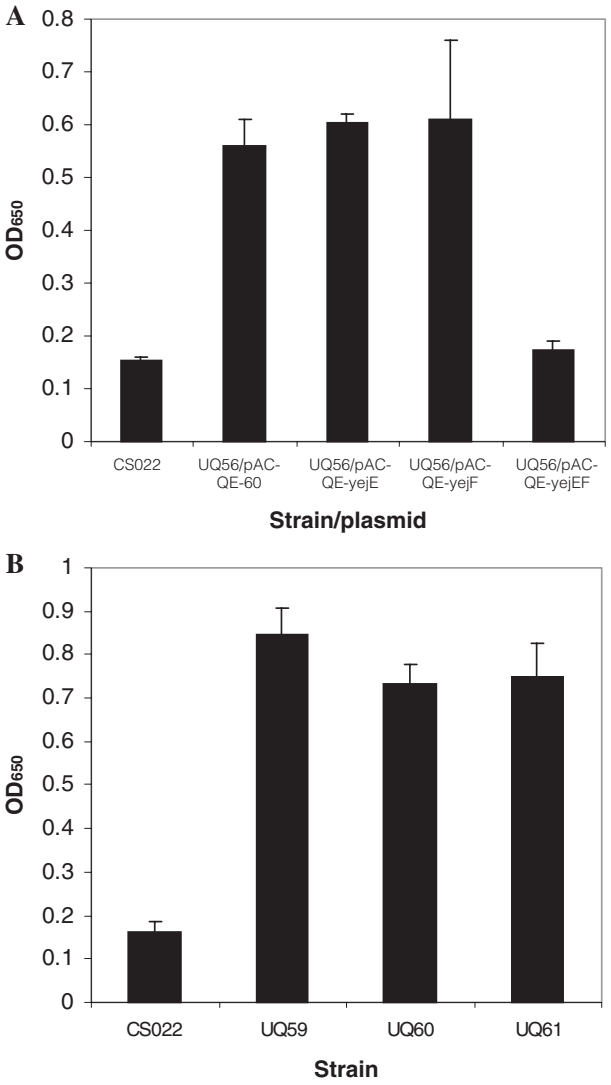

Fig. 3. Effects of gene complementation and specific gene disruption on presentation efficiency. Presentation assays were performed as described in 'Experimental procedures'. Panel A shows the effect of complementation of the indicated genes. All strains are also transformed with pQE-OVA8 that is compatible with the pAC-QE-based plasmids. Panel B shows the effect of the indicated gene-specific disruption. All strains are transformed with the pQE-OVA8. IL-2 secretion from B3Z is indicated in OD values of the ELISA. Results are from one representative experiment of two (A) or three (B) and are the average of two different colonies assayed in the same experiment in duplicates. Bars, ±SD. Differences between CS022 and each UQ56/pAC-QE-based plasmid (except for UQ56/pAC-QE-*yejEF*) gene-complemented strain are statistically significant with $P < 0.01$. Differences between CS022 and the UQ56/pAC-QE-*yejEF*-complemented strain are statistically insignificant, and between CS022 and each gene-disrupted strain are statistically significant with $P < 0.01$ (single-factor ANOVA test).

yejABEF is located at minute 49 of the *S. typhimurium* chromosome (Fig. 2). It is an operon containing four genes that encode for the components of an ABC-type dipeptide/oligopeptide/nickel transport system (NCBI accession number NC_003197). It is likely that the products of all four genes are required for assembly of the functional transporter, as seen in other bacterial ABC type transporters (Locher *et al*., 2002). Thus, we assume that the functional transporter and all of its components are associated with interference to MHC-I-mediated presentation.

The phenotype of yej *mutants is similar to the wild-type phenotype in antigen abundance and other parameters*

The higher presentation observed for the *yej* mutants could be a result of parameters not directly associated with MHC-I-mediated presentation. Therefore, we conducted the next set of experiments. We first determined whether presentation differences between the parental and *yej*-mutated strain were a result of differential antigen abundance in bacteria. We constructed a pQE60 vector encoding GFP under the same promoter of the pQE-OVA8 (pQE-GFP) and measured its expression in the parental and the *yej* mutant strains (CS093, UQ55, CS022 and UQ56). The steady-state level of the GFP, analysed by flow cytometry, in strains with and without the *yej* mutation was unaltered (Fig. 4A, shown for CS093 and UQ55). This indirect approach was used because neither Western blot analysis nor ELISA with anti-OVA8 mAb were able to efficiently detect the minute amount of OVA8 peptide in bacteria. To further validate this issue, we used a sensitive functional assay in which equal amounts of pQE-OVA8 harbouring bacteria were disrupted by repeated freeze–thaw lysis. Presentation of released OVA8 peptide (exogenously 'ready to bind') was titrated on BMA3.1A7 macrophages and assessed via IL-2 secretion by co-incubated B3Z cells. Results indicated that intrabacterial peptide amounts were similar (Fig. 4B, shown for CS093/pQE-OVA8 and UQ55/pQE-OVA8).

The observed phenotype of the *yej* mutants could be a result of total variations in MHC-I expression between macrophages infected with parental and mutated strains. For this reason, we compared total MHC-I (and MHC-II) expression on macrophages infected with the different parental and mutated strains. Expression of MHC-I and II was similar between non-infected macrophages and those infected with the parental strains and the *yej* mutants (Fig. 4C, shown for CS022 and UQ56).

We then questioned the possibility whether the enhanced presentation was a result of different invasion capacity or survival within the macrophages. We compared these two parameters between CS093 vs. UQ55 and CS022 vs. UQ56 strains. The invasion and survival experiments were done essentially as described in *Experimental procedures*. We used two approaches for assessing invasion and survival: a gentamicin protection assay and a flow cytometry-based method. No significant differ-

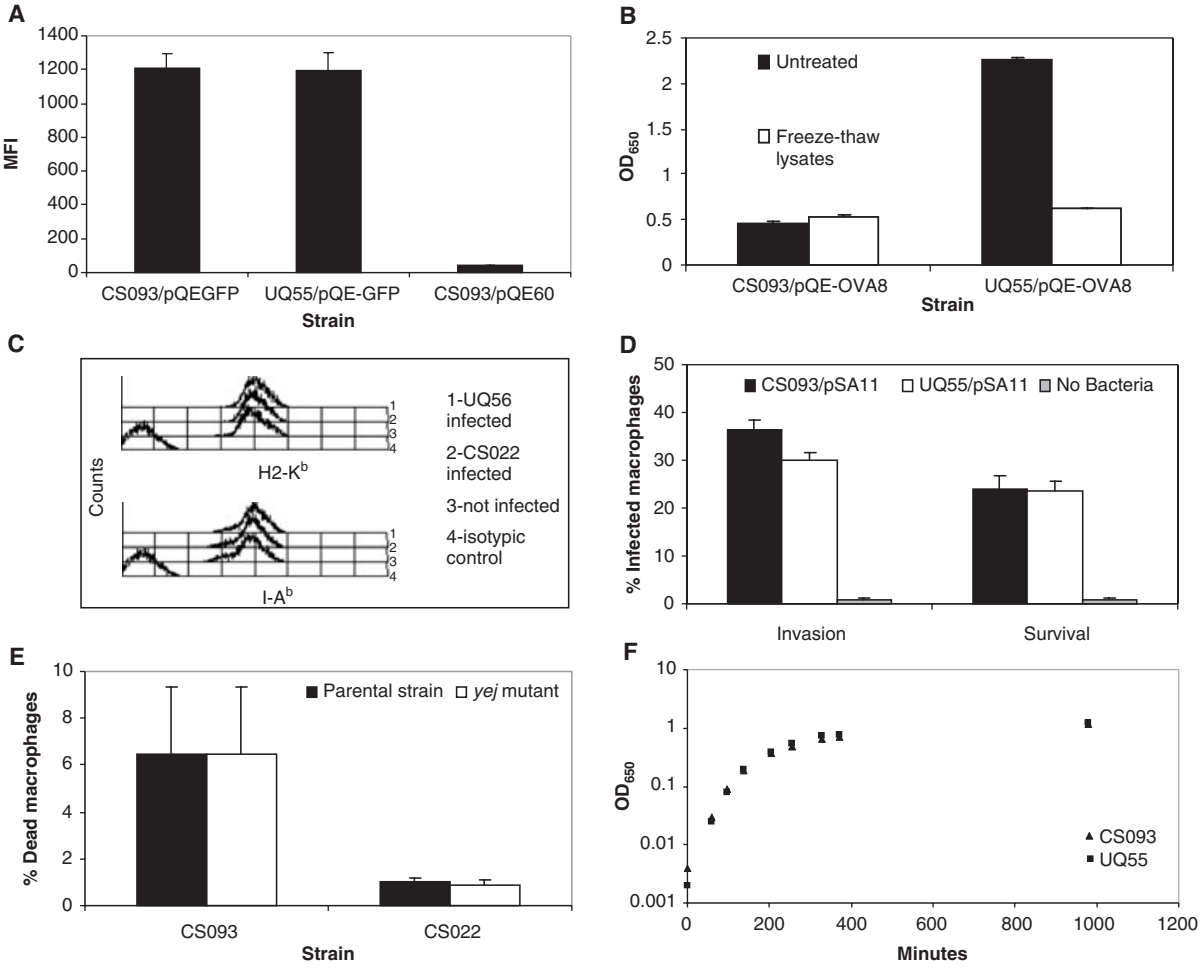

Fig. 4. Phenotype of *yej* mutants is similar to the wild-type phenotype in antigen abundance, bacterial growth rate, invasion into, survival within, total MHC expression and death induction of macrophages.
A and B. Antigen abundance in parental and *yej* mutant strains harbouring pQE-GFP or pQE-OVA8 was quantified by GFP expression levels using flow cytometry or by freeze–thaw lysis of bacteria and OVA8 presentation assay (IL-2 secretion indicated in OD values) respectively. Average ± SD.
C. MHC-I and II were verified on non-infected and infected macrophages, 18 h following infection with indicated strains. A representative 3D FACS histogram.
D. Invasion and survival (10 min or 24 h after co-incubation of bacteria with macrophages respectively) of indicated strains were determined as described in *Experimental procedures*. Average percentage of infected macrophages ±SD.
E. Death induction of indicated strain on macrophages was measured 30 min and 18 h following infection. Average percentage of dead (PI^+) macrophages ±SD is shown. Spontaneous death of macrophages was subtracted by calculating percentage PI^+ macrophages from uninfected samples. Black bars, CS093 and CS022; white bars, UQ55 and UQ56.
F. Bacterial growth rates were determined by measuring OD_{650} at indicated time points.

ences were found between the capacity of parental and mutant strains to invade or survive in macrophages (Fig. 4D, shown for CS093 vs. UQ55).

The enhanced MHC-I-mediated presentation phenotype could be the outcome of higher toxicity of the parental bacteria for macrophages, which reduces the quantity of available APCs. We thus compared both trypan blue and propidium iodide (PI) staining of parental- and *yej*-mutant-infected macrophages by cell counting and flow cytometry, respectively, 30 and 18 min after infection. In either case, no differences were observed with regard to macrophage viability between each pair of parental and mutant strain (Fig. 4E).

Finally, we determined growth rates of the wild-type strains and the *yej* mutants under different experimental conditions (different osmolarities, Mg^{2+}- and Fe^{3+}-deprivation which intimate intracellular growth) in order to exclude the possibility that growth rates were different and, consequently, during the coincubation with the macrophages one of the strains overgrew. In all cases growth curves were equivalent (Fig. 4F, shown for CS093 and UQ55).

The yej *operon is not regulated by the* phoPQ *regulon*

As the *phoPQ* regulon affects MHC-I-mediated presentation by infected macrophages we determined whether the *yej* operon is regulated by the *phoPQ*. We suspected that *yej* was not *phoPQ*-regulated (activated) because the *yej* mutation caused enhanced presentation on both CS022 and CS015 backgrounds (Fig. 1, Table 2). Yet, we performed a more robust assay in order to verify this finding. A GFP-encoding plasmid, $pP_{yej}GFP$, under the promoter of the *yej* operon was constructed. CS093, CS022 and CS015 strains were transformed with this plasmid and with $pP_{pagC}GFP\_OVA$, a plasmid containing GFP under the promoter of *pagC* (*phoP*-activated gene). These strains were analysed for GFP expression using flow cytometry. There were no differences in the expression of GFP in $pP_{yej}GFP$-transformed strains, while $pP_{pagC}GFP\_OVA$-transformed strains exhibited the expected phenotype of a positive-control (Fig. 5).

Effect of yej *mutation on the capacity to prime CD8⁺ T-cell responses in mice*

We evaluated the effect of the *yej* mutation on the *in-vivo* induction of $CD8^+$ T-cell responses by *Salmonella*. SL7207 was previously used as a preferred vaccine strain for recombinant *Salmonella*-based vaccines (Paglia *et al*., 1998; Xiang *et al*., 2001). Thus, we compared both immunogenicity and vaccine efficacy of SL7207 and the SL7207-*yej* mutant, UQ58. Both strains were transformed with the pQE-OVA8 plasmid.

To test whether our OVA8 plasmid-transformed *Salmonella* strains were able to induce OVA8-specific $CD8^+$ T cells, mice were intravenously infected with 5×10^5 *Salmonella* of both strains. $CD8^+$ spleen cells of infected mice and untreated control mice were analysed 11 days after infection using H-$2K^b$-OVA8 tetramers. In addition, cells were stained with anti-CD62L mAb. In the spleen, CD62L is expressed on naïve T cells. During activation, CD62L is downregulated from the T-cell surface and activated T cells acquire a $CD62L^{low}$ phenotype (Tripp *et al*., 1995). After infection with the OVA8-transformed SL7207 strain, we detected a small population of OVA8-specific $CD8^+$ T cells. Although frequencies were low, they were significantly enhanced when compared to background staining in non-infected control mice (Fig. 6). Infection of mice with the OVA8-transformed UQ58 mutant induced a distinct population of OVA8-specific $CD8^+$ T cells. Both frequencies and total numbers of OVA8-specific $CD8^+$ T cells in spleens of these mice were three- to fourfold enhanced compared to frequencies in mice infected with the OVA8-transformed SL7207 strain. These results indicate that the *yej* mutation augmented peptide-specific $CD8^+$ T-cell responses.

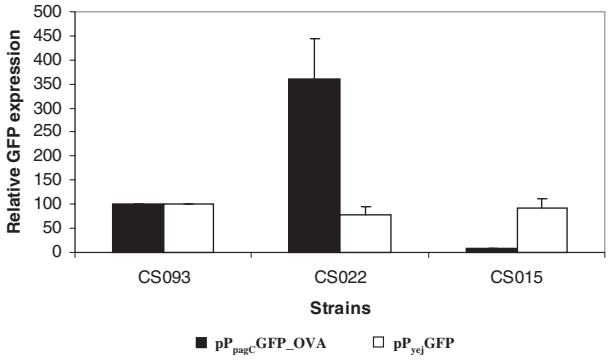

Fig. 5. The *yej* operon is not regulated by the *phoPQ* regulon. Indicated strains (CS093: wild type, CS022: $PhoP^c$ and CS015: $PhoP^-$) were transformed with either $pP_{yej}GFP$- or $pP_{pagC}GFP\_OVA$-encoding GFP under the *yej* promoter or the PhoP-activated gene C respectively. Fluorescence was assessed by flow cytometry. Results are expressed as mean fluorescence intensity of GFP relative to the values obtained for the parental strain. Average ± SD of four different colonies. GFP expression by $pP_{pagC}GFP\_OVA$ is significantly different between all strains with $P < 0.005$ (single-factor ANOVA test).

Next, we assessed vaccine efficacy of the OVA8-transformed *Salmonella* strains using a different immunization scheme. SL7207 carrying no plasmid was used as negative control. Mice were reconstituted with splenocytes from OT-1 mice. This adoptive transfer procedure enabled us to efficiently detect and quantify the specific $CD8^+$ T-cell response following vaccination with the different bacterial strains. On days 3 and 10 after adoptive transfer, mice were vaccinated with 2×10^9 of each of the specified bacterial strains p.o. Seven days after the second vaccination, groups of mice were challenged i.v. with 2.5×10^5 B16-OVA cells; B16-OVA is a metastatic tumour cell line, which expresses OVA, accumulates in the lung within minutes after i.v. injection and induces lymphocyte recruitment to the lung (Netland and Zetter, 1984). To quantify vaccination-induced responses against metastatic tumour challenge, lymphocytes were isolated from the lung 1 day after tumour challenge and analysed by flow cytometry for the presence of OVA8-H-$2K^b$-specific T cells by staining with fluorescent CD8-specific mAb and H-$2K^b$-OVA8 tetramers. The *yej*-mutated *Salmonella* UQ58 improved $CD8^+$ T-cell responses compared to unmutated SL7207 controls. The negative control, as expected, displayed a low background of adoptively transferred OT-1 cells (Fig. 7).

Finally, we determined the potency of these vaccine constructs against B16-OVA tumour growth. Mice were prime/boost-vaccinated, in intervals of 7 days, with 2×10^9 of each of the above-specified bacterial strains p.o. Seven days after the booster vaccination, groups of mice were challenged intrafootpad with 10^5 B16-OVA cells. Tumour size was monitored 30 days later by microcaliper measurements. The SL7207/pQE60 (mock plasmid) did not protect against B16-OVA tumour challenge. A minor pro-

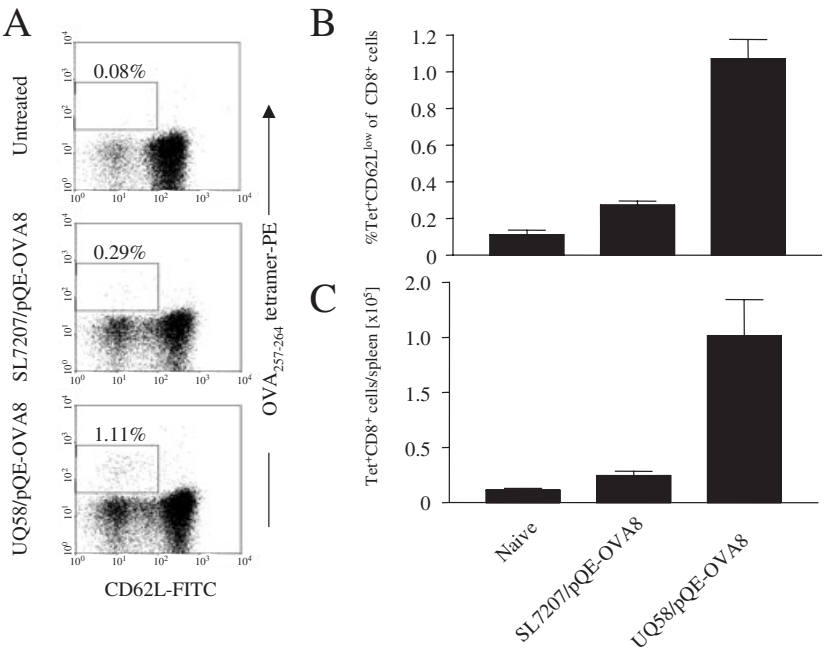

Fig. 6. Induction of OVA8-specific $CD8^+$ T-cell responses by mutant *Salmonella*. (C57BL/$6 \times 129)F_1$ mice were intravenously infected with 5×10^5 *Salmonella* of the strains SL7207/pQE-OVA8 or UQ58/pQE-OVA8, or were left untreated. After 11 days, spleen cells from infected mice were stained with FITC-conjugated anti-CD62L mAb, Cy5-conjugated anti-CD8 mAb and PE-conjugated OVA8-tetramers, and analysed by flow cytometry.
A. Representative results of $CD8^+$-gated cells. Numbers are the percentage value of cells in the region calculated for $CD8^+$ T cells only.
B. Percentage value of H-$2K^b$-OVA8-tetramer$^+$ $CD62L^{low}$ of $CD8^+$ T cells.
C. Total number of H-$2K^b$-OVA8-tetramer$^+$ $CD62L^{low}$ $CD8^+$ T cells per spleen (average ± SD of three individually analysed mice per group).

tection was observed in the SL7207/pQE-OVA8 vaccine group, whereas UQ58/pQE-OVA8 proved a significantly better vaccine, conferring potent protection against tumour challenge (Fig. 8). This protection was not a bacterial-non-specific effect as in addition to the SL7207/pQE60 control, the UQ58 strain carrying irrelevant plasmid was used in another experiment and shown not to confer any protection (data not shown). These results strongly support the *in-vitro* findings and corroborate the findings from two other *in-vivo* studies. These results also indicate that *yej* mutants are improved vectors for *Salmonella*-based vaccines.

Discussion

Identification of genes affecting MHC-I-mediated antigen presentation in *Salmonella* should improve our understanding of bacterial virulence and basic mechanisms underlying MHC-I presentation of exogenous antigens. These insights can pave the way for rational design of improved *Salmonella*-based vaccines. Therefore, we performed a systematic search for *Salmonella* genes, which influence, directly or indirectly, antigen presentation by MHC-I. The screening method employed proved to be efficient, because it picked up the *phoPQ* regulon, shown to affect MHC-I presentation (Fig. 1), in more than 3000 mutants analysed. Wick *et al*. (1995) and Svensson *et al*. (1997) have shown that MHC-II presentation to T cells occurred with greater efficiency in macrophages infected with $PhoP^-$ mutant strains compared to those infected with wild-type *Salmonella*. Yet, there are no reports on a specific gene that is regulated by this *phoPQ* regulon and interferes with presentation on MHC. Our screen identified two genes of the *yej* operon, which conferred to *Salmonella* a phenotype of specific MHC-I presentation interference independent of the *phoPQ* regulon (Figs 1 and 5).

The use of *ΔaroA S. typhimurium* as an heterologous antigen-delivery vector was most recently shown for OVA (Bachtiar *et al*., 2003). In addition, *Salmonella* vaccines have been successfully used to induce antitumour immunity (Pawelek *et al*., 1997; Medina *et al*., 1999). We have shown similar elicitation of cytotoxic T lymphocytes (CTLs) and tumour protection following prime/boost vaccination with *ΔaroA S. typhimurium* encoding the OVA epitope. Others have reported that CTL activity was not evident following infection with the *ΔaroA* strain alone and was only detectable following challenge with virulent *Salmonella* (Lo *et al*., 1999). Possible explanations for the differences between the studies might be the size of inocula (5×10^5 and 2×10^9 vs. 10^6), route of administration (i.v. and p.o. vs. i.p.), nature of antigen (heterologous vs. endogenous) and differences in *ΔaroA* strains utilized (SL7207 vs. SL3235). Moreover, we have shown that *ΔaroA S. typhimurium yej* mutants (UQ58) serve as a superior vaccine delivery vehicle than their parental strains in both activation of $CD8^+$ T cells and in protective antitumour immunity. Both frequencies and total numbers of OVA8-specific $CD8^+$ T cells expressing low levels of L-selectin in spleens of UQ58/pQE-OVA8-vaccinated mice were three- to fourfold enhanced compared to SL7207/pQE-OVA8-vaccinated mice (Fig. 6). UQ58/pQE-OVA8 compared to SL7207/pQE-OVA8 demonstrated a twofold

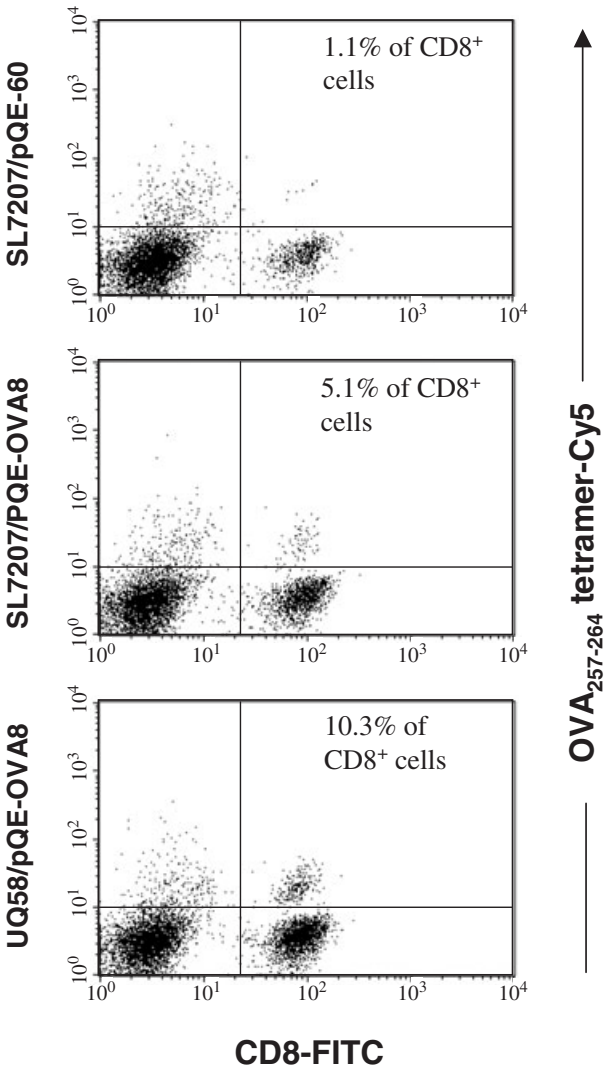

Fig. 7. Flow cytometry-based quantification of the efficacy of *yej*-mutant-based vaccine constructs. Splenocytes (2×10^7 cells) were isolated from OT-1 TCR transgenic mice and transferred i.v. to C57BL/6 mice. These mice were then vaccinated twice p.o. with indicated *Salmonella* vaccines and challenged i.v. with 2.5×10^5 B16-OVA tumour cells 7 days after the last vaccination. One day after the tumour inoculation, immunocytes were isolated from the lung and analyzed by flow cytometry. Lung immunocytes were stained with anti-CD8 and Cy5-conjugated H-2K^b/β2m/OVA8 tetramers. The percentages of CD8$^+$ cells (*x* axis) and tetramer$^+$ cells (*y* axis) of the total CD8$^+$ T-cell population are indicated in the dot plots. Results represent one of the two experiments performed.

enhancement of OVA8-specific CD8$^+$ T cells in the lung in a different experimental scheme utilizing OVA8-specific-CD8$^+$ adoptive transfer prior to vaccination (Fig. 7). Most importantly, the *yej* mutant UQ58/pQE-OVA8 conferred superior protection against a B16OVA tumour challenge (Fig. 8). Interestingly, the *yejE* and *yejF* genes are greatly upregulated (approximately two- and 10-fold, respectively) upon macrophage infection, according to a recent study by Eriksson *et al*. (2003).

Therefore, our data reveal a novel class of *Salmonella* virulence genes, which favour pathogen persistence in the host by affecting acquired (late) immunity rather than by directly impairing host integrity. The exact mechanism by which the *yej* operon operates needs to be uncovered, although the effects seen with the *yej* mutants are not related to antigen abundance, invasion, survival and other non-specific differences between the parental strains and the *yej* mutants (Fig. 4). Recent publications have provided evidence that phagosome-mediated MHC-I cross-

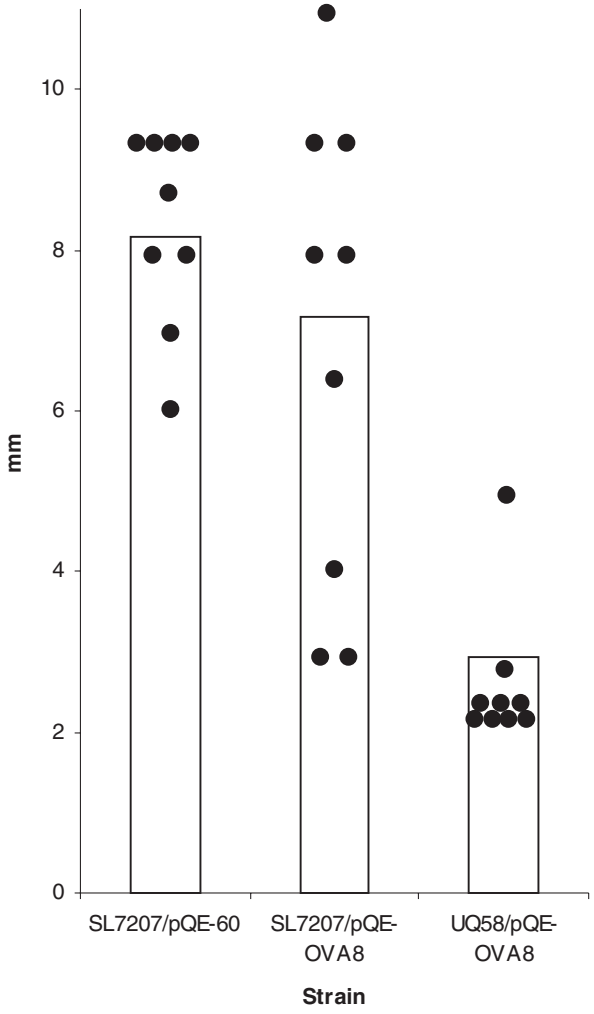

Fig. 8. Tumour protection efficacy of *yej*-mutant-based vaccine. C57BL/6 mice, four to five per group, were vaccinated twice p.o. with the indicated *Salmonella*-based vaccines at intervals of 7 days. Immunized mice were challenged s.c. in the footpad with 1×10^5 B16-OVA tumour cells 7 days after the second vaccination. The tumour size at the footpad (mm from foot dorsum to sole) was monitored at different time points using microcalipers. The measurement of results started on the day when the mean tumour size in the control vaccine group (SL7207/pQE60) reached >8 mm (days 48 and 59 after tumour challenge for the first and second experiments respectively). The results are from the two experiments performed. Each dot represents tumour size in a single mouse. Tumour size for the UQ58/pQE-OVA8 vaccine group was significantly different from each of the other two groups with $P < 0.005$ (single-factor ANOVA test).

presentation in macrophages involve antigen export from phagosome to cytosol, degradation by the proteasome, back-translocation of the processed peptides into the phagosomal lumen and loading on phagosomal MHC-I molecules (Houde *et al*., 2003). In dendritic cells, a similar pathway is driven by endoplasmic reticulum (ER)-phagosome fusion (Guermonprez *et al*., 2003). We proved that *yej* mutants interfere with presentation of OVA8 that is encoded either as a minigene or within fusion proteins (Table 2). Thus, the *yej* effect might be related to peptides loading on phagosomal MHC-I rather than to antigen export, proteasomal processing or back-translocation. As the *yej* operon is putatively involved in peptide transport, it might interfere with MHC-I presentation by secretion of short, 8–10 aa peptides that compete with immunogenic peptides for loading on phagosomal MHC-I. Manipulation of host immune responses via immunodominant epitope alteration is very common in eukaryotic viruses such as the human immunodeficiency virus, hepatitis B virus and other viruses (Klenerman and Zinkernagel, 1998), but is yet uncovered in bacteria. The *yej*-related phenotype of specific MHC-I presentation interference is evident also when bone marrow-derived dendritic cells (BMDCs) are used as APCs (A. Porgador, unpubl. data). Yet, BMDCs infected with *yej* mutants (UQ55) present peptides to T cells independently of IL-1β whereas wild-type (CS093)-infected BMDCs present peptides in an IL-1β-dependent fashion (A. Porgador, unpubl. data). IL-1β is a pro-inflammatory cytokine, which has been shown to enhance the proteolytic activity of cathepsins in human dendritic cells (Fiebiger *et al*., 2001). The observation that *yej*-mutant-infected BMDCs might not need enhancement of cathepsin activity for proper presentation of peptides suggests that the *yej*-interference mechanism is involved with the proteolytic activity of the cells. Additional studies are needed to elucidate the precise interactions of wild-type *Salmonella* and in particular the interactions of *yej* and *phoP* mutants with APCs. Already, on the basis of our findings, *Salmonella*-based vaccines can be constructed with the *yej*-operon deletion to greatly increase vaccine efficacy.

Experimental procedures

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. Strains were routinely grown at 37°C in LB medium containing 0.3 M NaCl, unless indicated otherwise. Ampicillin (100 μg ml^{-1}), tetracycline (10 μg ml^{-1}) or kanamycin (40 μg ml^{-1}) were added where appropriate.

Cell lines and MHC tetramers

The B16-OVA tumour cell line was a kind gift from Dr K. Rock (University of Massachusetts Medical School, Worchester, MA). The B16-OVA cell line is a mouse B16 melanoma cell line of H-2^b haplotype, transfected with the cDNA of chicken OVA. The cell line was cultured in 10% FCS–DMEM containing penicillin–streptomycin 1%, 50 μM 2-ME and HEPES 10 mM and supplemented with 1 mg ml^{-1} G418 and 50 μg ml^{-1} hygromycin. The C57BL/6 mouse (H-2^b)-derived murine bone marrow macrophage cell line, BMA3.1A7, has been described (Kovacsovics-Bankowski *et al*., 1993). The CTL hybridoma, B3Z, secretes IL-2 upon recognition of the OVA-derived peptide SIINFEKL when presented in association with the MHC-I H-2K^b molecule (Karttunen *et al*., 1992). Both cell lines were cultured in 10% FCS–RPMI-1640 containing penicillin–streptomycin 1%, 50 μM 2-ME and HEPES 10 mM.

The biotinylated H2-K^b/β2m/SIINFEKL monomer was obtained from the NIAID Tetramer Facility and the NIH AIDS Research and Reference Reagent Program. Tetramers were generated as described (Mittrucker *et al*., 2001). The resulting H-2K^b-OVA8 tetramers were used at 10 μg ml^{-1} to stain T cells specific for the OVA8 peptide presented by H-2K^b.

Plasmid constructions

The plasmids and oligonucleotides used in this study are listed in Tables 1 and 3 respectively. The pQE-OVA8, derived from pQE60 (Qiagen, Valencia, CA), encodes the OVA$_{257–265}$ SIINFEKL with an initiating methionine, under the T5 promoter. The pQE-OVA8 was constructed as follows: two complementary oligonucleotides, OVA8-*Nco*I and TEMPOVA8-*Bam*HI, encoding SIINFEKL with *S. typhimurium* commonly used amino acid codons, were synthesized. These oligos produce *Nco*I- and *Bam*HI-compatible ends after annealing. Following mixing at equimolar ratio, denaturation at 80°C for 5 min and annealing at 25°C for 60 min, the oligos were diluted and ligated to the linearized pQE60 that was cut with *Nco*I and *Bam*HI.

The pACQE60, which is a pQE-OVA8-compatible vector, was constructed as follows: the T5 promoter fragment from pQE60 was cut by *Xho*I and *Hin*dIII, blunted by Klenow and ligated into a *Sca*I linearized pACYC177 (Calgene, Davis, CA). This insertion disrupted the ampicillin resistance of pACYC177. The *yejE*, *yejF* and *yejE*F genes were ligated in frame to either the *Nco*I or the *Bgl*II unique restriction sites of the pACQE60 vector to obtain pACQE-*yejE*, pACQE-*yejF* and pACQE-*yejE*F. The genes were cloned using colony polymerase chain reaction (PCR) carried out with the following primers: *yejE-Nco*I-2319128 and *yejE-Nco*I-2320160 were used for *yejE* cloning; *yejF-Bcl*I-2320155 and *yejF-Bcl*I-2321750 were used for *yejF* cloning; and *yejE-Bcl*I-2319128 and *yejF-Bcl*I-2321750 were used for *yejEF* cloning. *Bcl*I restriction products share compatible ends with *Bgl*II digestions. *Bcl*I was used because *Bgl*II cuts the *yejF* and therefore could not be used in this case.

The pQE-GFP, which is a pQE60-derived vector encoding fluorescence-activated cell sorter (FACS)-optimized GFP protein under the T5 promoter was constructed as follows: the primers GFP-rev-*Bam*HI and GFP-for-*Bsp*HI were used in a PCR on pSA8. The PCR product, encoding GFP, was cut with *Bsp*HI and *Bam*HI. The fragment was then ligated to an *Nco*I- and *Bam*HI-cut pQE60. The pP$_{yej}$GFP plasmid is a derivative of pSA8. The promoter of the GFP was changed with the *yej* operon promoter as follows: Pro-*Bam*HI-2316090 and Pro-*Bam*HI-2316219 were used in a colony-PCR on CS093. The obtained fragment was cut with *Bam*HI and ligated to a modified pSA8, whose promoter was

Table 3. List of oligonucleotides used in this study

Oligonucleotide	Sequence	Note[a]
OVA8-*Nco*I	CATGAGCATCATCAACTTTGAAAAACTGTAAG	Compatible *Nco*I ends
TEMPOVA8-*Bam*HI	GATCCTTACAGTTTTTCAAAGTTGATGATGCT	Compatible *Bam*HI ends
yejE-*Nco*I-2319128	TACGCCATGGCGCCGCGATTAAGCCCGGT	*Nco*I restriction site
yejE-*Nco*I-2320160	GTCACCATGGTTATACCGCCTTAGCAGGATCAAAGG	*Nco*I restriction site
yejF-*Bcl*I-2320155	AAGGCGGTGATCAATGACATCTCCATTGCTTGCGATTG	*Bcl*I restriction site
yejF-*Bcl*I-2321750	TAGTCGTTGATCATAGGCGTCAGCTTAACGCGAGTAGCTGACG	*Bcl*I restriction site
yejE-*Bcl*I-2319128	GTAACTGTGATCAATGCCGCGATTAAGCCCGGTCAA	*Bcl*I restriction site
GFP-for-*Bsp*HI	AGATATACTCATGAGTAAAGGAGAAGAAC	*Bsp*HI restriction site
GFP-rev-*Bam*HI	TCTGGATCCTTATTTGTATAGTTCATC	*Bam*HI restriction site
Pro-*Bam*HI-2316090	ATAGGGATCCGCCTTCTGTGCCTGAATGGT	*Bam*HI restriction site
Pro-*Bam*HI-2316219	CGATGGATCCCTGAAGGCGGTTTCCTTATC	*Bam*HI restriction site
yejE-for-2319081	ATCAGCTATACGCTGGTCGATCCACGCATTGATTTTGAGGGACGATATGTAGGCTGGAGCTGCTTC	
yejE-rev-2320142	TAAATTTTCAATCGCAAGCAATGGAGATGTCATCTTATACCGCCTTATGGGAATTAGCCATGGTCC	
yejF-for-2320081	CGGCGAAGCGGTACGCGACGCCTTTGATCCTGCTAAGGCGGTATAAGTGTAGGCTGGAGCTGCTTC	
yejF-rev-2321745	CGTGGCGATAGCGGAACCTTTTTCTAACAATAATCCTTTTTAGGCGATGGGAATTAGCCATGGTCC	
TnA	GTTTTTGTTGTGATGTAGGCAT	
pKD4-1331	TTCTATGAAAGGTTGGGCTT	

a. Note refers to the underlined sequence.

removed by *Bam*HI. The pP_{pagC}GFP_OVA and pP_{tac}GFP_OVA plasmids were kind gifts from Dr D. Bumann (Max-Planck Institute for Infection Biology, Berlin, Germany). The pJLP-2H plasmid was a kind gift from Dr M.-J. Wick (Goteborg University, Goteborg, Sweden).

Gene disruptions

Specific *yejE*, *yejF*, *yejEF* gene disruptions were carried out as described (Datsenko and Wanner, 2000) with minor modifications: LB5010 *Salmonella* transformants carrying a Red helper plasmid were grown in 50 ml LB cultures with ampicillin and L-arabinose at 30°C to an OD_{600} of ≈ 0.6 and then made electrocompetent by concentrating 100-fold and washing three times with ice-cold 12% glycerol double-distilled water (DDW). PCR products were purified using High Pure PCR Product Purification Kit (Roche, Branchburg, NJ) and eluted in DDW. Electroporation was done by using a Cell-Porator with a voltage booster and 0.2 cm chambers according to the manufacturer's instructions (Bio-Rad, Hercules, CA) by using 50 μl of bacteria and 50–500 ng of PCR product. Shocked bacteria were added to 1 ml of SOC, incubated at 37°C for 1 h, and then spread onto LB-kan-agar plates to select kan^r transformants. PCR products containing the kan-resistance gene flanked by sequences upstream and downstream the disrupted genes were obtained with the following primers: *yejE*-for-2319081 and *yejE*-rev-2320142 were used to disrupt *yejE*; *yejF*-for-2320081 and *yejF*-rev-2321745 were used to disrupt *yejF*; and *yejE*-for-2319081 and *yejF*-rev-2321745 were used to disrupt *yejEF*. All PCR procedures were carried out according to standard protocols (Ausubel *et al*., 1999).

*Sequencing of Tn*10d-*Tet and kan insertion site*

The insertion site of Tn*10d* in the selected mutants was determined using a recently developed method (Qimron *et al*., 2003). Briefly, the pellet of 1.5 ml of an overnight culture of the *S. typhimurium* Tn*10d*-selected mutants was suspended in 567 μl of TE buffer. Then, 30 μl of 10% SDS and 4 μl of 20 mg ml^{-1} proteinase K were added and the cells were incubated for 60 min at 37°C. Thereafter, 100 μl of 5 M NaCl was added and mixed carefully, followed by 80 μl of 10% cetyltrimethylammonium bromide/0.7 M NaCl solution (Sigma, St. Louis, MI). After 10 min of incubation at 65°C, the DNA was extracted using chloroform/isoamyl alcohol (24 : 1) and phenol/chloroform/isoamyl alcohol (25 : 24 : 1). Following addition of 0.6 volume of 2-isopropanol, the DNA was pooled, washed with 70% ethanol, dried and resuspended in 30 μl of DDW (pH ≈ 8) to a concentration of 1–5 mg ml^{-1}. The obtained purified DNA was sequenced by particular cycling conditions without further manipulations, using the primer TnA. Gene disruptions were verified by direct sequencing using the primer pKD4-1331.

Transposon mutagenesis and back transductions

A P22 phage lysate grown on TT10423 (zzf-1831::Tn*10d*-Tet) was transduced into *S. typhimurium* CS093 carrying the plasmid pNK972 expressing the transposase gene from the tac promoter (Maloy *et al*., 1996). The tetracycline-resistant mutants selected on plates with LB medium and tetracycline (LB-tet) were collected in two pools, and samples of each pool were used to grow

P22 phage lysates. The resulting lysates carrying random chromosomal Tn*10d*-Tet insertions were used to infect overnight cultures of CS022 harbouring pQE-OVA8. Mutants obtained were screened for presentation efficiency. P22 phage-mediated transduction was used to transfer the Tn*10d*-Tet insertions. Selected mutants were streaked at least three times on new plates to avoid pseudolysogeny.

Screening for Salmonella *mutants with superior antigen presentation capacity*

BMA3.1A7 macrophages were seeded in a 96-well plate, 5×10^4 cells in 100 μl per well, in antibiotics-free RPMI medium and grown overnight. Freshly transduced mutant colonies were separately seeded into 50 μl LB medium containing 0.3 M NaCl, ampicillin and tetracycline in a 96-well plate, sealed with parafilm and grown overnight without shaking (condition permitting high invasive capacity; Leclerc *et al*., 1998). After overnight growth, bacteria were diluted 1 : 3 with 150 μl LB medium containing 0.3 M NaCl, ampicillin and tetracycline for 2 h to reach mid-log phase. Overnight-grown BMA3.1A7 macrophages were activated by 10 μg ml^{-1} of *Salmonella* lipopolysaccharide (LPS) (Sigma) for 2 h. Bacteria were then added, in 50 μl, to activated macrophages and coincubated for 30 min. Subsequently, macrophages were PBS-washed and the B3Z cell line, 5×10^4 cells in 200 μl, was added along with gentamicin (50 μg ml^{-1}) and chloramphenicol (100 μg ml^{-1}). IL-2 secretion by the B3Z hybridoma was measured after another overnight incubation by standard sandwich ELISA.

Upscaled measurements were performed with colonies selected from the screening procedure. These colonies were streaked on LB-tet-amp-agar for isolation and then separately seeded into 12 ml of LB medium containing 0.3 M NaCl, ampicillin and tetracycline in 15 ml sealed tubes. Bacteria were diluted by adding 3 ml of bacterial suspension into 9 ml of LB medium containing 0.3 M NaCl, ampicillin and tetracycline, and grown for 2 h in a new 15 ml tube. Following that, OD measurements were performed and bacterial cultures were adjusted to an moi of 10, 100 or 1000 in antibiotics-free RPMI medium. Bacteria (in 0.5 ml) were added to 5×10^5 LPS-activated macrophages in 0.5 ml of antibiotics-free medium per well in a 24-well plate. After 30 min of coincubation, macrophages were PBS-washed and the B3Z cell line (5×10^5 in 1 ml) was added, along with gentamicin (50 μg ml^{-1}) and chloramphenicol (100 μg ml^{-1}). IL-2 secretion by the B3Z hybridoma was measured after another overnight incubation by standard sandwich ELISA. In the case of pP_{pagC}GFP_OVA, BALB/c thioglycolate-induced peritoneal exudate cells served as APCs, and transgenic DO11.10 T cells recognizing $OVA323_{-339}$ on I-A^d were used as IL-2-secreting effector cells. C57BL/6 thioglycolate-induced peritoneal exudate cells and OT-1 transgenic T cells controlled the experiments of MHC-II presentation as an MHC-I parallel system. CCD was added in some experiments 60 min prior to addition of bacteria to macrophages at 10 μg ml^{-1} which was maintained throughout coincubation.

Invasion and survival assays

Bacteria harbouring GFP plasmids were grown overnight in LB medium containing 0.3 M NaCl, ampicillin with semi-anaerobic and non-shaking conditions. These bacteria were used to infect macrophages (BMA3.1A7 cell line) maintained in RPMI–10% FCS medium in a 5% CO_2 atmosphere at an moi of 100. Infection time was 30 min, and non-internalized bacteria were removed by extensive washing with PBS, pH 7.4. Fresh RPMI–10% FCS medium containing 50 μg ml^{-1} gentamicin was then added. After 10 min, 90 min or 24 h, infected macrophages were washed once more with PBS and then lysed with DDW and vigorous pipetting. The numbers of viable intracellular bacteria were determined by plating of serial dilutions on LB-amp-agar plates. Alternatively, the numbers of internalized bacteria were determined after 10 min, 90 min or 24 h by addition of 10 μg ml^{-1} PI (Sigma) to macrophages and flow cytometry analysis for GFP (indicating load of bacteria within macrophages) in live macrophages (PI negative).

Mice

Six- to eight-week old female C57BL/6 mice were purchased from Harlan Laboratories (Rehovot, Israel) and maintained under specific pathogen-free conditions. OT-1 mice, a kind gift from Dr M. Corr (UCSD, La Jolla, CA) with the permission of Dr M. Bevan (UW, Seattle, WA), are C57BL/6 T-cell receptor (TCR) transgenic mice that express a TCR specific for the OVA peptide SIINFEKL-H-2K^b complex (Hogquist *et al*., 1994). (C57BL/6 × 129)F_1 mice were bred at our facility in Berlin. All procedures with animals were carried out in accordance with institutionally approved protocols.

Adoptive transfer and immunizations

Splenocytes were isolated from OT-1 TCR transgenic mice and analysed by flow cytometry to ensure specificity and naïve phenotype of the TCR transgenic CD8$^+$ T cells. About 20% of the splenocytes were CD8$^+$ T cells and more than 96% of these CD8$^+$ T cells were Vα2$^+$, as well as stained positive with the H-2K^b/β2m/SIINFEKL tetramer. More than 95% of the Vα2$^+$, CD8$^+$ T cells were CD45RB^{high}, indicating a naïve phenotype (Xu-Amano *et al*., 1993). After lysis of red blood cells with tris-buffered ammonium chloride, 2×10^7 splenocytes were transferred to C57BL/6 mice by i.v. injection. Recipient mice were immunized twice on days 2 and 9 by feeding with 2×10^9 bacteria in 15 μl and challenged i.v. with 2.5×10^5 B16-OVA tumour cells 7 days after the last immunization. One day after the tumour inoculation, immunocytes were isolated from the lung and stained for flow cytometry. In another set of experiments, mice were similarly immunized twice on days 0 and 7. Seven days after the second immunization, mice were challenged with 10^5 B16-OVA cells in the footpad. Tumour diameter was monitored and measured with microcalipers in two dimensions.

Flow cytometry

Flow cytometry was performed using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA), and data were collected for 50 000 or 100 000 cells. Lung immunocytes were stained with FITC-conjugated anti-CD8 (clone: 53-6.7, BD PharMingen) and Cy5-conjugated H-2K^b/β2m/OVA8 tetramers. All incubations with antibodies or reagents were for 30 min on ice in PBS containing 2% FCS and 0.05% sodium azide. For flow

cytometry analysis of experiments involving i.v. inoculation of *Salmonella*, 4×10^6 spleen cells were incubated for 15 min at 4°C with purified rat IgG antibodies, anti-CD16/CD32 mAb (clone: 2.4G2) and streptavidin (Molecular Probes, Eugene, OR) in PBS, 0.5% BSA, 0.01% sodium azide. After incubation, cells were stained for 60 min at 4°C with Cy5-conjugated anti-CD8α mAb (YTS169), FITC-conjugated anti-CD62L mAb (Mel-14), and PE-conjugated H-2K^b/β2m/OVA8 tetramers. Subsequently, cells were washed with PBS, 0.5% BSA, 0.01% sodium azide and diluted in PBS. PI was added prior to some flow cytometry analysis. For MHC-I and II quantization after infection, the following antibodies were used separately: FITC-conjugated anti-H2-K^b (clone: AF6-88.5, BD PharMingen); FITC-conjugated anti-IA^b (AF6-120.1) and mouse IgG_{2a}kappa isotypic control (G155-178).

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