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Relationship of *Yersinia pseudotuberculosis* O Antigens IA, IIA, and IVB: the IIA Gene Cluster Was Derived from That of IVB

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O antigen is part of the lipopolysaccharide present in the outer membrane of gram-negative bacteria and is highly polymorphic. In this study, we obtained sequences of the O-antigen gene clusters for the *Yersinia pseudotuberculosis* antigens IA, IIA, and IVB. We propose that the IIA gene cluster was derived from the IVB cluster, one of the very few cases in which a parent gene cluster is identified, and that the IA gene cluster could be a hybrid of the IVB and IB gene clusters. All three O antigens contain 6-deoxy-D-mannoheptose, and we identified six genes for the biosynthetic pathway for the precursor of this sugar, GDP-6-deoxy-D-mannoheptose.

Lipopolysaccharide (LPS), an important component of the outer membrane of gram-negative bacteria, usually consists of three distinct regions: lipid A, core oligosaccharide, and O-specific polysaccharide (O antigen). The O antigen consists of repeats of an O unit of generally two to six sugars, and in most species it is highly polymorphic, with many forms that can vary in the types of sugars present, in their relative arrangement within the repeat unit, and in the linkage between repeat units (12). The genes for O-antigen synthesis are normally grouped together on the chromosome in a gene cluster which maps between *hemH* and *gsk* in *Yersinia pseudotuberculosis.*

We, among others, have undertaken an extensive study of the genetic basis of O-antigen variation by sequencing and identifying O-antigen genes, mostly in *Salmonella enterica* and *Escherichia coli* (see references 24 and 25 for reviews). Comparison of sequences has provided evidence for the origin of new O-antigen forms by recombination between gene clusters in *S. enterica* (5, 40) and evidence for recent interspecies transfer of an entire O-antigen gene cluster (28, 33).

Y. pseudotuberculosis O antigens are of particular interest, since the reported forms include all five naturally occurring 3,6-dideoxyhexose (DDH) sugars (abequose, colitose, paratose, tyvelose, and ascarylose) (6), otherwise rarely found in nature. The IA, IIA, and IVB O antigens have similar structures but differ in the presence of paratose, tyvelose, and abequose, respectively (Fig. 1). The biosynthetic pathways for the activated nucleotide forms of these DDH sugars are related (21), and the genes involved for all three have been identified in *S. enterica* (4, 14, 20, 35, 40) and *Y. pseudotuberculosis* (11, 15). Genes *ddhABCD* for the conversion of glucose-1-phosphate to CDP-4-keto-3,6-dideoxy-D-glucose are common to the three pathways. This compound can then be converted either to CDP-abequose by CDP-abequose synthetase (*abe*) or to CDP-paratose synthetase (*prt*). CDP-paratose can then be converted into CDP-tyvelose by CDP-tyvelose epimerase (*tyv*).

We previously reported sequences of the DDH genes from the IA and IIA O-antigen gene clusters (11, 15) and found the order to be the same as in *S. enterica*. In this study we report the complete DNA sequences of the O antigens of *Y. pseudotuberculosis* IA, IIA, and IVB. The sequences reveal DDH genes, 6-deoxy-D-mannoheptose pathway genes, the chain length determinant gene (*wzz*), the O-unit flippase gene (*wzx*), the O-antigen polymerase gene (*wzy*), and glycosyltransferase genes. Sequence comparisons suggest that the IIA gene cluster was formed by introducing an *abe* gene into a IVB gene cluster, providing one of the few clear cases of such a relationship. It also appears that the IA gene cluster is a hybrid of IVB and IB gene clusters.

Plasmids and bacterial strains. Plasmid pPR1670 was as described by Hobbs and Reeves (11). Plasmids constructed for sequencing were maintained in *E. coli* K-12 strain DH10B. The *Y. pseudotuberculosis* strains H892/87 (serotype IA, laboratory name M444) and H715/86 (IVB, M454) were obtained from S. Aleksic, Institute of Hygiene, Hamburg, Germany. *Y. pseudotuberculosis* IIA strain M85 was obtained from D. Hughes, NSW Dairy Corp. Lab (Australia).

DNA isolation, manipulation, and sequencing. We used the JUMPstart sequence (primer 412 [ATTGGTAGCTGTAAGC CAAGGGCGGTAGCGT]), which is a 39-bp element present upstream of many polysaccharide gene clusters (10), and the *gsk* sequence (primer 413 [GCGGGCATTGACTGGAAAGT AGTGTTTGGAC]), which is present downstream of O-antigen gene clusters in *Y. pseudotuberculosis*, to amplify the IIA and IVB O-antigen gene clusters by doing long PCR. PCR fragments of ~20 kb were obtained from strains M85 and M454, subjected to DNase I digestion, and cloned into pGEM-T to make banks for sequencing as described previously (39). The products of 32 individual PCRs were pooled to make each bank to limit the effect of PCR errors.

The IA sequence was obtained from three overlapping DNA fragments. The sequence from positions 4417 to 10136 was obtained from a partial *Sau*3A bank made from the insert of pPR1670 (11). The sequence from positions 1 to 4416 and from positions 10137 to 20511 was obtained from DNase I banks made from PCR products amplified from chromosomal DNA by using the JUMPstart or *gsk* primers and a primer based on the sequence of pPR1670.

The DNA template for sequencing was prepared by using the 96-well format plasmid DNA Miniprep kit from Advanced

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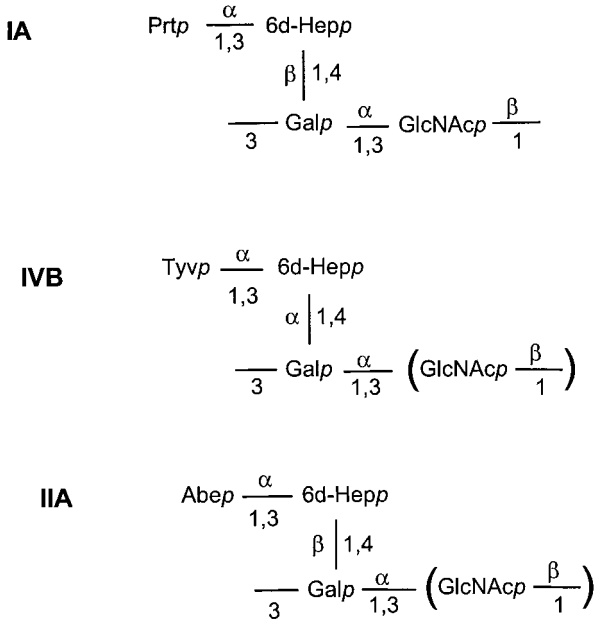

FIG. 1. Structures of *Y. pseudotuberculosis* O antigens IA, IVB, and IIA (17, 27, 30).

Genetic Technologies Corp. and the procedure developed by The Institute for Genome Research (34). Sequencing was performed with an Applied Biosystems 377 automated DNA sequencer. Sequence data were assembled by using the Phred/Phrap package of the University of Washington Genome Center, and the sequence annotation was done by using the program Artemis from the Sanger Centre. We used the algorithm described by Eisenberg et al. (8) to identify potential transmembrane segments from the amino acid sequence.

Sequences of 20,511, 19,535, and 19,457 bp, which cover the entire O-antigen gene cluster and the 5′ half of the *gsk* gene, were obtained from IA, IIA, and IVB, respectively. Analysis showed that there are 18 genes per gene cluster, including one obviously defective gene in IIA (Fig. 2). All were transcribed from JUMPstart to *gsk*. The nucleotide and amino acid sequences of each gene were used to search available databases for indications of function.

DDH biosynthetic pathway genes. The IIA, IA, and IVB O antigens contain abequose, paratose, and tyvelose, respectively (Fig. 1). We have previously sequenced the region from positions 1 to 6753 of the IIA O-antigen gene cluster and identified the *ddhABCD* and *abe* genes which are responsible for the synthesis of CDP-abequose (11). The *ddhABCD* genes are also present in the IA and IVB O-antigen gene clusters (Fig. 2) and are almost identical (99.4 to 100%) in the three gene clusters. The *prt* genes of IA and IVB are 100% identical to each other and *Y. pseudotuberculosis* IB and 99.8% identical to those of *Yersinia pestis* (29) (note that *Y. pestis* is a clone of *Y. pseudotuberculosis* [1]). The *tyv* gene of IVB is 68% identitical to that of *S. enterica* serovar Typhimurium (14). Thus, all of the genes necessary for the synthesis of CDP-paratose (*ddhABCD* and *prt*) and CDP-tyvelose (*ddhABCD*, *prt*, and *tyv*) are found in IA and IVB, respectively.

6dDHep biosynthetic pathway genes. 6-Deoxy-D-mannoheptose (6dDHep) is present in all three O antigens and is not otherwise found in *Y. pseudotuberculosis* (29). It is closely related to L-glycero-D-manno-heptose (LDHep), which is present in the LPS inner core of many species of gram-negative bacteria (23). D-Glycero-D-manno-heptose (DDHep) has also

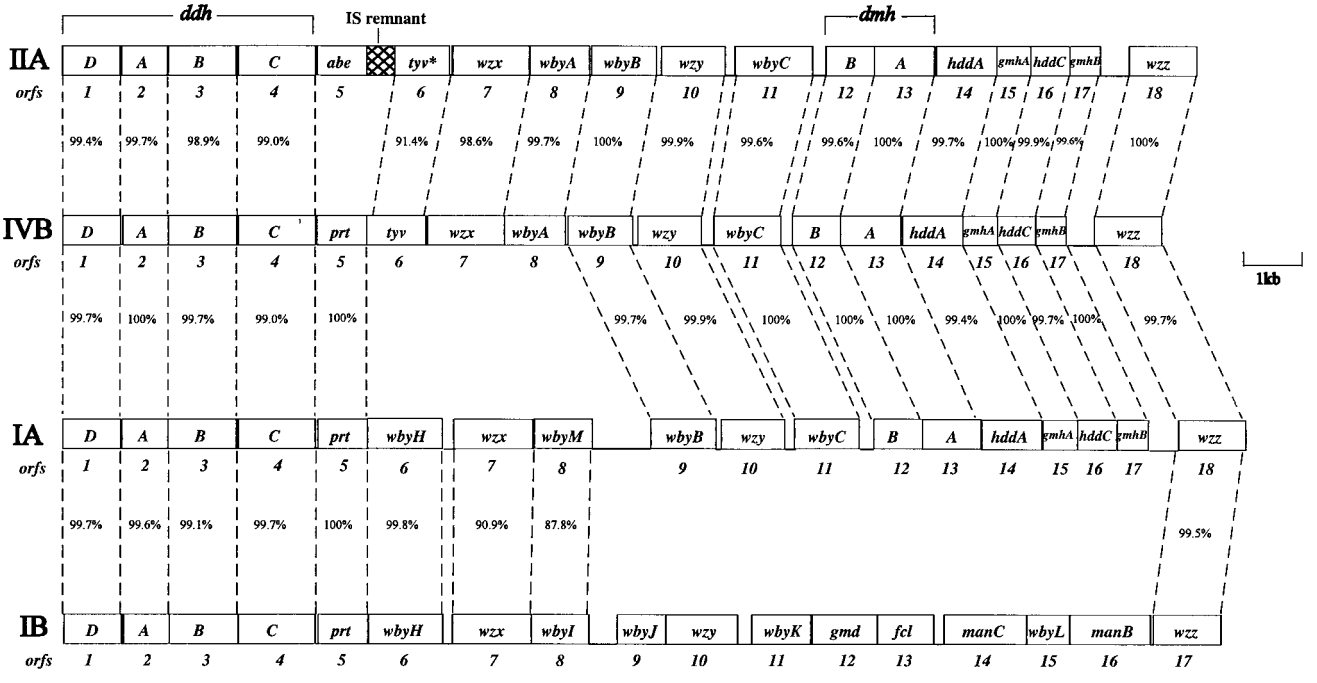

FIG. 2. O-antigen gene clusters of *Y. pseudotuberculosis* IVB, IIA, IA, and IB. The 18 open reading frames are numbered 1 to 18 in map order. The IB gene cluster (29) is included for comparison.

been reported in the LPS outer core region of some species and since carbon six, the asymmetric carbon atom that differentiates LDHep and DDHep, is reduced in 6dDHep and no longer asymmetric, one can envisage either LDHep or DDHep as the precursor for 6dDHep.

Kneidinger et al. (16) have recently shown that DDHep of *Aneurinibacillus thermoaerophilus* capsule is synthesized as precursor GDP-DDHep from sedoheptulose 7-phosphate via a four-enzyme pathway (see steps 1 to 4 of Fig. 3). Our three *Y. pseudotuberculosis* O-antigen gene clusters have in common Orf14 to Orf17, which are homologous to the GDP-DDHep pathway genes of *A. thermoaerophilus*, suggesting that it is the GDP-DDHep pathway that is extended to give the 6dDHep precursor (Fig. 3). Orf14, Orf15, Orf16, and Orf17 show 50, 61, 42, and 42% identity to HddA, GmhA, HddC, and GmhB of *A. thermoaerophilus*, respectively. It seems clear that the *Y. pseudotuberculosis* genes have the same functions as those of *A. thermoaerophilus* and have been given the same names.

Biosynthesis of 6-deoxyhexoses starts with nucleotide diphospho sugars and involves firstly oxidation of C-4 and then the reduction of C-6, followed by reduction of C-4 to give a specific 6-deoxyhexose (26). We suggest that GDP-6dDHep is formed in this way from GDP-DDHep (Fig. 3, steps 5 and 6). Orf13 of the three gene clusters is similar to Gmd, which carries out the oxidation of C-4 and the reduction of C-6 on GDP-mannose. GDP-DDHep differs from GDP-mannose only in having a CH_2OH substitution on C-6, and we suggest that Orf13 converts GDP-DDHep to GDP-4-keto-6-deoxy-D-mannoheptose. This leaves only the final C-4 reduction step, and we suggest that this is carried out by Orf12, which is similar to many sugar dehydratases, converting GDP-4-keto-6-deoxy-D-mannoheptose to GDP-6dDHep. We have named the Orf13 and Orf12 genes *dmhA* and *dmhB*, respectively.

The proposed GDP-6dDHep pathway includes four genes of the GDP-DDHep pathway and two additional genes. The six genes are present in the same order in a capsular gene cluster of *Burkholderia mallei* (7), with amino acid identity levels between *B. mallei* and *Y. pseudotuberculosis* gene products ranging from 35 to 53%. This capsule is a homopolymer of 6dDHep, and the presence of the set of six genes in the same order in *B. mallei* confirms our designation of genes for the GDP-6dDHep pathway.

Genes *wzz*, *wzx*, and *wzy*. A putative *wzz* gene was found in each cluster. The three genes (*orf18* in each case) are highly similar (Fig. 2) and show 91% identity to *wzz* of *Y. pestis* (29) and 92% identity to *wzz* of *Y. pseudotuberculosis* IIA, which was sequenced and studied by Stevenson et al. (32). The protein has two predicted transmembrane segments, as for many other Wzz proteins.

The presumptive *wzy* genes (*orf10*) are near identical in the three gene clusters (Fig. 2). The deduced proteins have many predicted transmembrane segments and were most similar to Wzy proteins of *E. coli* O157 (39), *E. coli* O111 (37), *S. enterica* O4 (14), and *E. coli* K-12 (31). BLOCKMAKER and PSI-BLAST operations were carried out (38) and, after four iterations, the four input proteins and many other distantly related Wzy proteins but no other proteins were retrieved (E value $\leq 4e \times 10^{-35}$). We propose that *orf10* is the *wzy* gene.

The presumptive *wzx* genes (*orf7*) were first identified as encoding an integral inner membrane protein with 12 pre-

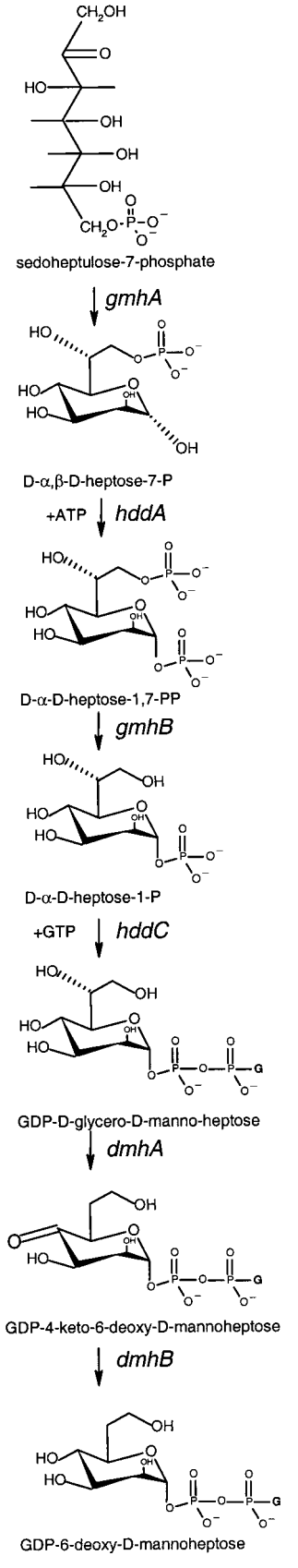

FIG. 3. Proposed biosynthetic pathway of GDP-6-deoxy-D-mannoheptose.

dicted transmembrane segments. Orf7 of IA shows 99% identity to Wzx of *Y. pestis* and 90% identity to Wzx of *Y. pseudotuberculosis* IB (29), and *orf7* of IA was identified as *wzx*. The *orf7* genes of IVB and IIA are 98.6% identical and were identified as *wzx* by carrying out BLOCKMAKER and PSI-BLAST operations as described above.

Transferase genes and comments on structures of IIA and IVB. Sugar tranferase genes can usually be identified by being most similar to other sugar transferase genes but often cannot be allocated to specific transferase specificities on this basis. We found three putative transferase genes for each of the three O-antigen gene clusters. None of the predicted proteins had the several putative transmembrane segments found in WecA and WbaP, which add a sugar phosphate to UndPP to initiate synthesis of the O unit (14, 22) and, since all structures include GlcNAc (30), we suggest that, as for *E. coli* GlcNAc-containing O antigens, synthesis is initiated by the transfer of GlcNAc-1-P to UndP by WecA (3), the same reaction that initiates synthesis of enterobacterial common antigen (ECA). Skurnik et al. (29) showed that WecA starts the O-antigen biosynthesis in *Y. enterocolitica* serotype O8 when the gene cluster was transferred to *E. coli* (41). It has also been shown that *Y. pestis* expresses ECA (36), and we found that the *wecA* gene was present in an ECA gene cluster in the *Y. pestis* genome (http://www.sanger.ac.uk/Projects/Y_pestis) (74% identical to *E. coli wecA*). It seems that in *Yersinia* sp. as in *E. coli*, WecA can act as first transferase for O antigens containing GlcNAc, and given that only three transferases were identified within the IA, IIA, and IVB O-antigen gene clusters, we propose that WecA initiates synthesis in all three.

The *wbyC* genes of the three clusters are virtually identical (Fig. 2) and were most similar to *wbbP* of *E. coli* Dysenteriae 1 (34% identity) (9), and WbyC could be the transferase for the same linkage in the three *Y. pseudotuberculosis* gene clusters.

WbyB, also common to all three gene clusters, is similar to many putative sugar transferases, and we suggest that it is the transferase for putting the 6dDHep residue onto galactose since this linkage is also common to all three structures.

The third putative sugar transferase genes, *wbyA* in IIA and IVB and *wbyM* in IA, are both between *wzx* and *wbyB* (Fig. 2). *wbyA* is similar to *wbaV* of *S. enterica* groups B and D (62 and 65% identity, respectively), which encode transferases for abequose or tyvelose (19), although both forms are nonspecific for the dideoxyhexose (19, 20). We suggest that WbyA is the tyvelose or abequose transferase of IVB and IIA. WbyM is only 21% identical to WbyA but is similar to several sugar transferases and is presumably the paratose transferase, since paratose is unique to IA and this is the only linkage specific to IA. It is of particular interest that *wbyM* is 87.8% identical to *wbyI*, a putative glycosyltransferase, of *Y. pseudotuberculosis* IB (29). IA and IB both have paratose 1-3 linked to mannose and 6dDHep, respectively, which are closely related sugars, adding strong support for *wbyM* and *wbyI* being paratose transferases.

The IIA gene cluster was derived from that of IVB. The IIA cluster differs from the IVB cluster by the presence of an *abe* gene in place of the *prt* gene of IVB and because the *tyv* gene of IIA is defective and not functional. The similarity between IIA and IVB sequences for all but *abe*, *tyv*, and *prt* indicates that one gene cluster is derived from the other, whereas the levels of identity, ranging from 98 to 100% (Fig. 2), indicate relatively recent divergence. The structures for IIA and IVB differ only in the presence of abequose or tyvelose, respectively, as the DDH sugar, and replacement of *prt* and *tyv* by *abe* is precisely what is required to change the DDH sugar. The *tyv* gene is useful only in the presence of a *prt* gene, and the defective *tyv* gene in IIA indicates that the ancestral form had both *tyv* and *prt*. The formation of the IIA cluster with *abe* must have involved replacement of *prt* and *tyv* by *abe* in a recombination event probably mediated by the insertion sequence (IS), of which a substantial remnant can still be seen. The defective *tyv* gene of IIA and the IS remnant adjacent to *abe* had been observed previously (11, 15) and led us to postulate an IS-mediated recombination event. The IVB gene cluster, for which we now present the sequence, has the characteristics expected of the ancestral form for the IIA gene cluster.

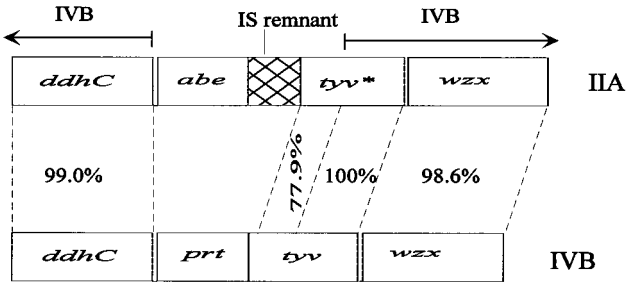

FIG. 4. The region around the *tyv* gene of the O-antigen gene clusters of *Y. pseudotuberculosis* IIA and IVB indicates the probable origin of the IIA gene cluster. Note that the 5′ end of *tyv** of IIA has accumulated many mutations, and this part shares 77.9% identity with the corresponding region of IVB. The asterisk in *tyv** indicates a defective gene.

Given that in both *S. enterica* and *Y. pseudotuberculosis* the *tyv*, *prt*, *abe*, and *asc* genes that code for the later steps in DDH synthesis always map downstream of the *ddh* genes, it is reasonable to assume this arrangement in the donor strain, which allows homologous recombination at one end and IS-mediated recombination at the other as proposed for the origin of the *S. enterica* D2 gene cluster (40).

The situation is complicated by the observation that, whereas the 3′ end of the *tyv* genes (bases 5820 to 6753 of IIA) is identical in the IIA and IVB gene clusters, the 5′ end of the IIA *tyv* gene is defective (Fig. 4). The first 76 bp are missing, and the remaining part of the 5′ end differs from the functional IVB gene by 18%, including several deletions and an overall frameshift. It appears that the two parts of the *tyv* gene may have entered at different times, suggesting two steps in the assembly of the IIA gene cluster.

The IA gene cluster is potentially a hybrid of IVB and IB gene clusters. The 5′ end of the IA gene cluster to *prt* and the 3′ end from *wbyB* are nearly identical to the corresponding segments of IVB (Fig. 2). However, the intervening segment of IA, including the *wbyH* and *wbyM* genes, is absent in IIA and IVB, but is present, with *wbyI* replacing *wbyM*, in IB (*wbyM* and the very similar *wbyI* are both putative paratose transferases as discussed above). The IB gene cluster also has the DDH genes common to IA, IIA, and IVB. The IA gene cluster is in effect a chimera of those of IVB and IB (see Fig. 2). There

is also a 1,012-bp intergenic region between *wbyI* and *wbyB* in IA that is not similar to any known sequence.

It seems clear that one or more of IA, IB, and IVB arose by recombination, with IVB perhaps being the recipient of the segment *wbyH* to *wbyM/I* from IB to form IA. If so, it is interesting that *wzx* and *wbyI/M* have undergone 10% divergence, whereas *wbyH* has undergone very little (Fig. 2). The Wzx proteins are responsible for the translocation of two different O units across the inner membrane, whereas WbyI and WbyM are proposed to put the same residue (paratose) onto different sugars in IA and IB, so the divergence may reflect functional differences.

wbyH is 96% identical to *wbyH* of *Y. pseudotuberculosis* IB and *Y. pestis*, which was proposed (29) to be a sugar pathway gene. As discussed above, we have identified the genes for the synthesis of CDP-paratose and GDP-6dDHep, and this leaves no obvious function for *wbyH*. Skurnik et al. (29) also did not assign a function to *wbyH* in the IB gene cluster, with all required functions accounted for by other genes.

Concluding remarks. There are 12 O-antigen forms characterized in *Y. pseudotuberculosis* (2), and we now have sequences for four of them, including the three presented in this study. The *ddhABCD* genes are located at the 5′ end of the gene cluster (Fig. 2) in all four clusters, and these four genes are highly conserved.

The three new sequences are all for 6-deoxy-D-mannoheptose containing O antigens, and this has enabled us to identify the biosynthetic pathway genes which are at the 3′ end of the gene cluster just upstream of *wzz* gene (Fig. 2). It is probably no coincidence that the *ddh* and DDHep genes are conserved and at the 5′ and 3′ ends, respectively, of the gene clusters, since both are present in several gene clusters. This pattern for such pathway genes is seen, for example, in *E. coli*, *S. enterica*, and *Streptococcus pneumoniae* for *rml* genes, which are quite commonly present in these species, where it facilitates recombination among gene clusters (13, 18).

Nucleotide sequence accession numbers. The *Y. pseudotuberculosis* IA, IIA, and IVB O-antigen gene cluster sequences have been deposited in GenBank under accession numbers AF461768, AF461770, and AF461769, respectively.

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