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The biosynthesis and biological role of 6-deoxyheptose in the lipopolysaccharide O-antigen of *Yersinia pseudotuberculosis*

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Summary

Yersinia pseudotuberculosis **O:2a harbours 6-deoxy-D-*manno*-heptose in its O-antigen. The biological function of 6-deoxyheptose and its role in virulence is unknown and its biosynthetic pathway has not been demonstrated experimentally. Here, we show that *dmhA* and *dmhB* are necessary for 6-deoxyheptose biosynthesis in *Y. pseudotuberculosis*. Their disruption resulted in the lack of 6-deoxyheptose in the O-unit and its replacement by D-*glycero*-D-*manno*-heptose, thus indicating relaxed specificity of the glycosyltransferases, polymerase and ligase involved in lipopolysaccharide synthesis. The *dmhB* mutant exhibited a lower content in ketooctonic acid (Ko)-containing core molecules and reduced ligation and polymerization of the O-unit. We also show that Tyr128 is essential for activity of DmhB, and that DmhB functions as an oligomer, based on the dominant negative effect of overexpression of DmhB Y128F in *dmhA*. Moreover, we demonstrate that 6-deoxyheptose is important for virulence-related functions of the outer membrane and its appendages *in vitro*, such as barrier function against bile salts, polymyxin and novobiocin, and flagella-mediated motility. Although both mutants colonized the mouse ceacum as well as the wild type, the *dmhB* mutant was impaired for colonization of the liver, suggesting that DmhB represents a potential therapeutic target.**

Introduction

Lipopolysaccharide (LPS) is one of the major virulence factors of *Yersinia pseudotuberculosis* (Bruneteau and Minka, 2003; Skurnik and Bengoechea, 2003), together with the flagella that confer bacterial motility (Venediktov *et al*., 1988), invasin that is necessary for bacterial invasion (Marra and Isberg, 1997), and the type III secretion system that can disable the host innate immune system (Cornelis, 2002a). The LPS is a major component of the outer membrane (OM) and is composed of three parts: the lipid A, the core oligosaccharide (core) and the O-polysaccharide (O-antigen). Like in other enterobacteria, the lipid A portion in *Y. pseudotuberculosis* is an endotoxin that elicits most of the pathological effects in the host (Brubaker, 1972).

In vitro, temperature regulates the level of O-antigen expression in *Y. pseudotuberculosis*, so that bacteria grown at 22°C express high levels of O-antigen, whereas O-antigen expression is decreased at 37°C (Straley and Perry, 1995; Vakorina *et al*., 2003). This would imply that LPS O-antigen is unnecessary for successful colonization of the host. However, there is increasing evidence that the *Y. pseudotuberculosis* O-antigen plays an important role in the resistance to complement mediated killing from the innate immune system (Porat *et al*., 1995) and in the colonization of host tissues. For example, signature tagged-mutagenesis experiments in *Y. pseudotuberculosis* have shown that genes that are involved in LPS O-antigen biosynthesis are essential for virulence *in vivo* (Karlyshev *et al*., 2001; Mecsas *et al*., 2001; Mecsas, 2002). Hence, temperature-induced downregulation of the O-antigen *in vivo* at 37°C likely occurs after the O-antigen has contributed to the successful evasion of the innate immune system and after colonization of the host is established. The specific components of the O-antigen responsible for this function have not yet been identified.

The O-antigen of *Y. pseudotuberculosis* serovars O:1a, O:2a and O:4b contains a 6-deoxyheptose branching off the main chain (Fig. 1A) (Samuelsson *et al*., 1974; Komandrova *et al*., 1984; Skurnik and Zhang, 1996). This uncommon sugar is not found in mammals, but is present in the capsule layer of a few other pathogens such as *Campylobacter jejuni*, the leading cause of enteritis worldwide (St Michael *et al*., 2002), *Burkholderia pseudomallei* and *Burkholderia mallei*, the causative agents of melioidosis and glanders (Knirel *et al*., 1992; Perry *et al*., 1995; DeShazer *et al*., 2001). In *C. jejuni* 81-176, the capsule is a virulence factor involved in serum resistance, epithelial

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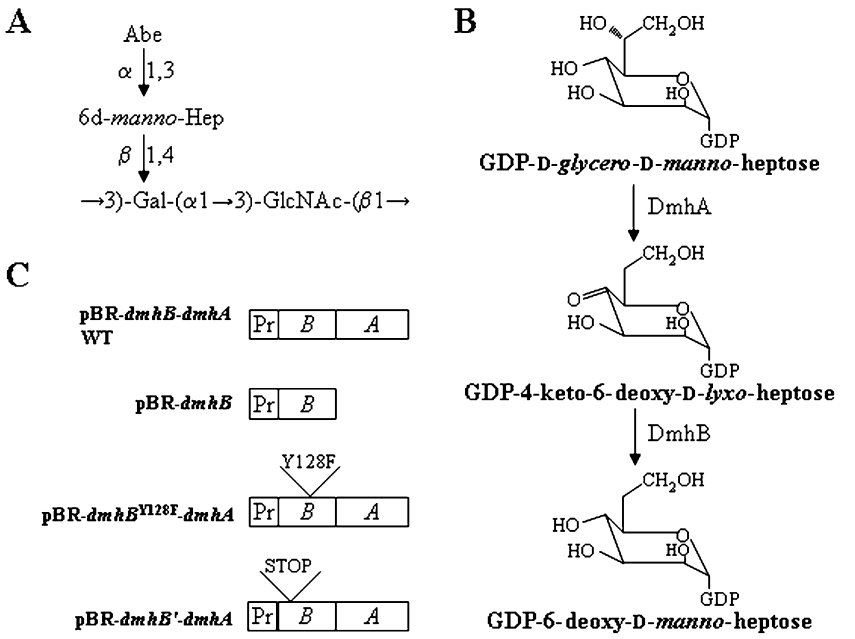

Fig. 1. The structural, enzymatic and genetic background of 6-deoxyheptose biosynthesis in the LPS O-antigen of *Y. pseudotuberculosis* O:2a.
A. Structure of the O-antigen of *Y. pseudotuberculosis* O:2a (Samuelsson *et al*., 1974; Skurnik and Zhang, 1996; this work). Abe, abequose; 6d-*manno*-Hep, 6-deoxy-D-*manno*-heptose; Gal, galactose; GlcNAc, *N*-acetylglucosamine.
B. Putative biosynthetic pathway for 6-deoxyheptose in *Y. pseudotuberculosis* O:2a (Pacinelli *et al*., 2002).
C. Genetic organization of the genes required for 6-deoxyheptose biosynthesis within the O-antigen gene cluster of *Y. pseudotuberculosis* O:2a and related constructs used in this study. Pr, promoter; *B*, *dmhB*; *A*, *dmhA*.

cell invasion and in the pathogen's ability to cause diarrhoeal disease (St Michael *et al*., 2002). In *B. mallei*, the capsule is critical for pathogenesis, and its disruption attenuates the bacterium in murine and hamster models (DeShazer *et al*., 2001). If in all these pathogens, 6-deoxyheptose contributes significantly to the function of the O-antigen or capsule as a virulence factor, then the enzymes necessary for its synthesis could be targets for the development of novel therapeutic agents with broad spectrum applications. To date, however, the molecular basis for the synthesis of such modified heptose is not clear.

In general, as reviewed previously (Raetz and Whitfield, 2002), the biosynthesis of O-antigens begins with the assembly of the O-antigen repeat unit (O-unit) by the sequential transfer of individual nucleoside diphosphate-activated precursor sugars onto undecaprenyl phosphate (Und-P), an inner membrane lipid carrier located at the cytoplasmic face of the inner membrane. Once assembled, the O-unit is translocated across the inner membrane by one of three known pathways to face the periplasmic space: the Wzy-dependent, the ABC-transporter-dependent or the synthase-dependent pathway. In *Y. pseudotuberculosis*, this is likely accomplished by the Wzy-dependent pathway, as the required genes have been identified within the O-antigen gene cluster (Skurnik and Zhang, 1996). Once the O-antigen subunits are translocated by Wzx, they are polymerized into long chains by Wzy, and the length of these chains is regulated by Wzz. The completed O-antigen chain is then ligated to the lipid A-core by the ligase WaaL, and is then transferred onto the OM by an unknown mechanism. Overall, LPS is composed of a mixed population of molecules: some lipid A-core molecules lack O-antigen, while others are capped by O-antigens of variable lengths, usually with a preferential number of repeats determined by Wzz (Barr *et al*., 1999).

Based on genomic analyses, the genes *dmhA* and *dmhB* found in the LPS gene cluster of *Y. pseudotuberculosis* O:2a have been identified as potentially involved in 6-deoxyheptose biosynthesis (Pacinelli *et al*., 2002). The *dmhA* gene encodes a putative C6 dehydratase thought to convert GDP-D-*glycero*-D-*manno*-heptose into GDP-4-keto-6-deoxy-D-*lyxo*-heptose. The *dmhB* gene encodes a putative reductase thought to reduce the DmhA reaction product into GDP-6-deoxy-D-*manno*-heptose (Fig. 1B) (Pacinelli *et al*., 2002). This GDP-activated precursor would then be used for the assembly of O-units. Although *glycero-manno* heptose can theoretically arrise from two distinct pathways as ADP-L-*glycero*-β-D-*manno*-heptose or GDP-D-*glycero*-α-D-*manno*-heptose (Kneidinger *et al*., 2002), the current assignment of the substrate for DmhA as GDP-activated heptose is consistent with the fact that the *Y. pseudotuberculosis* O:2a O-antigen cluster encodes for the four enzymatic activities necessary for GDP-D-*glycero*-α-D-*manno*-heptose synthesis and these genes are located immediately downstream of *dmhB.* Both DmhA and DmhB proteins belong to the Short chain Dehydrogenase and Reductase (SDR) family, which includes proteins from a diverse range of organisms that are involved in the metabolism of a wide variety of substrates (Jornvall *et al*., 1995). As SDR enzymes with different functions or different substrates often share identical catalytic and cofactor

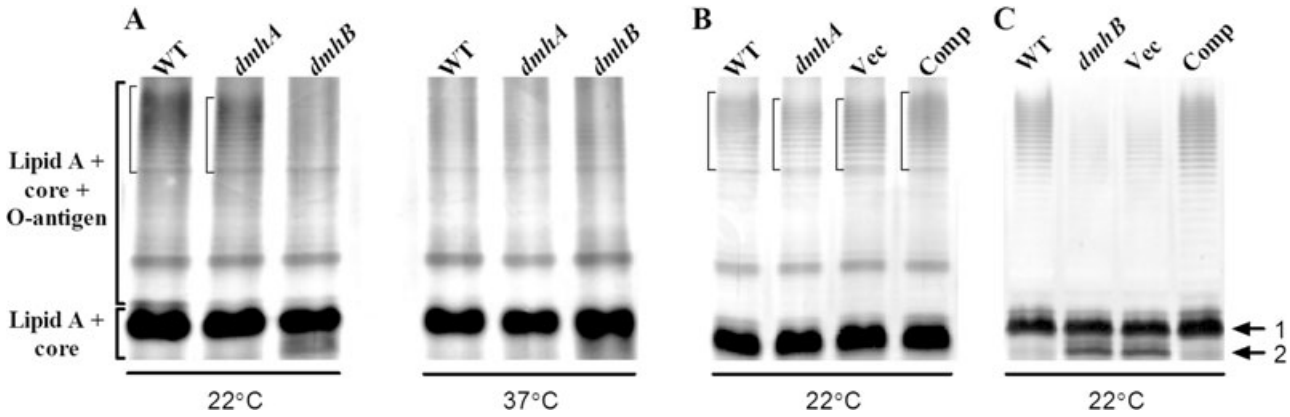

Fig. 2. SDS-PAGE analysis of *Y. pseudotuberculosis* O:2a LPS from WT, mutants and complemented strains grown at 22°C and 37°C. Detection was performed by silver staining. Vec indicates either the *dmhA* (B) or the *dmhB* (C) mutant harbouring the empty vector pBR322. Comp denotes the *dmhA* (B) or *dmhB* (C) mutant complemented *in trans*. Brackets in (A) and (B) illustrate the difference in the lengths of LPS isolated from the WT and mutants. Bands 1 and 2 in (C) were assigned as 'Core + one O-unit' and 'Core' only based on MS data. To ensure equal loadings, the bacteria were adjusted to OD_{600} of 1.0 and 1 ml of the suspension was pelleted. The pellet was re-suspended in 200 µl of solubilization buffer and treated according to the Hitchcock and Brown procedure (see *Experimental procedures*). Five microlitres of treated samples were loaded in each lane.

binding features, their functions are not definitive until established experimentally (Oppermann *et al*., 2003). As such, the functions and substrates of DmhA and DmhB described above are putative only and await experimental confirmation.

We hypothesize that the 6-deoxyheptose found in the O-antigen of *Y. pseudotuberculosis* contributes to the function of the O-antigen as a virulence factor. To test this hypothesis, and based on the proposed pathway mentioned above (Pacinelli *et al*., 2002), *dmhA* and *dmhB* were disrupted within *Y. pseudotuberculosis* O:2a. The composition and structure of the LPS of the resulting mutants were determined and the virulence-related properties of the mutants and their complemented counterparts were assessed *in vitro*. The role of LPS 6-deoxyheptoses in intestinal colonization and virulence was also investigated in a mouse model. The data presented in this study establish that *dmhA* and *dmhB* are involved in 6-deoxyheptose biosynthesis as their inactivation results in the production of a modified O-antigen devoid of 6-deoxyheptose. While inactivating *dmhA* or *dmhB* led to an unexpected increase in novobiocin resistance, both mutants exhibited increased susceptibility to important components of the innate immune system and one of them had impaired motility. Despite this, both mutants were still able to colonize the mouse ceacum but the *dmhB* mutant was not able to disseminate to the liver quite as efficiently as wild type (WT). Moreover, the data presented herein provide some insight into the mode of action of DmhB from the biochemical point of view, via the identification of Tyr 128 and oligomerization of DmhB as critical for activity.

Results

Inactivation of dmhA *or* dmhB *does not alter the growth rate of* Y. pseudotuberculosis

Successful inactivation of *dmhA* and *dmhB* via insertion of a kanamycin resistance cassette was achieved by homologous recombination, which demonstrates that neither gene is essential. Both mutants had identical growth rates compared with WT at 22°C and 37°C, and appeared morphologically similar to WT in shape, size and presence of peritrichous flagella as judged by electron microscopy (data not shown). This demonstrates that these mutations did not cause any adverse pleiotropic effects.

Inactivation of dmhA *or* dmhB *results in altered O-antigen profiles on SDS-PAGE gels*

The *dmhA* and *dmhB* genes were targeted for their putative role in the biosynthesis of 6-deoxyheptose (Fig. 1B), a sugar found branching off the main O-antigen chain in *Y. pseudotuberculosis* O:2a (Fig. 1A). To determine if O-antigen expression was altered by the inactivation of *dmhA* or *dmhB*, LPS from WT and mutants were analysed by SDS-PAGE and silver stain after extraction by SDS solubilization. Both mutants appeared to still express O-antigen (Fig. 2A), as a typical ladder-like pattern of high-molecular-weight (HMW) material was observed in both strains. However, the LPS profile was altered compared with the WT strain, indicating a definitive role of *dmhA* and *dmhB* in O-antigen synthesis (Fig. 2A). Indeed, the *dmhA* mutant expressed slightly less and shorter

O-antigen than WT, while the *dmhB* mutant expressed very little O-antigen. This was confirmed by densitometry analysis of silver-stained gels that revealed that the O-antigen/core ratio of *dmhA* and *dmhB* were on average 72% and 10% ($n = 4$) of that of the WT respectively. Also, a single strong fast migrating band was seen in the WT and *dmhA* strains whereas an additional faster migrating band was seen in the *dmhB* mutant (Fig. 2A and C). The band seen in WT and *dmhA* probably corresponds to lipid A-core + one O-unit as seen in *Yersinia enterocolitica* (Perez-Gutierrez *et al*., 2007) and as corroborated by mass spectrometry (MS) analysis (see below). The faster migrating band observed in the *dmhB* mutant possibly corresponds to lipid A-core only, which was also consistent with MS data (see below).

As mentioned above, O-antigen production is temperature regulated in *Y. pseudotuberculosis* (Straley and Perry, 1995; Vakorina *et al*., 2003). To determine whether temperature regulation of O-antigen production was affected by the inactivation of *dmhA* or *dmhB*, the WT and mutants were grown at 22°C and 37°C, and their LPS was analysed by SDS-PAGE and silver staining. As expected in the WT, O-antigen expression was strongly reduced at 37°C compared with expression at 22°C (Fig. 2A). The O-antigen/core ratio measured at 37°C by densitometry of silver-stained gels was only 6% of the ratio obtained at 22°C. The disruption of *dmhA* did not alter the temperature regulation of the O-antigen expression, as much higher levels of O-antigen were present at 22°C (Fig. 2A) than at 37°C. The O-antigen/lipid A ratio at 37°C was only 3.5% of that observed at 22°C. In contrast, temperature did not affect much O-antigen expression in *dmhB* as its O-antigen/core ratio remained constant at both temperatures. It is important to note that the O-antigen/lipid A ratio of the WT and *dmhA* mutants at 37°C were similar to that of *dmhB* grown at 37°C or 22°C.

Inactivation of dmhA *or* dmhB *results in the loss of 6-deoxyheptose in the O-antigen*

To characterize the specific changes in the LPS composition of the mutants, LPS from the WT and mutants were isolated by phenol/water extraction (Westphal and Jann, 1965) and the per-*O*-trimethylsilyl derivatives of the monosaccharide methyl glycosides produced from the samples by acidic methanolysis were analysed by gas chromatography/mass spectrometry (GC/MS). The LPS from WT and *trans*-complemented strains were also analysed by alditol acetate derivatization after hydrolysis in trifluoroacetic acid to resolve the 6-deoxyheptose and glucose peaks. The mole percentages of all the sugar residues isolated are presented in Table 1. Most notably, the data showed that 6-deoxyheptose was not present in the LPS of the mutants, indicating that both *dmhA* and *dmhB* are required for its biosynthesis.

Table 1. Composition analysis of LPS from WT, mutants and complemented strains based on trimethylsilyl derivatives analysis individual components obtained after acidic methanolysis of the whole LPS.

Residue	WT	*dmhA*	*dmhB*	*dmhA* complement	*dmhB* complement
Rib[a]	1.9	2.9	2.9	2.1	0.8
Man[a]	2.4	0.8	0.4	n.d.	n.d.
Abe	11.5	7.3	1.9	3.1	6.6
Gal	11.7	8.8	4.8	10.2	12.6
dHep	15.2	n.d.	n.d.	13.7	23.5
GlcNAc	19.8	6.9	10.9	36.6	20.1
Hep	18.6	46.1	47.1	22.5	20.6
Glc	6.1	11.9	10.1	3.7	3.4
Kdo	12.8	15.3	21.9	8.2	12.4
Total	100	100	100	100	100

a. Ribose and mannose residues were only present in trace amounts.
For the WT and complement, analysis of alditol acetates was also performed to allow quantification of heptose. The data are presented as the mole percentage of sugar residues found in the LPS.
Rib, ribose; Man, mannose; Abe, abequose; Gal, galactose; dHep, deoxyheptose; GlcNAc, *N*-acetylglucosamine; Hep, heptose; Glc, glucose; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; n.d., none detected.

Additional unexpected features were observed in the composition of the *dmhA* and *dmhB* mutants (Table 1). First, although both mutants lacked 6-deoxyheptose, their LPS still contained significant levels of abequose, especially in the *dmhA* mutant. This was surprising as abequose is attached to the main O-antigen chain through the 6-deoxyheptose in the WT (Fig. 1A). This suggests that the abequose present in the mutants might be attached to another – most likely similar – sugar. Second, much higher-than-normal levels of heptose were observed in the mutants compared with WT. It is possible that a heptose is incorporated into the O-antigen in place of the 6-deoxyheptose, thus allowing for abequose attachment.

To test this hypothesis, a more refined analysis of the LPS molecules was performed, which required establishing the complete structure and composition of the WT O-antigen-core molecule.

Determination of the WT O-antigen and LPS core composition and structure

The LPS of the WT strain cultivated at 22°C was first isolated by phenol/water extraction as above, but was further purified by precipitation of impurities by trichloroacetic acid (TCA). The lipid A was removed by mild acid hydrolysis, and the water-soluble supernatant was separated into a high molecular weight (HMW) fraction and a low-molecular-weight (LMW) fraction. The composition of both HMW and LMW fractions was determined by gas-

Table 2. Sugar analysis of the carbohydrate fractions released from the LPS of *Yersinia pseudotuberculosis* O:2a and derived mutants.

	Abe[a]	Glc	Gal	6dHep	GlcN	DDHep	LDHep
O-polysaccharides (HMW fractions)							
WT	0.17	0.40	1.00	0.93	0.92	0.16	0.13
dmhA	0.17	0.67	1.00	–	0.71	0.98	0.11
O-unit-core oligosaccharides (LMW fractions)							
WT	0.10	0.77	1.00	0.74	0.79	0.62	1.92
dmhA	0.29	0.72	1.00	–	0.68	1.13	1.18
dmhB	0.14	0.76	1.00	–	0.50	0.95	1.59

a. Abequose is significantly destroyed under the hydrolysis conditions used.
The data represent the detector response relatively to galactose in GLC of the alditol acetates obtained after hydrolysis with 1 M trifluoroacetic acid (120°C, 2 h).
Abe, abequose; 6dHep, 6-deoxy-D-*manno*-heptose; LDHep and DDHep, LD-heptose and DD-heptose respectively.

liquid chromatography (GLC) after hydrolysis by trifluoroacetic acid and derivatization to the alditol acetates. The data obtained for the HMW fraction that corresponds to the O-polysaccharide-core were consistent with the published O-antigen composition (Samuelsson *et al*., 1974; Skurnik and Zhang, 1996) and the data reported in Table 1, although the level of abequose detected was fairly low due to significant destruction during hydrolysis by trifluoroacetic acid (Table 2).

The HMW fraction was analysed also by ^{1}H NMR spectroscopy. The ^{1}H NMR spectrum of the O-antigen fraction showed, *inter alia*, characteristic signals for 6-deoxyheptose and abequose (δ 1.76 and 2.14 for H-6 of 6-deoxyheptose, δ 2.00, 2.09 for H-3 and δ 1.20 for H-6 of Abe) (Fig. 3, top). As some details of the O:2a antigen structure have not been established chemically but proposed based on genetic data (Reeves *et al*., 2003), full structural analysis of the WT HMW O-antigen was

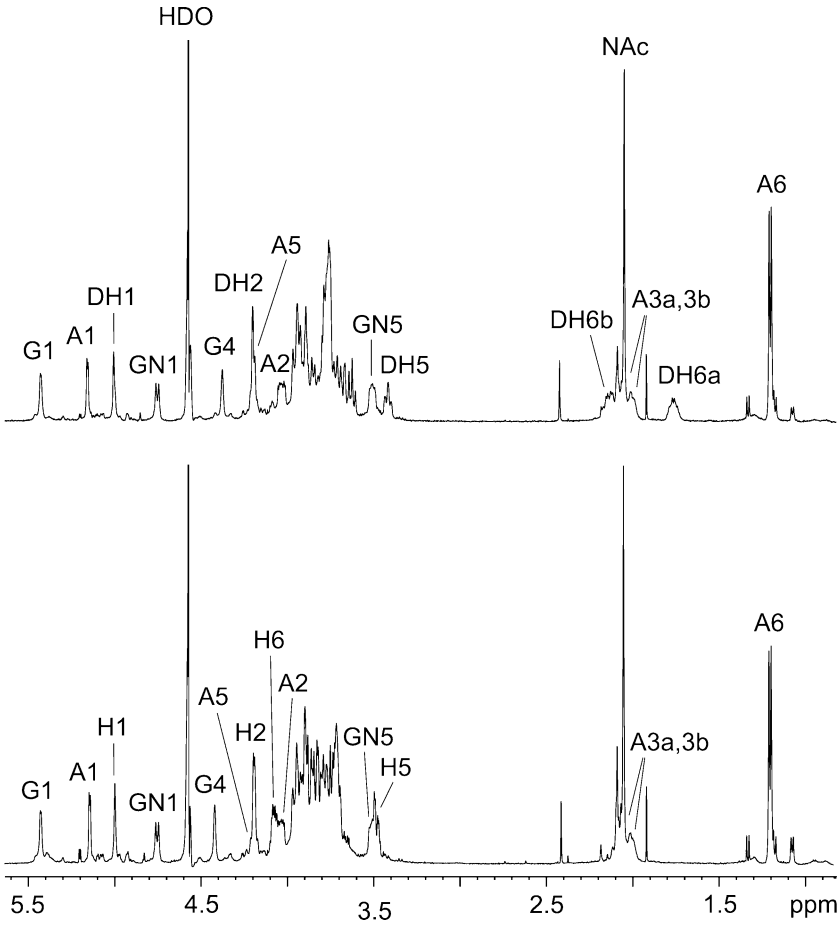

Fig. 3. ^{1}H NMR spectra of the HMW O-antigens from LPS of *Y. pseudotuberculosis* O:2a (top) and *dmhA* mutant (bottom). Numbers refer to protons in sugar residues denoted as follows: G, galactose; A, abequose; DH, 6-deoxy-D-*manno*-heptose; H, DD-heptose; GN, GlcNAc.

performed using two-dimensional ^{1}H and ^{13}C NMR spectroscopy (details of this study will be reported elsewhere). As a result, the earlier proposed structure shown in Fig. 1 was fully confirmed.

The LMW fraction was also analysed by high-resolution electrospray ionization MS, which showed that all oligosaccharides present consisted of the core moiety capped by one O-unit (Fig. 4, top). The observed molecular masses were in full agreement with the composition of the tetrasaccharide O-unit, as established by studies of the HMW fraction (see above), and with the composition of the hexasaccharide core, whose structure has been elucidated recently in *Y. pseudotuberculosis* O:3 and O:4b (Knirel *et al*., 2007, 2008) and found to be essentially the same as in *Yersinia pestis* (Knirel *et al*., 2005).

The mass spectrum of the LMW fraction showed two mass peak clusters (A and B) with the major peaks at 2075.70 and 1839.64 u for compounds depicted in the inset of Fig. 4 (top) (the calculated molecular masses are 2075.67 and 1839.62 u respectively). Cluster A belongs to oligosaccharides containing a lateral residue of octulosonic acid [either 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) or D-*glycero*-D-*talo*-oct-2-ulosonic acid (Ko)], whereas oligosaccharides of cluster B were derived by cleavage of the lateral Kdo during mild acid hydrolysis of the LPS (Ko does not cleave under these conditions) (Knirel *et al*., 2005). Further heterogeneity of the LMW fraction is associated with alternation of two pairs of terminal monosaccharides in the core, namely Kdo versus Ko (Δm 16 u) in cluster A and galactose versus D-*glycero*-D-*manno*-heptose (DD-heptose) (Δm 30 u) in both clusters. Furthermore, a minor fraction of the oligosaccharides from cluster A contained additional phosphoethanolamine, and the Kdo at the reducing end of cluster B oligosaccharides occurred in either normal or anhydro form (Δm 18 u). Most interestingly, in cluster B there was a minor peak at 1855.64 u (1839.64 + 16), which cannot be accounted for by heterogeneity in the core moiety but may arise from an O-unit in which 6-deoxyheptose is replaced by heptose. Then, the approximately 10:1 ratio of these peaks may reflect the ratio of activities of the glycosyl transferase involved with the different substrates (see below).

Inactivation of dmhA *or* dmhB *results in the substitution of 6-deoxyheptose with* DD*-heptose in the O-unit*

The LPS of the *dmhA* and *dmhB* mutants were also prepared from cultures grown at 22°C using phenol/water extraction and TCA precipitation. The same analyses were performed with HMW and LMW fractions from the *dmhA* mutant which, as in the WT, corresponded to the O-polysaccharide-core and O-unit-core respectively. The *dmhA* O-antigen did not contain any 6-deoxyheptose, as observed before with the trimethylsilyl derivatives. Also importantly, a significant increase in the DD-heptose content of the molecule was observed in the LMW fraction (Table 2), with an increase of the DD-heptose to L-*glycero*-D-*manno*-heptose (LD-heptose) molar ratio from ~1:3 in the WT to ~1:1 in the mutant.

The ^{1}H NMR spectrum of the HMW fraction (Fig. 3, bottom) was consistent with retaining of abequose and incorporation of DD-heptose in the O-unit in lieu of the 6-deoxyheptose. Thus, it contained signals for abequose (δ 2.00, 2.10 for H-3 and δ 1.21 for H-6) but lacked signals for 6-deoxyheptose. The spectrum was fully assigned using two-dimensional COSY, TOCSY and ROESY experiments and spin systems for Gal, GlcNAc, Abe and DD-heptose were identified. Comparison of the chemical shifts and $J_{1,2}$ coupling constants with those of the HMW fraction from WT showed that in the two polysaccharides the corresponding glycosidic linkages have the same configurations. The ROESY patterns of both polysaccharides were markedly similar too and demonstrated the same linkage positions and sequence of the constituent monosaccharides. Particularly, as 6-deoxyheptose in WT, DD-heptose in *dmhA* mutant was found to be β-linked at position 4 of Gal and glycosylated itself by α-Abe at position 3.

The mass spectrum of the LMW fraction (Fig. 4, middle) showed that the *dmhA* mutant core remained unaltered compared with that of the WT (Fig. 4, top) and, again, in all molecules the core moiety was capped with one O-unit. The MS patterns of the LMW fractions from both strains (WT and *dmhA* mutant) were essentially the same with two mass peak clusters A and B but masses of all major compounds from the *dmhA* mutant were higher by 16 u, which corresponds to a replacement of the 6-deoxyheptose with DD-heptose (e.g. compare masses 2075.70 and 2091.70 u, respectively, for the oligosaccharides with the full O-unit linked to the core containing lateral Ko and DD-heptose).

Similarly, compositional analysis and MS data demonstrated the lack of the 6-deoxyheptose in the O-unit found in the LMW fraction of the *dmhB* mutant and its replacement by DD-heptose (Table 2, Fig. 4, bottom). Significantly, in addition to clusters A and B, the mass spectrum showed the corresponding clusters C and D, and E and F, with prominent peaks for the core with an incomplete O-unit containing only Gal and GlcNAc, and the core without any O-unit respectively. These findings indicate reduced ligase activity and inefficient assembling of the O-unit probably due to altered activity of the heptosyltransferase. Also, the *dmhB* mutant contained a much lower proportion of Ko-containing core molecules compared with WT and the *dmhA* mutant, thus showing that the Ko synthesis pathway or transfer was affected too.

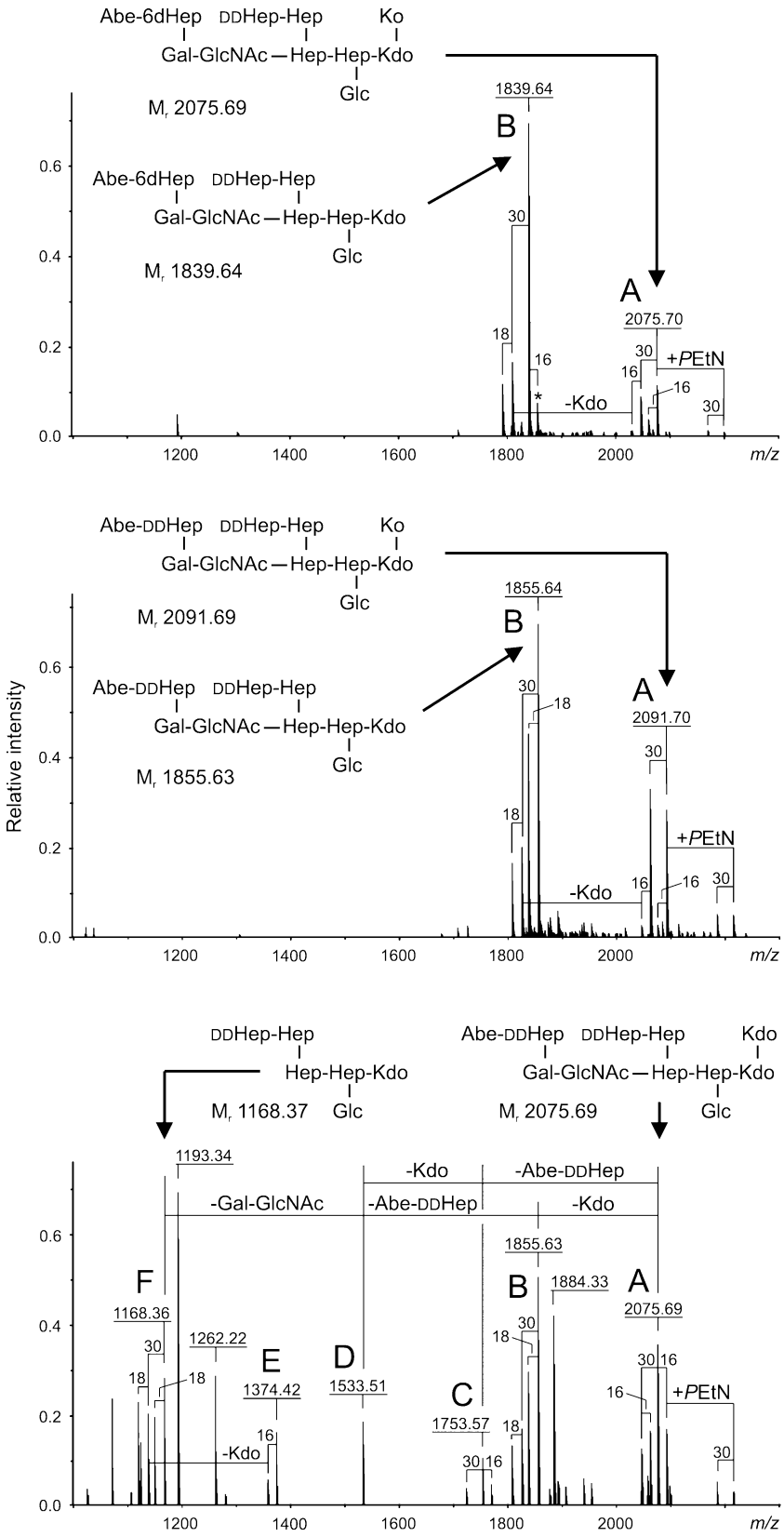

Fig. 4. Charge-deconvoluted negative ion electrospray ionization mass spectra of LMW fractions from LPS of *Y. pseudotuberculosis* O:2a (top), *dmhA* mutant (middle) and *dmhB* mutant (bottom). Mass differences of 16, 18 and 30 u correspond to substitution of Kdo for Ko, anhydro-Kdo for Kdo and Gal for DD-heptose in the core. Hep and DDHep, LD-heptose and DD-heptose, respectively; 6dHep, 6-deoxy-D-*manno*-heptose; *P*EtN, phosphoethanolamine. M_r values shown in the insets are calculated molecular masses whereas recorded masses are shown in the spectra.

Unexpectedly, no HMW O-antigen in an amount sufficient for NMR analysis could be obtained from the *dmhB* mutant LPS after purification by water/phenol extraction and TCA precipitation. This is in contrast with the observation of the ladder-like pattern on SDS-PAGE gels (Fig. 2) that indicated O-antigen production, although at a very low level. As *Y. pseudotuberculosis* also produces enterobacterial common antigen (ECA), the possibility that the HMW bands observed in *dmhB* by SDS-PAGE are not O-antigen and might rather correspond to ECA was tested by Western blot using an anti-ECA polyclonal antiserum. In the gel system used throughout these studies, ECA was readily detected in all strains as a fast-migrating LMW band located at the bottom of the gel, which did not overlap with the O-antigen bands and was not sensitive to temperature regulation (data not shown). Consequently, these data confirm that the HMW bands observed by SDS-PAGE analysis and silver staining of *dmhB* are indeed O-antigen. It is possible that the more refined purification method used for MS and NMR analyses of the LPS led to loss of the low level of O-polysaccharide-containing molecules produced by this mutant.

In summary, the LPS analyses support the hypothesis that *dmhA* and *dmhB* are involved in the biosynthesis of the 6-deoxyheptose found in the *Y. pseudotuberculosis* O:2a O-antigen, and demonstrate that the disruption of *dmhA* or *dmhB* leads to replacement of the 6-deoxyheptose by DD-heptose in the O-units, as well as reduced ligation and polymerization of the O-unit in the *dmhB* mutant. Disruption of *dmhA* did not alter the core structure or composition significantly, whereas that of *dmhB* led to a lower content of Ko-containing molecules. The impact of these compositional and structural differences on the function of the LPS molecule regarding virulence related traits was investigated both *in vitro* and *in vivo*.

Inactivation of dmhB *impairs motility of* Y. pseudotuberculosis

The LPS is a major component of the OM and has been demonstrated to interact directly with OM proteins (van Alphen *et al*., 1978; Schweizer *et al*., 1978; Gmeiner and Schlecht, 1980). The observed alterations in the LPS composition of both mutants could lead to altered interactions with OM proteins, which could in turn lead to OM destabilization. As the flagella are anchored to the OM, an unstable OM could negatively affect their function. Indeed, LPS alterations are often associated with impaired motility in a wide range of bacteria (Komeda *et al*., 1977; Merkx-Jacques *et al*., 2004). As an indirect means to determine if the inactivation of *dmhA* or *dmhB* affected OM stability, the motility of the mutants was compared with that of WT on soft agar plates. Although

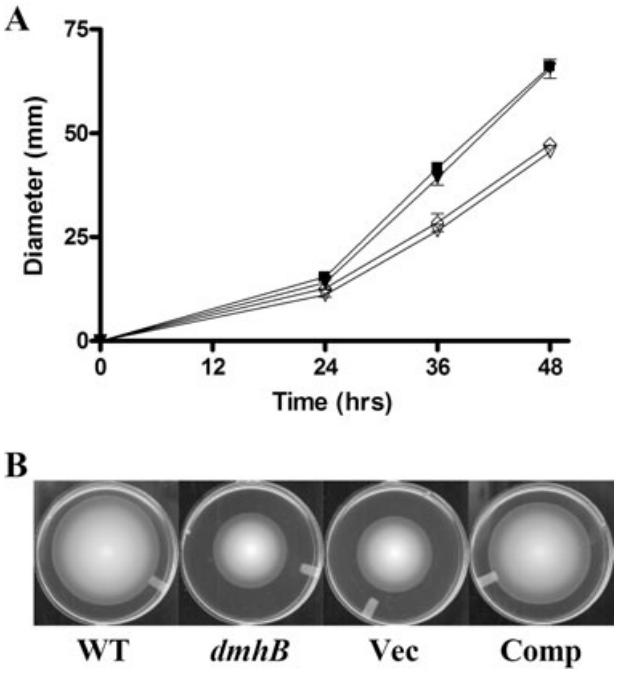

Fig. 5. Motility of WT, *dmhB* mutant and complemented strain.
A. Diameter of motility halo over 48 h at 22°C.
B. Snapshots of representative motility plates at 48 h.
■ and WT: wild type; ▽ and *dmhB*: *dmhB* mutant; ◇ and Vec: *dmhB* mutant + pBR322 empty vector; ▼ and Comp: *dmhB* complemented *in trans* with pBR-*dmhB*. Error bars indicate standard deviations of triplicate samples. A significant ($P < 0.05$) difference between WT and *dmhB* mutant was observed according to an unpaired *t*-test at 48 h.

morphological analysis by electron microscopy showed that both mutants expressed peritrichous flagella similar to WT (data not shown), a significant impairment in motility was seen in the *dmhB* mutant compared with WT (Fig. 5). In contrast, the *dmhA* mutant was fully motile (data not shown). The impaired motility observed only in the *dmhB* mutant correlates well with the lower level of capping of core by O-units and presence of limited amounts of HMW O-antigen chains observed in this mutant but not in the *dmhA* mutant (Fig. 2A).

Inactivation of dmhA *or* dmhB *increases sensitivity to lipophilic agents and antimicrobial peptides*

The OM protects the bacterium from antimicrobial agents. Its stability and barrier function are inherently linked to the composition of the LPS O-antigen and to the level of intermolecular bridging established through phosphorylated residues present in the core and the lipid A. Defects in the core or O-antigen often correlate with higher bacterial sensitivity to killing by detergents, lipophilic agents, cationic antimicrobial peptides as well as hydrophobic antibiotics (Schnaitman and Klena, 1993; Nesper *et al*., 2001; Raetz and Whitfield, 2002; Merkx-Jacques *et al*., 2004).

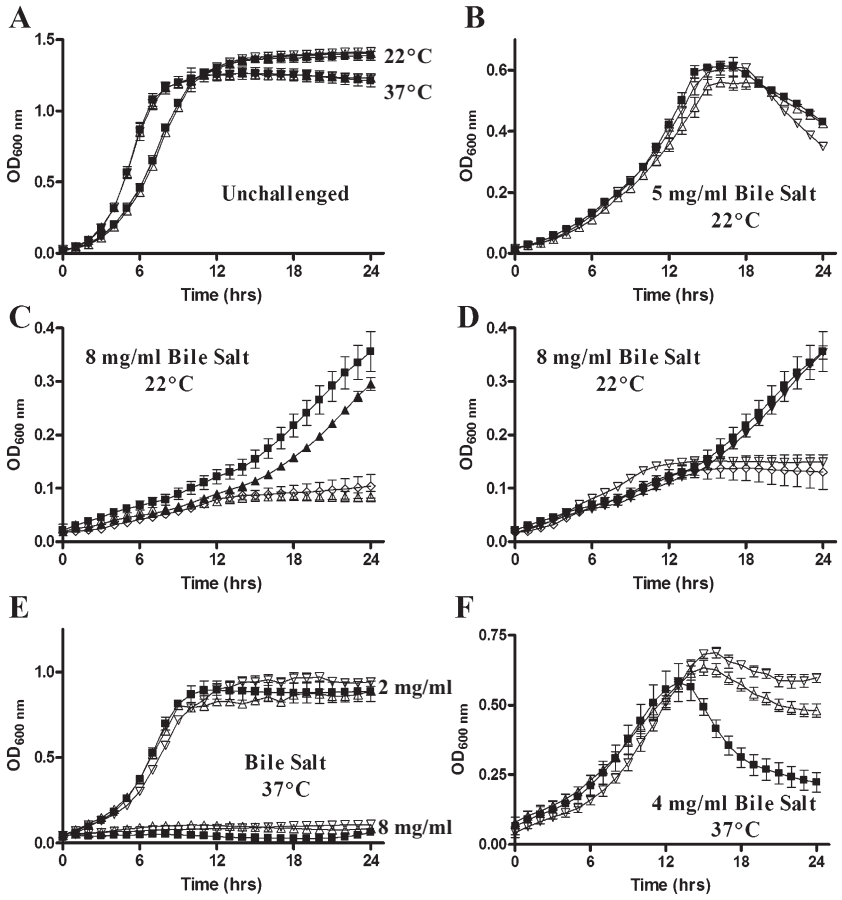

Fig. 6. Bile salt sensitivity assays at 22°C and 37°C. The WT, mutant and the complemented strains were challenged with 0 (A), 5 (B), 8 (C and D), 2 or 8 (E), 4 (F) mg ml^{-1} bile salt. All strains were monitored for growth by optical density at 600 nm (OD_{600}) at 22°C (A–D) or 37°C (A, E and F) over 24 h. ■, wild type; △, *dmhA* mutant; ▽, *dmhB* mutant; ◇, mutant + pBR322 empty vector, specifically *dmhA* mutant in (C), and *dmhB* mutant in (D); ▲, *dmhA* complement; ▼, *dmhB* complement. Error bars indicate standard deviations of triplicate samples. A significant ($P < 0.05$) difference between WT and mutants was observed according to an unpaired *t*-test at 24 h.

In various *Yersinia* species, the intermolecular bridging that occurs via the lipid A and core also plays a crucial role in the resistance to bile salts (Skurnik *et al.*, 1999), hydrophobic antibiotics such as novobiocin (Bengoechea *et al.*, 1998a; 2003; Skurnik *et al.*, 1999), as well as cationic antimicrobial peptides such as polymyxin B (Anisimov *et al.*, 2005; Winfield *et al.*, 2005). Based on the observed LPS alterations of both mutants mentioned above, the barrier function of the OM may be compromised in both mutants. Hence, the sensitivity of the *dmhA* and *dmhB* mutants to lipophilic agents (SDS and bile salts), cationic peptides (polymyxin B) and hydrophobic antibiotics (novobiocin) was investigated.

When WT and mutants were challenged with 0.01–0.2% SDS, growth was drastically and equally reduced in all strains, and the WT and mutants had similar growth rates (data not shown). This indicates that the modifications to the LPS in the mutants did not alter OM resistance towards this detergent at the concentrations tested.

No difference was observed between the growth rates of the WT and the mutants in the absence (Fig. 6A) or presence (Fig. 6B) of a low concentration of bile salts at 22°C, a temperature where O-antigen is produced in large amounts. However, differences in growth rates were observed upon challenge with higher concentrations of bile salts (Fig. 6C and D). Whereas very limited growth was observed when the *dmhA* or *dmhB* mutants were subjected to exposure to 8 mg ml^{-1} bile salts, the WT grew relatively well in the same conditions. Colony-forming unit (cfu) determinations indicated that all strains, including the mutants, were viable after the 24 h period (data not shown), despite the absence of further growth of the mutants (Fig. 6). The cfu counts matched approximately the cfu expected based on the OD_{600}. These data indicate that both mutants are less resistant to bile salts than WT at 22°C and that bile salts exert a stronger bacteriostatic effect on the mutants than on the WT.

To test whether the observed difference in resistance to bile salts was due to the difference in O-antigen composition in the mutants or not, the resistance of the mutants to bile salts was also tested at 37°C, a temperature that represses O-antigen expression (Fig. 2A). At this temperature, all strains grew equally well in the absence of challenge (Fig. 6A) but showed decreased resistance to bile salts, to the point where no growth was observed upon challenge with 8 mg ml^{-1} bile salts (Fig. 6E). When the WT and mutants were challenged with lower bile salts concentrations (4 and 2 mg ml^{-1}), both mutants were able

to grow to the same density as the WT (Fig. 6E and F). All strains stayed viable over a 24 h period as determined by cfu counting, although significant lysis of the WT strain was observed upon challenge with 4 mg ml^{-1} bile salts only (Fig. 6F).

Together with the data obtained at 22°C, this indicates that the O-antigen of *Y. pseudotuberculosis* contributes significantly to bile salts resistance, even when limited amounts of O-antigen are present as observed in the *dmhB* mutant. Also, the data indicate that the presence of 6-deoxyheptose in the O-antigen is a major contributor to bile salt resistance as both mutants which harbour O-antigen where 6-deoxyheptose is substituted by DD-heptose are much less resistant. Overall, it appears that a more hydrophobic bacterial surface generated via alteration to the O-antigen in the mutants or by growing all strains in conditions that repress O-antigen production results in less resistance to bile salts.

Upon challenge with 10 μg ml^{-1} polymyxin B at 22°C, the WT strain could grow to the same final density as in the absence of challenge (Fig. 7B), but there was a lag phase that probably corresponds to adaptation of the bacterium to exposure to polymyxin as observed in other *Yersinia* strains (Rebeil *et al.*, 2004; Anisimov *et al.*, 2005; Knirel *et al.*, 2005). Both mutants were able to grow to the same final density as the WT but exhibited a prolonged lag in growth compared with WT: 3 h extra for *dmhA* compared with WT (Fig. 7B) and 6 h extra for *dmhB* (Fig. 7C). This lag phase demonstrates higher sensitivity of the mutants to polymyxin, which would most likely offer opportunity for clearance by the host within the context of an infection. These differences were correlated to the production and composition of the O-antigen, as no difference in growth was observed between the WT and the mutants upon challenge with 10 μg ml^{-1} polymyxin at 37°C (Fig. 7A).

As a whole, both mutants are more sensitive to bile salts and polymyxin B than WT when grown under conditions where O-units are produced. The differences are essentially abrogated at temperatures that repress O-antigen production, indicating that the O-units and specifically their 6-deoxyheptose are essential to provide WT levels of resistance to bile salts and polymyxin. This demonstrates that 6-deoxyheptose plays a significant role in protection against bile salts from the digestive tract, as well as against cationic antimicrobial peptides from the innate immune system.

Inactivation of dmhA *or* dmhB *increases novobiocin resistance*

When the WT and mutants were challenged with novobiocin at 22°C, both mutants were unexpectedly more resistant than the WT. Furthermore, the *dmhB* mutant was even more resistant to novobiocin than the *dmhA* mutant

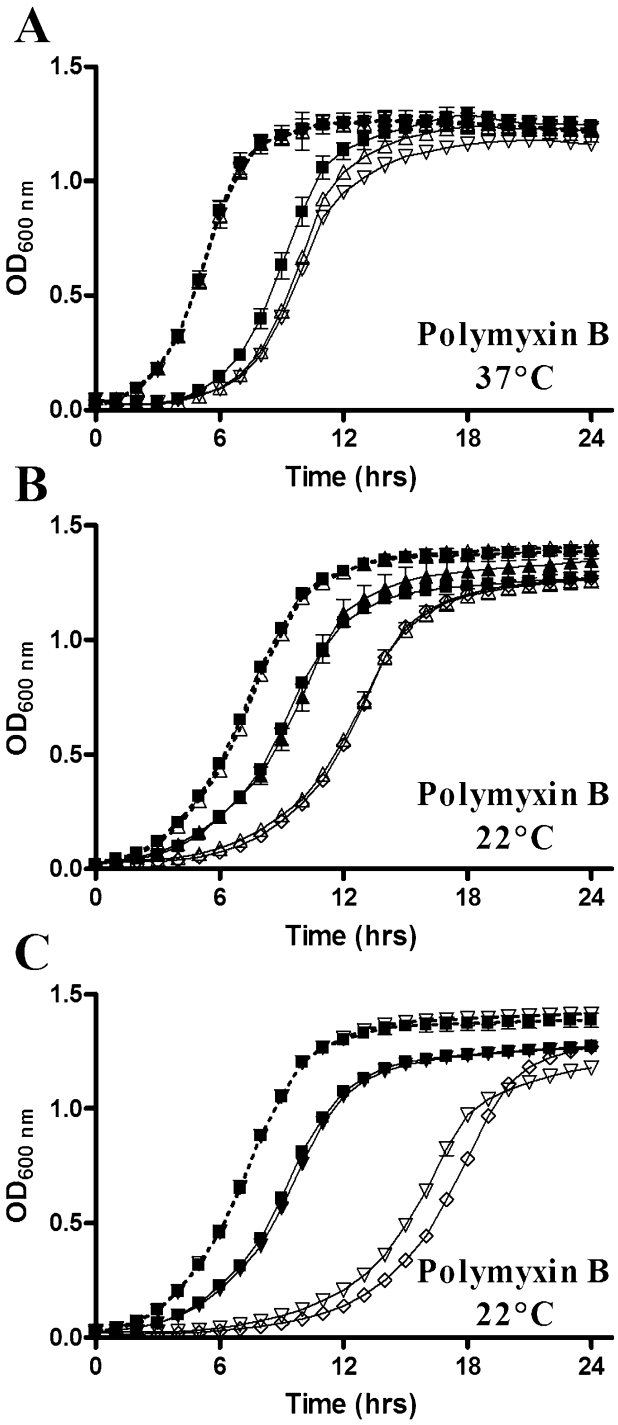

Fig. 7. Polymyxin B sensitivity assays at 37°C and 22°C. The WT, mutant and the complemented strains were challenged with 10 μg ml^{-1} polymyxin B. All strains were monitored for growth by optical density at 600 nm (OD_{600}) at 37°C (A) or 22°C (B and C) over 24 h. Plain lines: with polymyxin challenge. Dotted lines: without challenge. The symbols are the same as in Fig. 6 except that ◇ denotes *dmhA* mutant + empty vector pBR322 in (B) and *dmhB* mutant with empty vector in (C). Error bars indicate standard deviations of triplicate samples. A significant ($P < 0.05$) difference between WT and mutants were observed in the time required for each strain to reach mid-exponential growth (OD_{600} 0.6) according to an unpaired *t*-test.

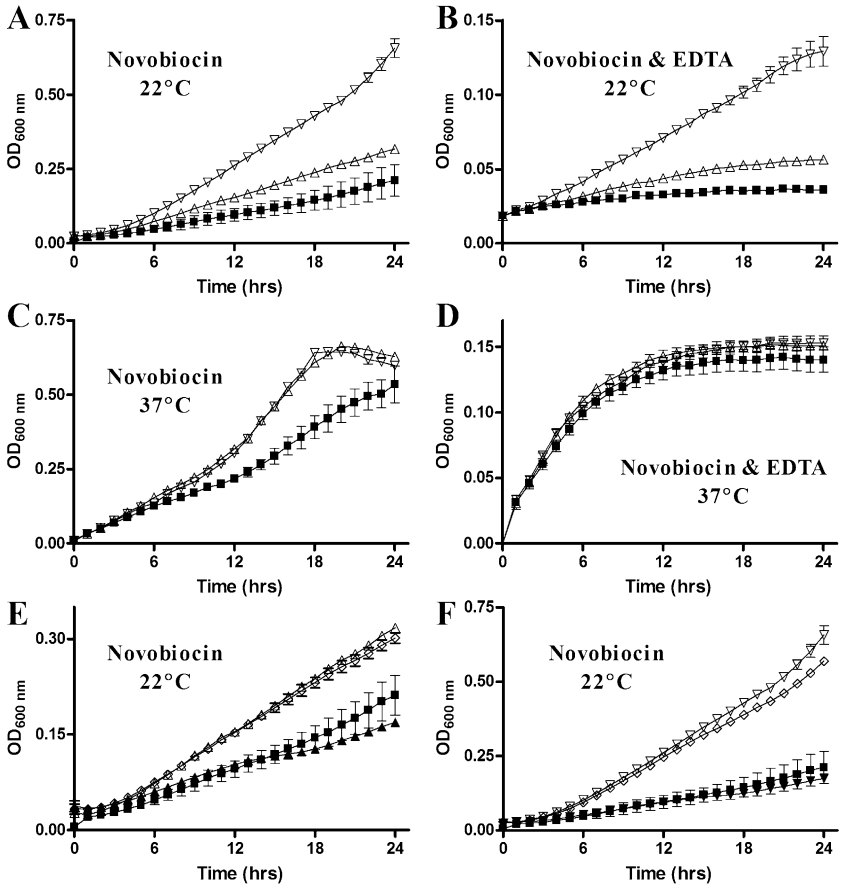

Fig. 8. Novobiocin and EDTA sensitivity assays at 22°C and 37°C. WT, mutants and complemented strains were monitored for growth by optical density at 600 nm (OD_{600}) over 24 h at 22°C (A, B, E and F) or at 37°C (C and D) in the presence of 100 μg ml^{-1} novobiocin with (B and D) or without (A, C, E and F) 1 mM EDTA. The symbols are the same as in Fig. 6 except that ◇ denotes *dmhA* mutant + empty vector pBR322 in (E) and *dmhB* mutant with empty vector in (F). A significant ($P < 0.05$) difference between WT and mutants was observed according to an unpaired *t*-test at 16 h.

(Fig. 8A). To delineate whether this effect was due to modifications of the composition of the O-antigen, EDTA was used to abolish the contribution of the lipid A-core to the OM stability via sequestering divalent cations that cross-link lipid A-core molecules to one another (Leive, 1965). The addition of EDTA during novobiocin challenge decreased growth in all strains compared with challenge with novobiocin only, indicating that lipid A-core bridging had a major contribution towards novobiocin resistance (Fig. 8B). However, both mutants were still significantly more resistant to novobiocin than the WT in the presence of EDTA, the *dmhB* mutant being much more resistant than the *dmhA* counterpart (Fig. 8B). This indicates that the differences seen between WT and mutants are primarily due to the O-antigen, and that the presence of O-antigen is unfavourable to resistance to novobiocin, as the *dmhB* mutant that produces very little O-antigen has the highest level of novobiocin resistance. Also, these data indicate that substitution of 6-deoxyheptose for DD-heptose improves slightly novobiocin resistance, as seen in the *dmhA* mutant.

To further delineate the respective contributions of the lipid A-core and O-antigen to novobiocin resistance, the experiments were repeated at 37°C to downregulate O-antigen expression in all strains (Fig. 2A). Under novobiocin challenge at 37°C, the difference in resistance between the *dmhA* and *dmhB* mutants previously seen at 22°C was alleviated, but both mutants remained more resistant than WT (compare Fig. 8A and C). While the resistance of the *dmhB* mutant was not considerably altered by the temperature shift, that of the *dmhA* mutant and of the WT increased significantly. These differences were not due to growth differences as the growth rates of all mutants were similar at 37°C, and not drastically higher at 37°C than at 22°C (Fig. 6A). At this temperature, all strains produce the same and limited amount of O-antigen (Fig. 2A), and the common feature of both mutants that differentiates them from WT is production of altered O-units with DD-heptose instead of 6-deoxyheptose, so that the *dmhA* mutant appears *dmhB*-like in terms of O-antigen production. Consequently, these data suggest that the concomitant decrease in O-antigen production and composition alteration of the *dmhA* mutant at 37°C results in its increased resistance to novobiocin at 37°C. The data are also consistent with an unfavourable role of WT O-antigen for novobiocin resistance as downregulation of its expression at 37°C allowed better resistance of the WT strain.

The addition of EDTA to novobiocin at 37°C decreased considerably the resistance of all strains, again verifying that LPS core bridging in the mutants is critical for novo-

biocin resistance (compare Fig. 8D and C). Also, the addition of EDTA abolished most of the differences in the resistance between WT and mutants. As observed in the absence of EDTA, the final level of resistance of *dmhB* was not affected by temperature upshift in the presence of EDTA (compare Fig. 8B and D) but that of the WT and *dmhA* mutant was increased to *dmhB* levels (compare Fig. 8B and D). This supports further that the O-antigen was not favourable for novobiocin resistance and that limitation of its production in *dmhA* and WT by temperature upshift allows improved resistance properties.

Altogether, these experiments demonstrate that WT resistance to novobiocin is dependent on both the core and the O-antigen, whereby the resistance conferred by core bridging is counterbalanced by the sensitivity conferred by the O-antigen. Inactivation of *dmhA* or *dmhB* led to modifications in the O-antigen composition which, surprisingly, increased the resistance of the mutants to novobiocin, especially when this modification was accompanied by reduced O-antigen expression as seen in *dmhB.*

Inactivation of dmhA *and* dmhB *modulates bacterial virulence in mouse*

When mouse were orally inoculated with 1×10^9 WT *Y. pseudotuberculosis* bacteria, significant levels of caecum colonization could be observed 4 days after inoculation (mean 0.9×10^3 cfu per gram of tissue) for the seven mouse tested (Fig. 9A). WT bacteria were also recovered from both the spleen (3.4×10^4 cfu per gram of tissue, Fig. 9B) and the liver (2.3×10^2 cfu per gram of tissue, Fig. 10C) in four of the mice tested, confirming that the strain tested is invasive and can disseminate to lymphoid organs and deeper tissues. All eight mice tested were colonized by the *dmhA* mutant, as bacteria were recovered for each mouse in at least one of the organs investigated (Fig. 9). The mean bacterial loads in the caecum (1.5×10^4 cfu g^{-1}) and liver (1.6×10^2 cfu g^{-1}) were similar to the loads observed for the WT strain but the load recovered in the spleen (2.5×10^5 cfu g^{-1}) was ~1 log higher in the *dmhA* mutant than in the WT strain. Also, the two mice that did not harbour bacteria in their caecum harboured bacteria in their spleen and liver, which suggests a faster dissemination process followed by clearance from the original site of infection. Dissemination to the spleen and/or liver was observed for three-quarters (six out of eight) of the mice inoculated with the *dmhA* mutant, as opposed to approximately half (four out of seven) of the mice infected with the WT strain. Overall these data indicate that the *dmhA* mutant is not impaired for colonization and is slightly more invasive than WT. Six of the seven mice tested were colonized with the *dmhB* mutant, with mean bacterial loads similar to those observed for WT in the caecum (2.6×10^3 cfu g^{-1}, Fig. 9A). The frequency and

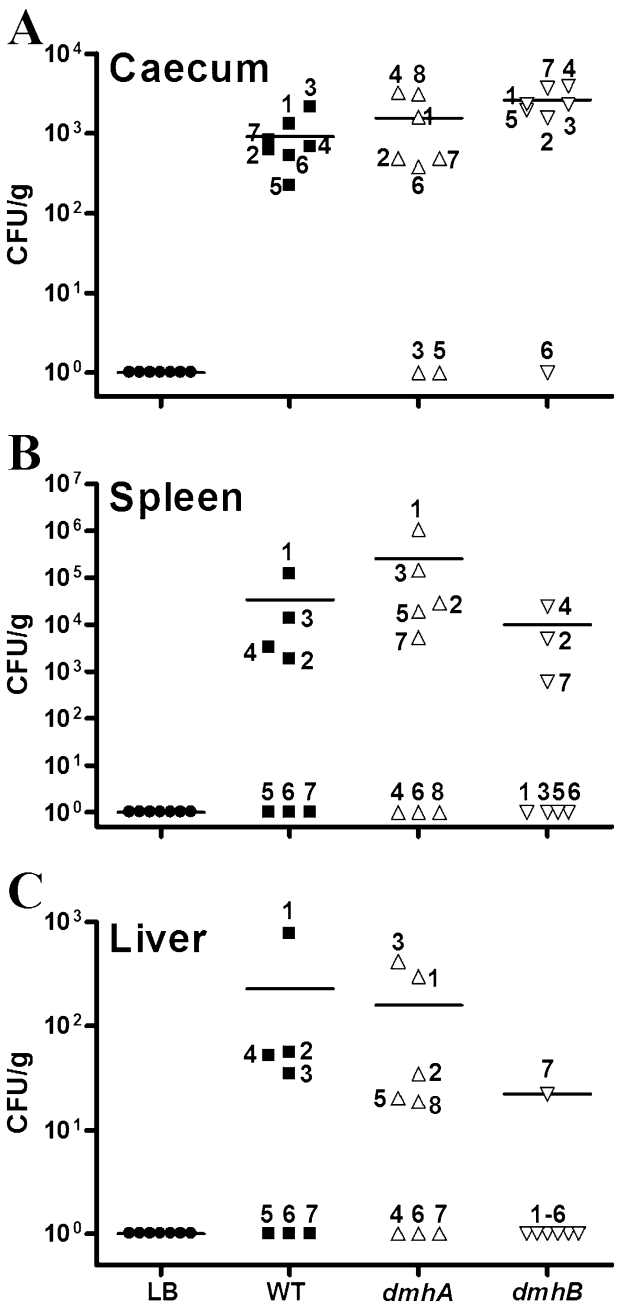

Fig. 9. Bacterial load obtained in the caecum, spleen and liver 4 days after oro-gastric inoculation of C57BL/6 mice with *Y. pseudotuberculosis* O:2a WT or mutants. The bacteria were enumerated by plating serial dilutions of the homogenized organs on *Yersinia* Selective Agar and the data are expressed as cfu per gram of tissue. The bars represent the average bacterial loads found in the colonized mice. The numbers refer to each individual mice tested. ●: LB broth. ■: WT. △: *dmhA*. ▽: *dmhB*.

level of dissemination to the spleen (9.9×10^3 cfu g^{-1}) were also similar to WT (Fig. 9B). However, dissemination to the liver was only observed for one mouse, and at a much lower level (2.1×10^1 cfu g^{-1}) than observed in WT (2.3×10^2 cfu g^{-1}). These data indicate that, like *dmhA*,

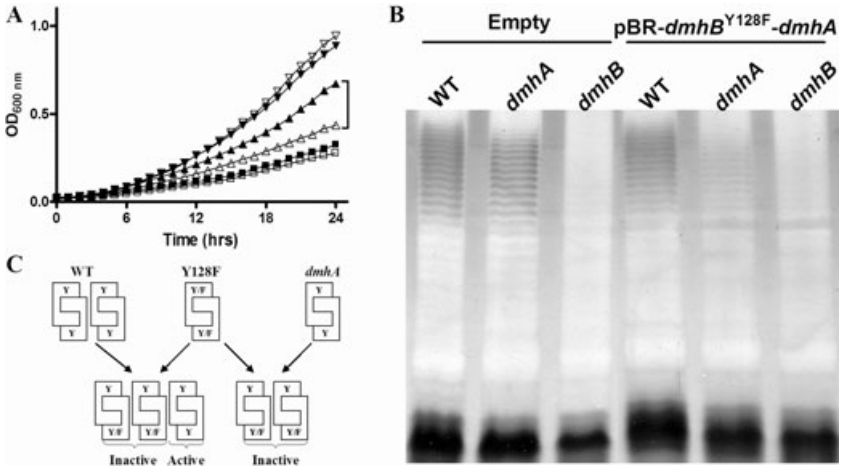

Fig. 10. Dominant negative effect seen with the catalytically inactive DmhB (Y128F) mutant.
A. Novobiocin sensitivity assay of WT and mutants with or without the pBR-*dmhB*^{Y128F}-*dmhA* construct. Strains were challenged with 100 μg ml^{-1} novobiocin and monitored for growth by optical density at 600 nm (OD_{600}) at 22°C over 24 h. □ and ■: wild type; △ and ▲: *dmhA* mutant; ▽ and ▼: *dmhB* mutant. Black symbols: strains containing the pBR-*dmhB*^{Y128F}-*dmhA* plasmid. Open symbols: strains lacking the complementation construct. Error bars indicate standard deviations of triplicate samples. The bracket illustrates the difference in growth between the *dmhA* mutant with and without the pBR-*dmhB*^{Y128F}-*dmhA* plasmid.
B. SDS-PAGE gel analysis of the LPS produced by WT and mutants in the presence or absence of the pBR-*dmhB*^{Y128F}-*dmhA* complementation construct.
C. Diagram of the dominant negative effect seen when the catalytically inactive DmhB Y128F mutant titrates away the chromosomally encoded active DmhB protein upon introduction of the pBR-*dmhB*^{Y128F}-*dmhA* construct into the WT and the *dmhA* strains.

dhmB is not significantly impaired for colonization or dissemination to the spleen. However, unlike *dmhA*, *dmhB* is impaired for further dissemination to (or persistence in) the liver. This would potentially suggest that the spread of the infection could be contained by the activity of lymphoid organs and macrophages in the case of the *dmhB* mutant. No pathological effects were observed 4 days post inoculation with any of the mice or any of the strains tested, even when high levels of colonization and dissemination to internal organs were observed.

Complementing the mutants demonstrates gene specificity of observed phenotypes

The kanamycin resistance cassette used to disrupt the *dmhA* and *dmhB* genes is non-polar by design and has been used in a variety of other bacteria without polarity effects (Heuermann and Haas, 1998; Gudlavalleti *et al*., 2004; Merkx-Jacques *et al*., 2004). However, in order to prove unambiguously that the phenotypes observed in the mutants were due to a single gene mutation in each case, and not to any unexpected polarity effect, complementation of the mutants was performed.

To complement the mutants, the genes were supplied *in trans* on a low-copy-number plasmid along with the upstream native promoter region of the *dmhA/B* operon. The *dmhA* and *dmhB* mutants were complemented with pBR-*dmhB-dmhA* and pBR-*dmhB* respectively (Fig. 1C). Upon introduction of the complementation constructs into the mutants, resistance to bile salts (Fig. 6A and D), polymyxin B (Fig. 7B and C) and novobiocin (Fig. 8E and F) reverted back to WT levels, and the expression of WT-like LPS was restored (Fig. 2B and C). Full motility was also restored in the *dmhB* mutant (Fig. 5).

The LPS from the complemented mutants was also purified and analysed by GC/MS, and showed a global reversion to WT composition of most components (Table 1). For example, the levels of galactose and GlcNAc reverted back to WT amounts. Furthermore, and most significantly, 6-deoxyheptose synthesis and incorporation in the O-antigen was restored in both strains.

Globally, these data indicate that the constructs successfully complemented the mutants by restoring WT phenotypes, and demonstrate that the phenotypes seen in the mutants were specifically due to the loss of function of each individual gene, with no polarity effect on downstream genes.

Expression of DmhA is dependent on DmhB encoded in cis

Surprisingly, complementation of *dmhA* could not be achieved with the pBR-*dmhA* construct in which the *dmhA* gene was directly coupled to the native promoter of the *dmhA*/*dmhB* operon, a strategy that successfully complemented the *dmhB* mutant. Possible reasons for this could be (i) because the *dmhB* gene may contain a translational enhancer required for efficient expression of the *dmhA*

product, or (ii) because a functional DmhB protein encoded *in cis* was required to allow expression of DmhA. To test for these two possibilities, the putative catalytic site of DmhB was disrupted through site-directed mutagenesis. Based on sequence alignments with other reductases of the SDR family, Tyr 128 was predicted to belong to the conserved SYK catalytic triad, and was mutated into a phenylalanine (Y128F) in the pBR-*dmhB-dmhA* complementation construct, yielding pBR-*dmhB*^{Y128F}-*dmhA*. This was anticipated to maintain the potential translational enhancer. As a control, the pBR-*dmhB*^{Y128F}-*dmhA* construct was first introduced into the *dmhB* mutant and assessed for function by monitoring the novobiocin resistance phenotype. This construct yielded no discernible difference in novobiocin resistance of the *dmhB* mutant, which demonstrates that the DmhB Y128F product is functionally inactive (Fig. 9A) and that Tyr 128 is important for DmhB function. However, introduction of the construct into the *dmhA* mutant also failed to restore WT-like novobiocin sensitivity. The construct actually further increased the novobiocin resistance of the *dmhA* mutant, as if also inhibiting the activity of the chromosomally encoded DmhB protein that is present in this mutant (Fig. 10A). Similarly, this construct led to reduced production of O-antigen when introduced in the *dmhA* mutant (Fig. 10B). Such phenomenon was not observed when the pBR-*dmhB*^{Y128F}-*dmhA* construct was introduced in the WT.

As a member of the SDR family of enzymes, DmhB is anticipated to function as an oligomer. This offers the potential for a dominant negative effect via the titration and inactivation of endogenous DmhB expressed from the chromosome by the plasmid-encoded and inactive DmhB Y128F mutant. To avoid this phenomenon, a premature stop codon was inserted at the 5′ end of *dmhB* in the pBR-*dmhB′-dmhA* construct, which results in the expression of a severely truncated DmhB protein of 16 amino acids in length instead of 278. As a control, when the pBR-*dmhB′-dmhA* construct was introduced into the *dmhB* mutant, no alteration to the novobiocin resistance was seen, verifying that the construct does not supply any (active) DmhB protein (data not shown). However, introduction of this construct into the *dmhA* mutant still did not affect its novobiocin resistance and also failed to complement the LPS profile as seen through SDS-PAGE analysis (data not shown). Altogether, these data indicate that the production of active DmhB protein *in cis* is necessary to achieve complementation of the *dmhA* mutant but the reason for this phenomenon is not clear at this stage.

Discussion

The goals of this study were to elucidate the roles of *dmhA* and *dmhB* in the biosynthesis of 6-deoxyheptose and to assess the impact of 6-deoxyheptose on LPS function and on the virulence of *Y. pseudotuberculosis*. As 6-deoxyheptose is not found in mammals, but is present in the virulence factors of several pathogenic bacteria, demonstrating the role of 6-deoxyheptose in virulence would implicate the enzymes responsible for its synthesis as targets for antimicrobial treatment. However, the biosynthetic pathway for 6-deoxyheptose in these organisms is not known, and only a putative pathway has been proposed for *Y. pseudotuberculosis* (Pacinelli *et al.*, 2002). The data provided in this study show that both *dmhA* and *dmhB* are required for the biosynthesis of 6-deoxyheptose and play a critical role in the expression and function of LPS in *Y. pseudotuberculosis* O:2a.

Validation of the mutants via complementation strategy leads to discovery of dominant negative effect

As DmhA is hypothesized to work upstream of DmhB in the 6-deoxyheptose biosynthetic pathway (Fig. 1B) (Pacinelli *et al.*, 2002), it was expected that the same phenotype would be observed in the *dmhA* and *dmhB* mutants, or if any differences were to be seen, a more dramatic phenotype change could be expected in the *dmhA* mutant. Furthermore, the spontaneous reduction of the GDP-4-keto-6-deoxy-D-*lyxo*-heptose intermediate produced by DmhA could supply 6-deoxyheptose residues in the absence of DmhB. However, contrary to all expectations, more drastic phenotypic differences were observed in the *dmhB* mutant than in the *dmhA* one. Hence, this raised the question of a polarity effect of inactivation of *dmhB* on the downstream *dmhA* gene. Complementation of the *dmhB* mutant *in trans* using pBR-*dmhB* confirmed that the inactivation of the *dmhB* gene did not cause a polarity effect on *dmhA* as all phenotypes seen in the *dmhB* mutant reverted back to WT levels. The slightly higher-than-normal levels of heptose observed in the *dmhB* complemented strain could be due to the presence of more abundant DmhB in the complemented strain than in the WT as the pBR322 complementation vector used has a copy number of ~15–20 copies per cell (Sambrook and Russell, 2001). This suggests that the 6-deoxyheptose biosynthetic pathway is finely tuned, and the incorporation of its product in the O-antigen is dependent on the precise amount of biosynthetic enzymes expressed.

Complementation of the *dmhA* mutant could only be achieved with the pBR-*dmhB-dmhA* construct and not with pBR-*dmhB*^{Y128F}-*dmhA*, pBR-*dmhB′-dmhA* or pBR-*dmhA*. This indicated that production of DmhA is not regulated by a translational enhancer present on the upstream mRNA sequence, and that production of a full-length and functional DmhB protein, encoded *in cis*, is required for proper DmhA production.

The complementation attempt using the pBR-*dmhB*^{Y128F}-*dmhA* construct revealed the existence of a

dominant negative effect involving DmhB. This enzyme belongs to the SDR family of proteins which commonly form functional oligomers (Oppermann *et al*., 2003). The enhanced resistance to novobiocin observed upon introduction of the pBR-*dmhB*^{Y128F}-*dmhA* construct into the *dmhA* mutant could indicate a dominant negative effect that could occur if non-functional DmhB Y128F monomers interact with and titrate out the functional copies of DmhB, thus resulting in the formation of inactive complexes (Fig. 10C). This could further enhance the resistance to novobiocin in the *dmhA* mutant by titrating out the functional DmhB protein encoded by the chromosome. As expected, no such effect was observed upon introduction of the pBR-*dmhB′*-*dmhA* construct in the *dmhA* mutant as this construct only supplies severely truncated DmhB protein. The fact that no change in novobiocin resistance was seen when the pBR-*dmhB*^{Y128F}-*dmhA* construct was introduced into the WT indicates that there may be lower levels of chromosomally encoded DmhB produced in the *dmhA* mutant than in the WT (Fig. 10C). Hence introduction of the pBR-*dmhB*^{Y128F}-*dmhA* construct in this mutant could lead to a complete titration of functional DmhB, while only a partial titration would occur in the WT and would not be sufficient to affect novobiocin resistance. Previous studies have demonstrated dominant negative effects in other SDR enzymes such as the dehydrogenases *RDH5* and *scully* (Torroja *et al*., 1998; Liden *et al*., 2001) and the epimerase *GALE* (Quimby *et al*., 1997). However, to the best of our knowledge, this is the first time a dominant negative effect has been seen in a putative reductase.

Fundamental implications with regards to LPS synthesis in Y. pseudotuberculosis

Until recently, little and sometimes conflicting information was available about the core composition and structure in *Y. pseudotuberculosis* (Samuelsson *et al*., 1974; Ovodov and Gorshkova, 1988). Our study presents the first complete description of the core structure for *Y. pseudotuberculosis* O:2a. Essentially the same LPS core structure has been reported for *Y. pseudotuberculosis* O:3 and O:4b (Knirel *et al*., 2007; 2008) and for *Y. pestis* (Knirel *et al*., 2005). The similarities comprise the alternation of the distal DD-heptose and Gal, as well as the presence of Ko, which was until recently considered unique to *Burkholderia cepacia* and *Acinetobacter calcoaceticus* (Kawahara *et al*., 1987; Isshiki *et al*., 1998).

The present study also has fundamental implications with regards to LPS synthesis. The Wzy-dependent biosynthetic pathway involves multiple functionally conserved proteins such as Wzx, Wzy, Wzz and WaaL. Of these proteins, only Wzx and Wzy are specific for the cognate O-antigen unit, while the others recognize the lipid A-core or Und-P (Schnaitman and Klena, 1993; Whitfield and Valvano, 1993; Whitfield, 1995; Skurnik and Bengoechea, 2003). The biosynthesis of complete LPS molecules occurred in both *dmhA* and *dmhB* mutants (Fig. 2A), but the O-antigen units of the mutants had incorporated DD-heptose in place of 6-deoxy-D-*manno*-heptose, which allowed for the attachment of abequose. This suggests that the 6-deoxyheptosyltransferase was not strictly specific for its substrate and was able to recognize and incorporate DD-heptose into the O-antigen instead of 6-deoxyheptose. This was already suggested by the presence of O-units containing DD-heptose instead of 6-deoxyheptose in the WT strain. However, their approximate ratio of 1:10 suggests that the activity of the glycosyl transferase involved is much lower with DD-heptose than with 6-deoxyheptose. The data also indicate that the abequosyltransferase has relaxed acceptor specificity as it can attach an abequose residue to a sugar other than 6-deoxyheptose. In addition, the altered O-units were still polymerized efficiently into long chains by Wzy in the *dmhA* mutant, although Wzy is thought to recognize the residues of the O-unit specifically. This indicates either (i) that Wzy in *Y. pseudotuberculosis* O:2a only recognizes residues found in the O-antigen backbone, galactose and GlcNAc, with little input from the branching residues such as 6-deoxyheptose, or (ii) that replacement of 6-deoxyheptose with isosteric DD-heptose has no significant impact on the activity of Wzy. However, very little polymerization of the O-units was observed in the *dmhB* mutant despite the fact that their composition is identical to the *dmhA* mutant O-units. This suggests that inactivation of *dmhB* somehow downregulates the production or activity of Wzy independently of its O-antigen composition. This could be due to the lower availability of its substrate, the Und-P-O-unit.

Also, the disruption of *dmhB* resulted in decreased lipid A-core capping by the O-unit compared with WT and *dmhA* mutant. This indicates that the activity of the ligase WaaL was decreased in the *dmhB* mutant. As WaaL mainly recognizes the O-antigen through the Und-P carrier (Raetz and Whitfield, 2002), no direct effect on WaaL was anticipated in either mutant. As Wzx, Wzy, Wzz and also potentially WaaL are believed to form a complex required for efficient assembly of LPS molecules in the periplasm (Raetz and Whitfield, 2002; Marolda *et al*., 2006), it is possible that inactivation of *dmhB* has pleiotropic effects that affect optimal formation or activity of this complex. Such effects would result in less efficient assembly and presentation of O-antigens on the OM, which is consistent with the more pronounced changes in OM barrier function observed in the *dmhB* mutant. It cannot be excluded, however, that inactivation of *dmhB* caused decreased availability of O-units that would also result in the lower capping of the *dmhB* lipid A-core,

without any direct effect on the catalytic properties of WaaL.

Another difference between the two mutants is their core composition, whereby the core of the *dmhB* mutant exhibits a higher proportion of Kdo. It is not clear how mutations in the O-antigen biosynthetic cluster could affect core biosynthesis as both loci are independent. However, the formation of an altered core could certainly alter directly the activity of LPS assembly enzymes that recognize the core structure and composition, such as the ligase WaaL (Raetz and Whitfield, 2002). This would explain the least amount of ligation seen in the *dmhB* mutant and a reduced O-antigen expression in *Y. pseudotuberculosis* at 37°C (Straley and Perry, 1995; Vakorina *et al*., 2003; this work), i.e. under the conditions when Ko is not incorporated into the LPS core of *Yersinia* (Knirel *et al*., 2005; 2008). This is not anticipated to have any feedback effect on the enzymes involved in the assembly and polymerization of the O-units as these steps occur before ligation to the core, unless all enzymes are present in a single complex where perturbations affecting the activity of one enzyme also affect the activity of all others.

Role of dmhA *and* dmhB *on virulence*

As mentioned above, the differences observed in the composition of the LPS between the WT and mutants could explain the phenotypes observed in both mutants *in vitro*. Past studies have shown that LPS core modifications result in decreased motility in a wide variety of bacteria (Toguchi *et al*., 2000; Izquierdo *et al*., 2002; Nagy *et al*., 2006). *Y. pseudotuberculosis* appears to be no different as motility was impaired in the *dmhB* mutant, which, unlike the *dmhA* mutant, exhibits slightly altered and often uncapped lipid A-core molecules.

It was previously shown in *Y. enterocolitica* that lipid A and core bridging plays a crucial role in the resistance against bile salts (Skurnik *et al*., 1999). The data presented in this study show that the WT O-antigen in *Y. pseudotuberculosis* also contributes to bile salts resistance, as growth of both mutants was severely impaired upon challenge with bile salts at 22°C (Fig. 6C and D), and sensitivity was enhanced upon challenge at 37°C (Fig. 6E). Although the experiments were performed with bile salts concentrations slightly higher than the reported physiological level of 5 mM (~2.2 mg ml^{-1}) (Perez de la Cruz Moreno *et al*., 2006), the significant differences observed suggest a potential role *in vivo.*

Several Gram-negative bacteria, including *Yersinia* species (Anisimov *et al*., 2005; Winfield *et al*., 2005), adapt to polymyxin exposure by modifying their LPS with 4-aminoarabinose which repels cationic peptides (Rebeil *et al*., 2004). Protection via addition of glycine to the core has also been proposed (Anisimov *et al*., 2005). Our data suggest that the WT *Y. pseudotuberculosis* strain adapts to the challenge although no data are available at this stage to determine whether this involves modification of the LPS by 4-aminoarabinose or glycine. Importantly though, the mutants were eventually able to adapt to the polymyxin challenge too but in a delayed fashion. This indicates that their LPS composition is somehow suboptimal for this adaptation process to take place.

In previous studies, resistance to novobiocin had largely been attributed to a stable lipid A-core in Gram-negative bacteria (Schnaitman and Klena, 1993; Walsh *et al*., 2000). Bridging between phosphorylated residues within the lipid A and core by divalent cations creates a barrier that prevents hydrophobic compounds such as novobiocin from entering into the bacteria (Bengoechea *et al*., 1998b; Skurnik *et al*., 1999; Frirdich *et al*., 2003; Ramjeet *et al*., 2005). Recent studies have shown that the O-antigen also contributes to novobiocin resistance (Allen *et al*., 1998; Merkx-Jacques *et al*., 2004). Our data demonstrate that in *Y. pseudotuberculosis* O:2a, lipid A-core bridging also plays a major role in novobiocin resistance but that the presence and composition of the WT O-antigen is unfavourable to novobiocin resistance. The resistance patterns observed in our mutants correlate well with the LPS composition analysis which showed that DD-heptose replaces 6-deoxyheptose in both mutants. Heptose is more hydrophilic than 6-deoxyheptose, and may aid in repelling the hydrophobic antibiotic novobiocin. As a result, both mutants, and especially the *dmhB* mutant that makes hardly any O-antigen, were more resistant to novobiocin than WT (Fig. 8A).

In *Y. pseudotuberculosis* (Rebeil *et al*., 2004), the lipid A switches from an hexa- to a tetra-acylated form upon temperature upshift. By enhancing membrane fluidity, this could facilitate entry of hydrophobic compounds and enhance novobiocin sensitivity. However, our data indicate that this phenomenon did not affect novobiocin sensitivity significantly as, once O-antigen production was downregulated by temperature upshift, the WT and *dmhA* mutant were more resistant to novobiocin than at 22°C.

Mouse infection experiments were performed to test the role of *dmhA* and *dmhB* on bacterial virulence *in vivo*. For these experiments, the bacteria were grown at 26°C, a temperature that promotes good growth and supports production of HMW O-antigen. It should be noted that upshift to 37°C, as encountered in the host, does not totally abolish O-antigen formation in any of the strains and does not alter the amount of lipid A-core + one O-unit molecules produced, as seen by SDS-PAGE gels (Fig. 2A). The maintenance of one O-unit at 37°C is consistent with prior observations made in *Y. enterocolitica* (Perez-Gutierrez *et al*., 2007). Consequently, the differences in O-unit composition present in the mutants could potentially affect not

only the initial steps of intestinal colonization but also the subsequent dissemination to lymphoid organs.

Mouse infection experiments demonstrated that the colonization potential of *Y. pseudotuberculosis* O:2a was not affected by the replacement of the 6-deoxyheptose by DD-heptose in the O-antigen, or by the amount of O-antigen present on the bacterial surface. However, the replacement of the 6-deoxyheptose by DD-heptose did modulate the invasiveness of *Y. pseudotuberculosis* O:2a, with an unexpectedly enhanced invasive character of the *dmhA* mutant. This effect was dependent on the presence of WT-like levels of O-antigen, as it was not observed in the *dmhB* mutant. The combined effect of the substitution of DD-heptose for 6-deoxyheptose, the reduction of O-antigen production and impaired flagella-mediated motility resulted in the limited ability of the *dmhB* mutant to reach or persist in the liver, despite its WT-like ability to colonize the caecum and reach the spleen. This indicates that targeting DmhB for therapeutic purposes might be beneficial.

As determined by PCR (data not shown), the WT and mutant bacteria harboured the pYV plasmid that is essential for virulence. This plasmid encodes Yop proteins that promote extracellular replication of the bacterium in lymphoid tissues by inhibiting phagocytosis by polymorphonuclear leukocytes and macrophages (Cornelis, 2002b). The differences observed between both mutants indicate that the effect is largely dependent on the LPS composition and amount of O-antigen. Hence, the higher load of *dmhA* mutant bacteria in the spleen suggests that this mutant shows enhanced resistance to phagocytosis- or complement-mediated killing and the lower recovery of *dmhB* in the liver suggests the reverse for *dmhB*. It is likely that, over time, the *dmhA* mutant would be recovered from the spleen longer than the WT whereas the *dmhB* mutant would be cleared faster than the WT. No data could be obtained beyond 4 days post inoculation to confirm this because of practical and humane considerations.

In *Y. enterocolitica*, expression of O-antigen is co-ordinated with the expression of other virulence factors, including that of the Yops. The absence of O-antigen downregulates transcription of the Yop virulon in *Y. enterocolitica* (Perez-Gutierrez *et al*., 2007). If this also applied to *Y. pseudotuberculosis*, the *dmhB* mutant would have less Yop-related antiphagocytic properties than the WT or *dmhA* mutant as it produces much less O-antigen and also produces significant amounts of uncapped lipid A-core molecules. Hence, it would not be expected to disseminate efficiently to internal lymphoid organs. Our results obtained 4 days post inoculation would suggest that this is not the case as dissemination to and survival in the spleen was not affected.

In summary, we demonstrate herein that *dmhA* and *dmhB* are required for the biosynthesis of 6-deoxyheptose in *Y. pseudotuberculosis*, and that disruption of these genes affects primarily the composition of the LPS O-units, and this in turn affects the resistance of *Y. pseudotuberculosis* to antimicrobial agents and its motility. Based on the observed decreased resistance to bile salts and polymyxin B of both mutants and impaired motility of the *dmhB* mutant, the mutants were expected to be less virulent *in vivo*, which would validate DmhA and DmhB as therapeutic targets. The results of mouse infection experiments cautions against the use of DmhA as a therapeutic target as this mutant was slightly more invasive than WT. However, the *dmhB* mutant showed promising reduction of colonization of the liver relatively to the WT, which suggests that it might be cleared more efficiently over time and that DmhB could potentially be targeted for therapeutic benefit. Furthermore, the mechanism of biosynthesis of 6-deoxyheptose is not currently understood and the data presented herein already provide a few insights into the mode of action of DmhB by showing that Tyr 128 and potentially the oligomerization of DmhB are essential for its activity. In addition to providing fundamental insights into LPS biosynthesis, the knowledge gained from this study could be applied to other organisms which also harbour 6-deoxyheptose within their surface polysaccharides, and help elucidation and targeting of their 6-deoxyheptose biosynthetic pathway.

Experimental procedures

Bacterial strains, growth conditions and reagents

Bacterial strains and plasmids used in this study are listed in Table 3. *Y. pseudotuberculosis* was grown at 22°C on *Yersinia* Selective Agar (Oxoid) or Luria–Bertani (LB) media supplemented with CIN antibiotics: cefsulodin (15 μg ml^{-1}), irgasan (4 μg ml^{-1}) and novobiocin (2.5 μg ml^{-1}) (Schiemann, 1979; Swaminathan *et al*., 1982). *Escherichia coli* was grown at 37°C on LB media supplemented with ampicillin (100 μg ml^{-1}), kanamycin (30 μg ml^{-1}) and tetracycline (15 μg ml^{-1}) as required. For selection of recombinant *Y. pseudotuberculosis* on solid agar media, kanamycin (100 μg ml^{-1}) and tetracycline (10 μg ml^{-1}) were used, while kanamycin (20 μg ml^{-1}) and tetracycline (5 μg ml^{-1}) were used for selection in liquid media (Bengoechea *et al*., 2002). All restriction enzymes were purchased from Invitrogen unless otherwise noted.

Cloning of the dmhA *and* dmhB *genes*

The promoter, *dmhB* and *dmhA* genes were PCR amplified with Expand Long Range DNA polymerase (Roche) using chromosomal DNA from *Y. pseudotuberculosis* O:2a (Fig. 1C) and primers BP1 and AP1. The sequences of the primers are provided in Table 4. The PCR product, as well as the pUC18 plasmid, were digested using BamHI and KpnI, and the resulting fragments were ligated together using Rapid DNA ligase (Fermentas) to yield pUC-*dmhB-dmhA*

Table 3. List of bacterial strains and plasmids used in this work.

Strains and plasmids	Description	Reference
Strains		
Y. pseudotuberculosis		
O:2a strain 208	Wild-type strain used in this study	Bogdanovich *et al.* (2003)
dmhA::Kan	*dmhA* disrupted by kanamycin resistance cassette	This study
dmhB::Kan	*dmhB* disrupted by kanamycin resistance cassette	This study
dmhA::Kan/pBR-*dmhB-dmhA*	*dmhA* knockout strain complemented *in trans* using pBR-*dmhB-dmhA*	This study
dmhB::Kan/pBR-*dmhB*	*dmhB* knockout strain complemented *in trans* using pBR-*dmhB*	This study
E. coli		
DH5α	*supE* Δ*lac U169 (Φ80lacZΔM15) hsdR recA endA gyrA hri relA*	Hanahan (1983)
SM10 *λpir*	*Thi thr leu sup tonA lacy recA*::RP4-2-Tc::mukm *λpir*	Simon *et al.* (1983)
Plasmids		
pHel3	Shuttle vector containing kanamycin resistance cassette; Kan^r	Heuermann and Haas (1998)
pUC18	Cloning vector; Ap^r	Yanisch-Perron *et al.* (1985)
pUC-*dmhB/A*	2.2 kb BamHI and KpnI fragment from genomic DNA of *Y. pseudotuberculosis* O:2a, containing the *dmhA*, *dmhB* genes and their 5′ promoter, cloned into pUC18	This study
pUC-*dmhA*::Kan	Removal of 300 bp within the *dmhA* gene and insertion of a 1.2 kb kanamycin resistance cassette	This study
pUC-*dmhB*::Kan	Removal of 600 bp within the *dmhB* gene and insertion of a 1.2 kb kanamycin resistance cassette	This study
pCVD442	Suicide vector containing the counter-selectable marker *sacB*, Ap^r	Donnenberg and Kaper (1991)
pCVD-*dmhA*::Kan	3.2 kb *dmhA* disrupted fragment from pUC-*dmhA*::Kan cloned into pCVD442	This study
pCVD-*dmhB*::Kan	3.0 kb *dmhB* disrupted fragment from pUC-*dmhB*::Kan cloned into pCVD442	This study
pBR322	Complementation vector; Ap^r Tet^r	Bolivar *et al.* (1977)
pBR-*dmhB-dmhA*	2.2 kb fragment from pUC-*dmhB-dmhA*, containing the *dmhA*, *dmhB* genes and their 5′ promoter, cloned into the pBR322 PstI and EcoRI sites	This study
pBR-*dmhA*	1.4 kb fragment from pUC-*dmhB-dmhA* containing the *dmhA* gene and its upstream promoter cloned into the pBR322 PstI and EcoRI sites	This study
pBR-*dmhB*	1.2 kb fragment from pUC-*dmhB-dmhA* containing the *dmhB* gene and its upstream promoter cloned into the pBR322 PstI and EcoRI sites	This study
pBR-*dmhB*^{Y128F}-*dmhA*	2.2 kb fragment containing the *dmhA*, *dmhB* genes and their 5′ promoter. The *dmhB* gene has a point mutation Y128F designed to disable the catalytic function of the product	This study
pBR-*dmhB′-dmhA*	2.2 kb fragment containing the *dmhA*, *dmhB* genes and their 5′ promoter. The *dmhB* gene has a premature stop codon inserted at the 5′ end creating a severely truncated protein of 16 amino acids in length	This study

plasmid. After transformation on *E. coli* DH5α, transformants were recovered via selection for ampicillin resistance. Positive clones were verified by PCR, restriction enzyme digestion and DNA sequencing performed at the Robarts Institute Sequencing Facility (London, Canada).

Gene disruption

A kanamycin resistance cassette was PCR amplified out of the pHel3 plasmid (Heuermann and Haas, 1998) using primers Aph3P1 and Aph3P3 (Table 4). The pUC-*dmhB-dmhA* plasmid was used as a template for inverse PCR using primers BP2 and BP3 which removed 600 bp from the middle of the *dmhB* gene, as well as AP2 and AP3 which removed 300 bp from the middle of the *dmhA* gene. The PCR products and kanamycin resistance cassette were digested using BglII and XbaI, and then ligated together to yield pUC-*dmhA*::Kan and pUC-*dmhB*::Kan plasmids. After transformation in *E. coli* DH5α, transformants were selected for ampicillin and kanamycin resistance. Positive clones were verified by PCR and restriction enzyme digestion.

Transferring disrupted genes to the pCVD442 suicide vector

The disrupted genes were PCR amplified out of the pUC-*dmhA/B*::Kan plasmids mentioned above using primers BP1 and AP1. The PCR product was then blunted using T4 DNA polymerase (New England Biolabs). The pCVD442 suicide vector (Donnenberg and Kaper, 1991) was digested using SmaI, and the two fragments were ligated. The plasmids were recovered in *E. coli* SM10 *λpir* with kanamycin and ampicillin selection. Positive clones were verified by PCR and restriction enzyme digestion.

Creating knockout mutants of Y. pseudotuberculosis *O:2a*

The knockout mutants were created following previously described procedures (Bengoechea *et al.*, 2002). Briefly summarized, pCVD442-*dmhA/B*::Kan plasmids were conjugated from *E. coli* SM10 *λpir* into *Y. pseudotuberculosis* on LB agar at a recipient : donor ratio of 1:100 and allowed to

Table 4. Nucleotide sequences for all primers used.

Primer	Sequence (5′→3′)
Forward primer	
AP2	GCTCTAGAACTTTTGTGACTCGAATG
AP4	AGGGTCCATGGGCATGAATAATGTTTTAATTACAGGT
AP7	AGGGTCCATGGGCAAACTATTAACTGGCGAA
ApH 3P1	GAAGATCTGATAAACCCAGCGAACCA
BP1	CGGGATCCTGTCGAGGAAAAATTTTCTGA
BP2	GCTCTAGATATGTGATTGGGATAGTATC
BP4	AGGGTCCATGGGCATGACAAAGGTGTTTATATTAGG
BTruncTop	ACG**C**CTATATTGGTAATAATTTAATGG
Y128FTop	ATGAAAATTCTGAAAAAAAACCATTTGGAAAATT**T**TGGAGAAATGAAGTCCG
Reverse primer	
AP1	GGGGTACCTTAGCGATTTAATGGAATGCG
AP3	GAAGATCTGATACACTCTAATAGATTAG
AP5	GCGACGGATCCTTAGCGATTTAATGGAATGCG
ApH 3P3	GCTCTAGAGACATCTAAATCTAGGTAC
BP3	GAAGATCTGGTGATGATATTGCTGA
BP5	GCGTCGGATCCTTATTCCTTAATTACTTCCAGATATGG
BP7	AGGGTCCATGGTTATTCCTTAATTACTTCCAGAT
BTruncBot	AG**G**CGTTGGAACCTAATATAAACA
Y128FBot	CGGACTTCATTTCTCCA**A**ATTTTCCAAATGGTTTTTTTTCAGAATTTTCAT

Restriction sites are underlined
Mutated nucleotides are in bold

recover O/N at 37°C on LB agar. Transconjugates were selected on *Yersinia* Selective Agar supplemented with ampicillin and kanamycin and grown at 37°C for 3 days. The merodiploids recovered were grown in LB with kanamycin for three passages at 25°C to allow for the second cross-over event. The bacterial culture from the last passage was diluted 1:5 (w/v) into fresh LB supplemented with kanamycin and 15% sucrose, incubated at room temperature for 3 h, and then recovered on *Yersinia* Selective Agar supplemented with kanamycin O/N at 37°C. The recombinants that survived 15% sucrose exposure were checked for their sensitivity to ampicillin. The appropriate replacement of the WT *dmhA* and *dmhB* genes was confirmed by Southern blot and PCR for genes *dmhA*, and *dmhB* using primers AP4 and AP5, and BP4 and BP5 respectively.

Southern blotting

Southern blotting was performed using the digoxigenin-labelling method with detection with antidigoxigenin-alkaline phosphatase-CSPD [disodium 3-(4-methoxyspiro-4-yl)phenyl phosphate] substrate as specified by the manufacturer (Roche). Chromosomal DNA was extracted from *Y. pseudotuberculosis* using DNAzol (Invitrogen) and cut with EcoRI/StuI. DIG-labelled probes of *dmhA*, *dmhB*, and the kanamycin resistance cassette were created using primers AP4 and AP5, BP4 and BP5, ApH 3P1 and ApH 3P3 respectively.

Creating complementation constructs

To create a complementation plasmid that contains either *dmhA* or *dmhB* under its native promoter, a DNA fragment containing both genes as well as the upstream promoter was PCR amplified from pUC-*dmhB-dmhA* and cloned into the PstI and EcoRI sites of pBR322 to yield pBR-*dmhB-dmhA* (Fig. 1C). This construct was used to complement the *dmhA* mutant directly or after further modification by site-directed mutagenesis. Site-directed mutagenesis was performed using the QuikChange method (Stratagene). Primers BTruncTop and BTruncBot were used to insert a premature stop codon into the 5′ end of *dmhB* in pBR-*dmhB-dmhA* to generate pBR-*dmhB′-dmhA*. Also, the conserved $SY_{128}K$ catalytic triad was mutated to $SF_{128}K$ using primers Y128FTop and Y128FBot, generating the pBR-*dmhB*^{Y128F}-*dmhA* complementation vector. The resulting constructs were verified by DNA sequencing. To complement the *dmhB* mutant, the downstream *dmhA* gene from the pBR-*dmhB-dmhA* construct was removed using inverse PCR and primers BP7 and AP7.

Complementing knockout mutants

The *dmhA* and *dmhB* mutants were complemented *in trans* by introducing the *trans*-complementation constructs mentioned above in each mutant by electroporation (Conchas and Carniel, 1990). Briefly, 2 μl (containing 1–2 μg) of DNA, and 38 μl of competent cells at OD_{600} 0.6 were placed in an ice-cold 0.2 cm electroporation cuvette, and electroporated using a Bio-Rad electroporator set to 50 μF, 200 Ω and 2.5 kV. Tranformants were allowed to recover for 3 h in LB broth with vigorous shaking, and then selected on LB agar containing kanamycin and tetracycline.

Electron microscopy

Yersinia pseudotuberculosis WT and mutants were grown O/N at 22°C on *Yersinia* Selective Agar containing CIN and

appropriate selective antibiotics. Samples negatively stained with 0.5% (w/v) uranyl acetate were observed under a Philips CM10 Electron Microscope in the Microbiology and Immunology departmental electron microscopy facility.

Lipopolysaccharide isolation and chemical analyses

Lipopolysaccharide was prepared by SDS solubilization of whole cells and proteinase K treatment as described previously (Hitchcock and Brown, 1983) and was analysed by SDS-PAGE (15% polyacrylamide). Detection was performed by silver staining (Fomsgaard *et al*., 1990) or Western blot with anti-*Y. pseudotuberculosis* O:2a antibody (Oxoid) or with anti-O14 *E. coli* antibody (Staten's Serum Institut, Copenhagen, Denmark) which reacts with ECA.

For composition analysis presented in Table 1, LPS was isolated by phenol-water extraction (Westphal, 1965) and analysed by The Complex Carbohydrate Research Center in Georgia (USA). Glycosyl composition analysis was performed by combined GC/MS of the per-*O*-trimethylsilyl (TMS) derivatives of the monosaccharide methyl glycosides or the alditol acetate derivatives to resolve the 6-deoxyheptose and glucose peaks. Methyl glycosides were prepared from dry LPS by methanolysis in 1 M HCl in methanol at 80°C (18–22 h), followed by re-N-acetylation with pyridine and acetic anhydride in methanol (for detection of amino sugars). The samples were then per-*O*-trimethylsilylated by treatment with Tri-Sil (Pierce) at 80°C (0.5 h). These procedures were carried out as previously described (Merkle and Poppe, 1994). GC/MS analysis of the TMS methyl glycosides was performed on an HP 5890 GC interfaced to a 5970 MSD, using a All Tech EC-1 fused silica capillary column (30 m × 0.25 mm inside diameter). The samples (500 μg of each) were hydrolysed using 2 M trifluoroacetic acid (TFA) (2 h in a sealed tube at 121°C), reduced with $NaBD_4$ and acetylated using acetanhydride/TFA. The resulting alditol acetates were analysed on a Hewlett Packard 6890 GC interfaced to a 5973 MSD (mass selective detector, electron impact ionization mode); separation was performed on a 30 m DB-1 capillary column with ammonia as the carrier gas.

For compositional and structural analyses presented in Table 2 and Figs 3 and 4, the LPS was also isolated by phenol-water extraction but subsequently purified by precipitation of impurities with TCA. The lipid A component was removed by mild acid hydrolysis (0.1 M NaOAc buffer pH 4.5 at 100°C for 2 h) followed by centrifugation (13000 *g*, 20 min). The water-soluble supernatant was fractionated by gel-permeation chromatography on Sephadex G-50 (S) in 0.05 M pyridinium acetate buffer pH 4.5 to generate HMW and LMW fractions. Compositional analyses were performed by GLC after hydrolysis of the fractions with 1 M TFA (120°C, 2 h), reduction with $NaBH_4$ and acetylation with acetanhydride in pyridine (1:1.5, v/v, 100°C, 30 min). GLC of the derived alditol acetates was carried out on a Hewlett-Packard 5880 chromatograph using a HP-5ms capillary column and a temperature gradient of 150°C (3 min) to 320°C at 5°C min^{-1}.

NMR spectroscopy and MS

Samples were deuterium-exchanged by freeze-drying twice from 99.90% D_2O and then examined as solutions in 99.96% D_2O at 40°C on a Bruker DRX-500 NMR spectrometer (Germany) using internal acetone (δ_H 2.225, δ_C 31.45) as reference. Mixing times of 200 and 100 ms were used in TOCSY and ROESY experiments respectively.

Negative ion electrospray ionization ion-cyclotron resonance Fourier transform MS analyses were performed on a hybrid Apex Qe instrument (Bruker Daltonics, USA) equipped with an actively shielded 7 Tesla magnet and an Appollo II Dual ESI/MALDI ion source. The drying gas temperature was set to 200°C, and the Q-voltage in the Q-cell was set to 4 V. Samples (~10 ng μl^{-1}) were dissolved in a 50:50:0.001 (v/v/v) mixture of 2-propanol, water and triethylamine pH ~8.5 and sprayed at a flow rate of 2 μl min^{-1}. The spectra showing several charge states for each component were charge deconvoluted, and mass numbers given in the charge-deconvoluted spectra refer to the monoisotopic molecular masses.

Mouse infection assays

All mice infection experiments were performed according to the rules and recommendations of the Animal Care and Veterinarian Services of the University of Western Ontario. The experimental protocol (# 2007-079-10) has been approved by the Animal Use Subcommittee. Seven C57BL/6 male mice (8 weeks) were orally inoculated with 100 μl of bacterial suspension containing 1×10^9 cfu of bacteria after 6 h of fasting. The mice were then fed sterile water and food *ad libitum* and monitored daily for 4 days. After 4 days, the mice were sacrificed by barbiturate injection and the spleen, liver and ceacum were recovered and homogenized. Serial dilutions were plated out in triplicates on *Yersinia* selective Agar (Oxoid) for cfu determination. The plates were incubated at room temperature for 36 h before enumeration.

Growth curves

Growth curves were performed in a 100-well microtitre plate using a Bioscreen C automated microbiology growth curve analysis system (MTX Laboratory Systems, Vienna, VA). *Y. pseudotuberculosis* strains were grown at 22°C or 37°C for 8 h in LB containing CIN and appropriate selective antibiotics. The samples were then washed twice in LB broth to remove all antibiotics, diluted to a final OD_{600} of 0.05 and grown at 22°C or 37°C under constant shaking and monitored for growth (OD_{600}) every hour for 24 h. Samples were tested in triplicates for each experiment. Three independent experiments were performed. The data are presented for one representative experiment.

Sensitivity assays

SDS, bile salts, polymyxin B and novobiocin sensitivity assays were also performed using the Bioscreen C machine. The sample preparation was the same as for the growth curves except that the samples were supplemented with either sublethal concentrations of SDS (0.01% to 0.2%), bile salts (2–8 mg ml^{-1}) (50% cholate and 50% deoxycholate), polymyxin B (10 μg ml^{-1}) or novobiocin (100 μg ml^{-1}) with or without EDTA (1 mM). In order to mimic the natural process of

host infection for the bile salt and polymyxin experiments performed at 37°C, cells were first grown at 22°C. Cells were kept at a constant 37°C for the novobiocin sensitivity experiments to mimic usage of novobiocin as a therapeutic in an infected individual and ensure maximal repression of O-antigen synthesis prior to exposure to novobiocin. Samples were tested in triplicate and OD_{600} measurements were taken every hour for 24 h.

Motility assays

Yersinia pseudotuberculosis strains were grown for 8 h at 22°C in LB broth with CIN and selective antibiotic. The cells were washed twice in LB, diluted to a final OD_{600} of 0.6 and stab-inoculated into LB CIN soft agar plates containing 0.25% bacto agar (DIFCO), and incubated at 22°C. Samples were tested in triplicate, and measurements of the diameter of the motility halo were taken at 24, 36 and 48 h.

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