Differential effect of auxotrophies on the release of macromolecules by *Salmonella enterica* vaccine strains

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keywords : auxotrophic attenuation, bacterial vaccine, DNA release, protein release, *Salmonella* vaccine

running title : Auxotrophic *Salmonella* vaccine strains

Abstract

Attenuated *Salmonella enterica* strains have been widely used as live carriers for vaccines and therapeutic molecules. Appropriate attenuation has been introduced into such bacteria for safety reasons and the improvement of strain properties. Here, we compared two strains that were rendered auxotroph for diaminopimelic acid (DAP) or thymidine monophosphate precursors by deletion of the genes *asd* or *thyA,* respectively. Upon removal of the complementing compound from bacterial cultures, both strains quickly loose their property to form colonies. However, while the *Δasd* bacteria lysed almost immediately under such conditions, *ΔthyA* bacteria remained physically intact during the observation period. As a consequence, the *Δasd* bacteria released their intracellular content such as proteins or plasmids into the supernatant. In contrast, no intracellular component, neither proteins nor plasmids, could be recovered from supernatants of *ΔthyA* bacteria upon depletion of thymidine. Thus, release of macromolecules from the bacterial carrier occurs as a consequence of the appropriate lethal attenuation. This might substitute for sophisticated secretion systems.

1. Introduction

Bacteria constitute efficient carriers for the *in vivo* delivery of prophylactic and therapeutic macromolecules. Commensal as well as pathogen derived bacteria have been employed for this purpose (Roland *et al.*, 2005; Baker, 2005). *Salmonella enterica* spp. have been tested in this context, due to their widely known physiology and well-established molecular genetics (Levine *et al.*, 1996; Sirard *et al.*, 1999). One prerequisite for a *Salmonella* vaccine strain is appropriate attenuation rendering the carrier safe in patients and animals (Levine *et al.*, 1996). Whether attenuation differentially influences delivery properties, has not been studied systematically so far.

Delivery of antigens or DNA vaccines requires liberation of the particular macromolecule from the bacteria after invasion of the host. Various secretion systems have been utilized for that purpose (Gentschev *et al.*, 2002; Russmann, 2004). However, export from the bacteria may encounter limitations regarding size, structure and amount of the cargo.

A simple alternative would be the release of protein or DNA from *Salmonella* vectors upon rupture of the cell wall. Therefore, we compared two vaccine strains harboring attenuating mutations in either the *asd* or the *thyA* gene with respect to their potential of macromolecule release upon cell death. *asd* encodes aspartate ß-semialdehyde dehydrogenase (EC 1.2.1.11) and *thyA* encodes thymidylate synthase (EC 2.1.1.45). Asd represents an essential enzyme for the bacterial cell wall synthesis while ThyA is a key enzyme in DNA synthesis. Mutation of the respective gene renders the strain either auxotroph for DAP or thymidine monophosphate precursors. Upon deprivation of the complementing substrates, bacteria of both strains should die quickly, phenomena known as DAP-less and thymineless death (Curtiss III, 1978; Cohen & Barner, 1954; Bazill, 1967). Here we demonstrate that Δ*asd* bacteria undergoing DAP-less death release large amounts of proteins and plasmid DNA while no macromolecules are released from *ΔthyA* mutants upon thymidine starvation.

2. Materials and Methods

2.1. Bacterial strains and plasmids

S. typhimurium strain SL7207 (*hisG*, Δ*aroA*) was kindly provided by Bruce Stocker (Hoiseth & Stocker, 1981). *S. typhimurium* strain χ8977 carries a deletion in *asd* (*ΔasdA16*) but otherwise is isogenic to SL7207. The *ΔasdA16* deletion was confirmed by PCR as previously described (Kang et al. 2002). An *asd* primer set was used to amplify 1564- and 322-bp DNA fragments from colonies of SL7207 (Asd^+) and the χ8799 (*ΔasdA16*) mutant, respectively (Supplementary Figure 1A). The DAP requirement of strain χ8799 was complemented by introducing plasmid pYA280 (Galan et al. 1990), which carries fuctional *asd*. This strain grew at similar rate in medium devoid of DAP as the wildtype (wt) strain SL7207 also harbouring pYA280. No bacterial cell death and release of plasmid DNA was observed (data not shown).For disruption of chromosomal *thyA* in SL7207, an established method using phage lambda Red recombinase was employed (Datsenko & Wanner, 2000). Bacteria, harbouring the curable Red expression plasmid pKD46, were electroporated with a linear DNA fragment encoding a selectable chloramphenicol resistance gene (Cm^R) flanked by *thyA* sequences. This cassette was generated as follows. Cm^R was amplified from pKD3 with primers 5'-ATGAAACAGTATTTAGAACTGATGCAAAAAGTGCTCGACGAATGTGTAGGCTGGAGCTGCTTC-3' and 5'-TTAGATAGCGACCGGCGCTTTAATGCCCGGATGCGGATCGTACATATGAATATCCTCCTTAG-3'. *thyA* was amplified by colony PCR of strain SL7207 using primers 5'-GGATATCATATGAAACAGTATTTAGAACTG-3' and 5'-GGATATCAGGCCTTTAGATAGCGACCGG-3'. Both products were ligated into the linear pCR2.1-Topo vector (Invitrogen, Germany), yielding plasmids pAEN1 and pAEN3f, respectively. Cm^R fragment from pAEN1 was obtained by *Eco*RI digest and inserted into the *Afl*II site of plasmid pAEN3f, located within *thyA*, giving rise to plasmid pAEN4. Klenow enzyme was used to fill ends. pAEN4 was digested with *Eco*RV and the 1937-bp fragment gel

purified for electroporation. The disruption of chromosomal *thyA* was verified by PCR using the *thyA* primer set 5'-AGGCACACAGAAAAACGACC-3' and 5'-CGAAATCATCGAACCGGTAG-3'. An 1829-bp and 395-bp product was obtained with the SL7207Δ*thyA* mutant whereas a 701-bp product was obtained with strain SL7207 ($ThyA^+$) (Supplementary Figure 1B). The thymidine requirement of strain SL7207*ΔthyA* was complemented by introducing plasmid pBTAH (Belfort et al. 1983), which carries functional *thyA*. This strain grew at similar rate in medium devoid of thymidine as the wildtype (wt) strain SL7207 also harbouring pBTAH. No bacterial cell death and release of plasmid DNA was observed (data not shown). Plasmid pHL222 encodes *luc* (firefly luciferase) derived from plasmid pGL3-basic (Promega, Germany) placed under control of the *Escherichia coli* (*E.coli*) beta-lactamase promoter in the background of plasmid pT7T3-19U (Pharmacia, Germany). Plasmid pHL232 encodes *hly* (listeriolysin O, LLO) under control of the constitutive *tetA* promoter in the background of plasmid pET11d (Novagen, Germany).

2.2. Growth conditions

S. typhimurium strain χ8977 was grown in Luria-Bertani medium (LB) supplemented with 50 μg mL^{-1} DAP, 30 μg mL^{-1} streptomycin and when appropriate 100 μg mL^{-1} ampicillin. *S. typhimurium* strain SL7207Δ*thyA* was grown in minimal medium (Sambrook *et al.*, 1989) supplemented with 40 μg mL^{-1} histidine, 40 μg mL^{-1} phenylalanine, 40 μg mL^{-1} tryptophane, 40 μg mL^{-1} tyrosine, 10 μg mL^{-1} 4-aminobenzoic acid and 10 μg mL^{-1} 2,3-dihydroxybenzoate and when indicated with 250 μg mL^{-1} thymidine. For removal of DAP or thymidine, bacteria were washed once and then resuspended in medium with or without supplementing substrate.

2.3. Determination of optical density, number and status of bacterial cells

Bacterial growth was monitored by optical density at 600 nm (OD_{600}) and the number of viable bacteria was determined by plating. Bacterial live/dead staining was carried out in

parallel using the BD Cell Viability Kit (BD Bioscience, Germany) that contains the DNA intercalating dyes thiazole orange and propidium iodide.

2.4. Quantification of luciferase and plasmid DNA in culture supernatant

After deprivation of DAP or thymidine samples were taken at consecutive time points and centrifuged. Supernatant was passed through a 0.22 µm syringe filter (Millipore, Germany) to remove remnant bacteria. Luciferase activity was determined using the Luciferase Assay System (Promega, Germany). Plasmid DNA from supernatants was isolated using the Qiaprep Spin Miniprep Kit (Qiagen, Germany). Plasmids were transformed by the heat shock method into chemical competent *E.coli* DH5α (Invitrogen, Germany).

2.5. LLO hemolysis assay

The hemolytic activity of LLO released from bacteria carrying plasmid pHL232 was analyzed by a hemolysis assay essentially as previously described (Portnoy *et al.*, 1988). Percentage of lysis was calculated by correlating experimental values to values of complete erythrocyte lysis in 0.25 % (v/v) Triton X-100.

2.6. Scanning and transmission electron microscopy

Samples were fixed in growth medium by adding 5 % formaldehyde and 2 % glutardialdehyde for 1 h at 4 °C. After washing three times with cacodylate buffer (0.1 M cacodylate, 0.09 M sucrose, 0.01 M $MgCl_2$, 0.01 M $CaCl_2$, pH 6.9) bacteria were settled onto poly-L-lysine coated glass cover slips for scanning electron microscopy and fixed again with 2 % glutardialdehyde in cacodylate buffer for 10 minutes at 25 °C. After washing with TE buffer (10 mM TRIS, 1 mM EDTA) samples were dehydrated with a graded series of acetone (10, 30, 50, 70, 90, 100 %), each step for 15 minutes on ice. After critical-point drying with liquid CO_2 (Bal-Tec CPD 030) samples were sputter coated with a thin gold film (Balzers Union SCD 040). Samples were examined in a Zeiss field emission scanning electron microscope DSM 982 Gemini at an acceleration voltage of 5 kV applying the Everhart-

Thornley SE-detector and the inlens detector at a 50:50 ratio. Images were stored on MO-disks and contrast and brightness was adjusted with Adobe Photoshop 6.0.

For ultrastructural anaylsis samples were further fixed with 1 % aqueous osmium tetroxide for 1 h at 25 °C, washed with cacodylate buffer and embedded in 1.75 % water agar. Small cubes were cut, dehydrated in a graded series of acetone (10, 30, 50, 70, 90, 100 %), each step for 30 minutes on ice. For the 100 % acetone step the samples were incubated at 25 °C. Embedding in epoxy resin (Spurr's resin) was done according to the described procedure (Spurr, 1969). Ultrathin sections were cut with a diamond knife, collected onto formvar-coated copper grids (300 mesh), and counterstained with 4 % aqueous uranyl acetate for 2 minutes and lead citrate for 1 minute. Samples were examined in a Zeiss transmission electron microscope TEM910 at an acceleration voltage of 80 kV and calibrated magnifications.

3. Results

3.1. Rapid bacterial cell death of *S. typhimurium ΔasdA* and *ΔthyA* mutants upon removal of the complementing compound

The attenuating mutations in the *asd* as well as in the *thyA* gene should result in rapid death of the bacteria as soon as the complementing compound is removed from the culture. This was the case when plating was used as assay. A brief increase in Cfu's was observed over 1 to 2 hours that was followed by a rapid decline (Fig. 1A). This indicates that bacterial cell death starts roughly at the same time for both attenuated strains.

Plating is a very gross assay that gives little information on the kinetics of inactivation. Therefore, we applied live/dead staining at various time points after removal of the complementing compound. This assay is based on staining the bacteria with two reagents : the membrane permeable dye thiazole orange (TO) and the membrane impermeable dye propidium iodide (PI). Both dyes intercalate into the DNA thereby gaining fluorescence intensity. Bacteria from the *S. typhimurium ΔasdA* strain changed from live to dead as expected (Fig. 1B; upper panels). Immediately after removal of DAP the bacteria were mainly stained by TO while after 1 and 4 hours an increasing portion of the bacteria became PI positive. Intermediate stages that represent "injured" stages, and are stained by both reagents, were also observed.

The *S. typhimurium ΔthyA* strain showed a completely different pattern (Fig. 1B; lower panels). Immediately after removal of thymidine, the majority of the bacteria were stained with TO as expected. But no PI positive cells became detectable even after 4 hours. Instead, the staining intensity with TO increased drastically despite the fact that bacteria were no longer platable.

Electron microscopy (EM) was employed to directly compare phenotypes of bacteria either undergoing DAP-less or thymineless death. Scanning EM revealed signs of lysis of *ΔasdA*

bacteria quickly following onset of DAP starvation. Particularly at equatorial regions large bulges could be observed already at 1 hour (Fig. 2). Such bulges were expected since similar structures have been observed when bacteria were treated with antibiotics that also block cell wall synthesis (Bayer, 1967; Staugaard *et al.*, 1976). At this early time point spheres were observed that probably represent bacterial debris.

Transmission EM was applied to further examine the bulges. As apparent in Fig. 3, blebs could be observed in DAP-starved cells, which do not contain cytoplasm whereas the bigger bulges were found to contain cytoplasm. This most likely represents a snapshot of the process of bacterial lysis.

Ultrastructural analysis confirmed the differences between the *Δasd* and the *ΔthyA* mutant observed by live/dead staining. Even at late time points after removal of thymidine no sign of cell wall damage could be observed with *S. typhimurium ΔthyA* using scanning EM (Fig. 2), although the bacteria exhibited an elongated shape and lack of septa formation. Transmission EM confirmed the maintenance of bacterial cell wall integrity of *ΔthyA* during thymidine starvation. However, at four hours of growth without thymidine, plasmolysis became detectable. Nevertheless, the cell wall remained intact (Fig. 3).

3.2. Recovery of macromolecules from supernatants of *S. typhimurium Δasd* and *ΔthyA* strains

The difference between *S. typhimurium Δasd* and *S. typhimurium ΔthyA* after removal of the complementing compound should have consequences with regard to the release of intracellular content of the bacteria. *S. typhimurium Δasd* should liberate its content while *S. typhimurium ΔthyA* should not. Therefore, we equipped bacteria of both strains with an expression plasmid encoding firefly luciferase as reporter. When both bacteria were transferred to medium without the complementing substrate, a rapid decrease in optical density was observed for *S. typhimurium Δasd,* while the OD in cultures of *S. typhimurium*

ΔthyA increased for at least 2 hours and only then decreased slowly (Fig. 4A). Since the bacteria most likely do not proliferate any longer, this might reflect the increase in size as observed by electronmicroscopy before.

The consequence of the different reactions after depletion of DAP or thymidine, respectively, is, that the intracellular content is released by *S. typhimurium Δasd* but not by *S. typhimurium ΔthyA*. As can be seen in Fig. 4B, 1 hour after removal of DAP, significant amounts of luciferase can be found in the supernatants of *S. typhimurium Δasd*. The activity of this enzyme decreased with time, most likely due to degradation by proteases that are co-released from dying bacteria. Similar observations were made when release of plasmid DNA was tested (Fig. 4C). Already after 1 hour, intact plasmids could be recovered from the supernatants of *S. typhimurium Δasd* bacteria depleted of DAP. Again, the amount decreased with time, most likely due to nucleases released from the bacteria. In contrast, no macromolecules, neither protein nor DNA, could be recovered from supernatants of thymidine depleted *S. typhimurium ΔthyA* (Fig. 4B and C). Therefore, both attenuated vaccine strains differ dramatically in their potential to release heterologous protein or DNA into their environment.

Similar observations were made when LLO was used instead of the luciferase reporter. LLO is a pore-forming cytolysin of the intracellular bacterial pathogen *Listeria monocytogenes* (Gaillard *et al.*, 1987). To test whether active LLO would be released from the attenuated bacteria we equipped both strains with a constitutive LLO expression plasmid. Upon removal of the DAP from *S. typhimurium Δasd* cultures, significant amounts of hemolytic activity could be detected almost immediately in supernatants. In contrast, no activity was found in cultures containing the complementing compound. As expected, no hemolytic activity was recovered from supernatants of *S. typhimurium ΔthyA* independent of whether thymidine was present or not. Thus, lysis induced by the auxotrophy of the *Δasd* mutant equips the carrier bacteria with properties that otherwise would require sophisticated secretion systems.

4. Discussion

Balanced attenuation is a crucial step during the conversion of pathogens into live vaccines or vaccine carriers. The particular attenuated strain needs to persist for a period of time in the host in order to induce an immune response. On the other hand, it should be applicable to individuals with a low immune response status. Besides safety, additional properties might be imposed upon a vaccine strain by the appropriate attenuation. For instance, the $PhoP^c$ phenotype of *S. typhimurium* vaccine strains resulting from a point mutation of the *phoQ* gene (Gunn *et al*., 1996) was shown to be essential for the induction of antibody responses to human papillomavirus virus-like particles (Baud *et al.*, 2004).

An impact of attenuation on other properties of the bacteria has not been studied systematically so far. Here, we compared release of macromolecules during bacterial cell death of two auxotrophic *S. typhimurium* strains *Δasd* or *ΔthyA*. Deficiency in *asd* was expected to interfere with the intactness of the bacteria since the mechanism of DAP-less death is well established (Bayer, 1967; Curtiss III, 1978). Shortly after removal of the complementing compound, the bacteria were no longer platable. This indicates terminal damage of the bacteria. Data derived from live/dead staining and the electron micrographs were consistent with this. The block in DAP synthesis results in the interruption of the cell wall synthesis and consequently in the bulging of the cytoplasm from the rupture. Scanning and transmission EM showed that bulges were formed by cytoplasm and cell wall resembling bulges found after treatment with other antibiotics interfering with cell wall synthesis (Bayer, 1967). The cellular content was released at early time points, as predicted.

In contrast, thymineless death is still not fully understood (Ahmad *et al.*, 1998). *S. typhimurium* SL7207*ΔthyA* bacteria remained physically intact during thymidine starvation for extended periods of time. The bacteria were terminally damaged shortly after depletion, since they no longer could form colonies. However, thymidine starvation of *ΔthyA* bacteria

did not result in PI positive cells. Thus, membrane integrity was maintained throughout the observation period. In addition, TO live staining of bacteria gained intensity. TO binds with high affinity to dsDNA that is accompanied by high fluorescence quantum yields. Binding to ssDNA is less pronounced (Nygren *et al.*, 1998). Therefore, the increased TO fluorescence intensity is most likely due to an increased accessibility of aberrant dsDNA in the dying bacteria.

We did not observe a size difference by forward scatter in cell populations with different TO fluorescence intensity (data not shown). On the other hand, the optical density increased for some time and the electron micrographs revealed an elongation of the bacteria. This is in agreement with previous findings using *E.coli* defective in *thyA* where an increase in cell volume and so-called filamentation was observed (Bazill, 1967; Meacock *et al.*, 1978).

The cause of terminal damage during thymine starvation is still unknown. In *E.coli,* induction of lysogenic prophages (Medoff & Swartz, 1969) or induction of the toxin-antitoxin system *mazEF* (Sat *et al.*, 2003) has been shown to contribute to thymineless death. Since SL7207*ΔthyA* harbours such elements, their contribution to bacterial death is possible although in our experiments we did not observe bacterial lysis as a result of prophage induction during starvation or internal phage like structures (data not shown). Surprisingly, at late time points *ΔthyA* bacteria remained physically intact despite severe plasmolysis-like cytoplasmic shrinkage was observed.

The differential reaction upon depletion of the complementing compounds had a dramatic differential effect on the release of macromolecules. Neither protein nor plasmid DNA could be detected in the supernatants of *ΔthyA* bacteria while such macromolecules could be found almost immediately in depleted cultures of *Δasd* bacteria. The instability of luciferase and expression plasmids is most likely due to the co-release of proteases and nucleases from bacteria during lysis. This could be avoided in future by generating strains devoid of such enzymes as described before (Jain & Mekalanos, 2000).

Disruption of the DAP-synthesis pathway in bacterial vectors could facilitate macromolecule transfer across the bacterial envelop. Such property might be used to substitute for secretion systems like the *E. coli* hemolysin secretion system (Gentschev *et al.*, 2002). Exemplarily, this system was employed to secrete LLO by a *Salmonella* vaccine strain and enabled the carrier bacteria to escape into the host cell cytosol (Gentschev *et al.*, 1995). As alternative we show that active LLO is efficiently released from *S. typhimurium Δasd* strain χ8977 undergoing DAP-less death. The same principle has been used recently to deliver DNA into the cytoplasm of cultured mammalian cells via invasive *E.coli* (Critchley *et al.*, 2004). Mutant *Salmonella Δasd* quickly undergo lysis in environments lacking DAP. For this reason such strains might be overattenuated *in vivo* with regard to their ability to colonize host tissues (Curtiss III *et al.*, 1987). Thus, conditional *asd* mutants might have to be constructed in order to allow the use of the full potential of this delivery system.

Acknowledgement :

The authors wish to thank I. Schleicher for expert technical assistance. This work was supported in part by grants from the German Research Council (DFG) and the Bundesministerium fuer Wirtschaft to S.W..

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Figure legends

Fig. 1. Colony formation and live/dead staining of attenuated bacteria. Strains harbouring mutation in *asd* or *thyA* genes were initally grown in complemented medium. a) The viability of bacteria in the culture was determined after washing and resuspension of cells in either complemented medium (filled bars) or non-complemented medium (open bars). Serial dilutions of cultures were plated on solid medium. b) From the cultures grown without complementing substrate samples were subjected to live/dead staining and subsequent flowcytometric analysis. Fluorescence of cells stained by thiazole orange (TO) is detected primarily in the FL-1 channel and fluorescence of propidium iodide (PI) stained cells primarily in the FL-3 channel. Samples were obtained at the indicated time points. Data from one representative experiment of several are presented.

Fig. 2. Scanning electron micrographs of *Δasd* and *ΔthyA* bacteria. Samples were obtained from equivalent cultures as in Fig.1. *Δasd* bacteria undergoing DAP-less death show typical signs of cell wall rupture already one hour post medium change (upper right panel). Cells with large bulges (black arrow) respresent most likely precursor states of spherical bacterial debris (white arrow head). During thymidine starvation Δ*thyA* bacteria elongate without signs of cell division.

Fig. 3. Transmission electron micrographs of *Δasd* and *ΔthyA* bacteria. Samples where obtained from equivalent cultures as in Fig. 1 and 2.. Small blebs (black arrows) and also larger bulges (white arrow head) are visible on disintegrating *Δasd* bacteria one hour after DAP deprivation (upper right panel). *ΔthyA* bacteria maintain the integrity of their cell wall even after four hours of thymidine starvation (lower right panel). Detachment of inner and

outer membranes is visible (black arrow heads) and the lack of septa formation at this time point is evident. Scale bars represent 1 µm.

Fig. 4. Release of protein and plasmid DNA from auxotrophic bacterial strains harbouring the firefly luciferase plasmid for constitutive expression. Bacteria were grown in complemented medium into logarithmic phase, washed and resuspended in either complemented (filled symbols) or non-complemented medium (open symbols). a) Optical densities of bacterial cultures. b) Activity of firefly luciferase released into the culture medium determined by a bioluminometric assay and expressed as relative light units (RLU). c) Plasmid DNA release from bacteria into the culture medium. Isolated DNA from supernatants was retransformed into compentent *E.coli* cells and resulting transformant colonies were enumerated. One representative of several similar experiments is shown.

Fig. 5. Release of listeriolysin O from auxotrophic bacterial strains. Strain Δ*asd* (quadrangle symboles) and strain Δ*thyA* (triangle symboles) harbouring the LLO expression plasmid pHL232 were grown in complemented medium (filled symbols) or non-complemented medium (open symbols). Culture supernatants were analyzed for LLO activity using the sheep erythrocyte hemolysis assay. Filtered supernatants were diluted fourfold in erythrocyte suspension and incubated at 37 °C for one hour. For relative comparison erythrocytes were completely lysed with 0.25% (v/v) Triton X-100. One representative of several similar experiments is shown.

Supplementary Fig. 1.

Molecular characterization of *S. typhimurium* strains by colony PCR. a) An *asd* primer set was used for PCR with strain SL7207 (wt) and strain χ8799 (*Δasd*) that encompasses the deletion. b) A *thyA* primer set was used for PCR with strain SL7207 (wt) and strain

SL7207*ΔthyA* that encompasses the disruption. Due to the strategy of disruption the two amplification products observed are expected. Numbers indicate sizes of PCR products in bp. M indicates size marker: ΦX174 *Hae*III.